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A pH Study of Four Media Inoculated with Bacteria and Protozoa

BY ROBERT F. MOTE ¹

Abstract. Fifteen species of protozoa were used for a 36 day period to test the pH of four protezoa media. During this experiment the pH was determined every three days for control media, the media with bacteria, and the media with bacteria and protozoa. The pH curve included a lag phase, an acceleration phase, a plateau, and a decline with three of the four media during the 36 days. The fourth medium did not have a decline from the plateau during the 36 days. The protozoa and the four media are described.

The ability of a protozoan species to survive and reproduce in a specific medium provides interesting questions. This experiment was designed to test four media that have become quite useful for stock protozoan cultures. What pH does a medium retain for any given length of time without bacteria or protozoa? What pH differences exist between the medium, the bacteria, and various species of protozoa? In this experiment, species-pure cultures were used to test the four media with various protozoan species. A species-pure culture is defined as a single protozoan species with bacteria.

LITERATURE

The literature is divided into two parts, that pertaining to the media and that pertaining to the pH of the media.

Noyes (1916) prepared a starch peptone medium for the cultivation of bacteria. Sandon (1927) used agar and hay infusion to cultivate protozoa. Needham (1937) referred to several types of cultural infusions, namely: hay and rye, rice and timothy hay, timothy and distilled water, hay-flour and distilled water, boiled hay, chopped timothy hay boiled well water.

Various nutrients such as rice, wheat, rye, and timothy have provided an energy factor for bacteria in protozoan cultures.

In this study separate pH curves for the media, the media and bacteria, and the media with bacteria and protozoa were obtained. Several factors may influence the sigmoid curves derived. Hall (1953) stated that inoculation of the protozoa may directly influence the density of a population and the rate of growth. Johnson (1933) stated that in cultures of *Oxytricha fallax*, the initial concentration of bacteria may determine whether the culture is to show the Robertson effect. Richards (1941) showed that growth rate may or may not follow a sigmoid curve.

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MATERIALS AND METHODS

This experiment consisted of the following; (1) Experimentation leading to development of a medium to be used in this experiment; (2) Use of 15 protozoan species from pure cultures; (3) Providing for a ready supply of biotic bacteria for protozoan culture inoculation; (4) Deriving a method for obtaining pH value without a great reduction in the fluid content of the medium; (5) Examining the cultures, obtaining the pH, and recording the media water loss during the 36-day experimentation.

The four media used for this experiment were starch-agar peptone-tryptophane, alfalfa distilled-water rice, soil-filtrate peptone-tryptophane rice, and hay-filtrate rice.

The starch-agar peptone-tryptophane medium was prepared in two steps.

- A. Difco Bacto agar 7.5 gm.
Argo cornstarch 0.25 gm.
Pyrex distilled water 500 c.c.
- B. Difco Bacto peptone 0.05 gm.
Tryptophane 0.05 gm.
Pyrex distilled water 500 c.c.

In preparation of this medium, the agar and cornstarch were placed with distilled water and brought to a boil. Seven c.c. of the boiling starch agar were placed in washed, auto-claved, and cotton-stoppered biological shell glass test tubes measuring 25x95mm. The test tubes with the 7cc of agar and cornstarch were then autoclaved at 15 pounds pressure for 20 minutes. After autoclaving when the agar medium had cooled, 24 cc of the peptone-tryptophane distilled water were added to each test tube. These aseptic test tubes were then set aside.

Alfalfa distilled-water rice medium: Test tubes 25x95mm were washed, cotton stoppered and autoclaved. Twenty-four cc of distilled water were added to each test tube, then each test tube was autoclaved. After autoclaving, additional distilled water was added to equal exactly 24 cc. Three alfalfa stems 25-30 mm in length, which had been boiled for 15 minutes, were added to each of the test tubes. Also added was one rice particle which had been boiled for 5 minutes. These aseptic test tubes were set aside.

The soil-filtrate peptone-tryptophane rice medium consisted of 200 gm of loam top soil and 600 cc of distilled water. This was air exposed for 48 hours. The top fluid of the infusion was then filtered. This was autoclaved at 15 pounds pressure for 20 minutes. Placed in previously washed and autoclaved shell glass test tubes were 12 cc of the autoclaved soil infusion filtrate. Also

added were 12 cc of peptone-tryptophane with distilled water. This made 24 cc of the autoclaved soil filtrate and peptone-tryptophane medium. One rice particle boiled for 5 minutes, was added. These test tubes, also aseptic were, then set aside.

The concentration of the peptone and tryptophane for this medium was 0.10 gm. peptone and 0.10 tryptophane per 500 cc of distilled water. When diluted with the soil filtrate, this concentration became 0.05 peptone and 0.05 tryptophane.

The hay-filtrate rice medium was made as follows; 15 gm of fresh straw were added to 850 cc of distilled water. This was boiled for 15 minutes, filtered and placed in two one-pint jars for 48 hours. Twenty-four cc of the filtered infusion were placed in test tubes and cotton stoppered after 48 hours air exposure. These were then autoclaved at 15 pounds pressure for 20 minutes. Additional hay filtrate was autoclaved so that each test tube would have 24 cc. This addition provided filtrate lost in autoclaving. The size of these test tubes was 25x95 mm. After autoclaving, one rice particle that had been boiled for 5 minutes was added. These aseptic test tubes were set aside for future use.

To test the 15 protozoan species the following test tubes were needed. Starch-agar peptone-tryptophane: five. Alfalfa distilled water rice: three. Soil-filtrate peptone-tryptophane rice: three, and hay-filtrate rice: four. Four test tubes were used to test each of the aseptic media, and four each for the bacteria and the media. This number was adequate and provided additional data during the 36-day experimentation.

All cultures of the four different media required bacteria except the control to test the media. Two drops of bacteria from a micropipette with an opening diameter of 1 mm were introduced into each test tube. The bacteria for this experiment were previously cultured from air-borne bacteria (Mote, 1966). These bacteria were cultured with autoclaved infusion filtrate from the soil, alfalfa, and hay. To inoculate the starch-agar peptone-tryptophane medium, bacteria that had been cultured in soil and hay filtrate were used. To inoculate the alfalfa distilled-water rice medium, bacteria cultured in alfalfa filtrate were used. The hay-filtrate rice medium was inoculated with bacteria that had been cultured in hay filtrate. Soil-filtrate peptone-tryptophane rice medium received bacteria cultured in soil filtrate.

The time from bacterial inoculation until the protozoa were added was twelve hours. The beginning pH readings for the various media were taken before the introduction of bacteria or protozoa. Table 1 gives the protozoan species used in this experiment, the medium used, and the number of protozoan species used to inoculate the cultures at the beginning of the experiment.

Table 1. Protozoan species, media, and number of protozoa inoculated into culture media.

Species	Medium	Number of Protozoa introduced into Cultures
<i>Spirostomum teres</i>	starch-agar peptone-tryptophane	6
<i>Paramecium multimicronucleatum</i>	" "	6
<i>Leucophrys patella</i>	" "	20-30
<i>Oikomonas termo</i>	" "	200
<i>Vorticello microstoma</i>	" "	3 Teletrech, 4 adult
<i>Chilomonas oblonga</i>	Alfalfa-distilled-water rice	30-35
<i>Colpoda steini</i>	" "	14
<i>Cryptomonas ovata</i>	" "	50
<i>Blepharisma undulans</i>	Hay filtrate rice	5
<i>Oxytricha fallax</i>	" "	5
<i>Ochromonas mutabilis</i>	" "	75-100
<i>Colpidium campylum</i>	" "	6
<i>Coleps bicuspis</i>	Soil-filtrate peptone-tryptophane rice	10
<i>Halteria grandinella</i>	" "	30-35
<i>Urocentrum turbo</i>	" "	4

Every third day the pH readings were taken for all the cultures by means of Accutint¹ pH test paper. Every ninth day the water loss from the cultures was determined. This provided information concerning loss of water from the micropipette withdrawal to obtain pH data and evaporation loss. The 25x95 flat-bottom biological shell glass test tubes provided an easy method to determine water volume loss. This was done by correlating water loss against a millimeter ruler. A table had previously been prepared converting millimeters into cubic centimeters. To minimize the water loss during the experiment, aluminum caps were placed over the cotton stoppers of the test tubes. None of the four media were buffered to a beginning pH.

Examination was either by microscope, or a 10X hand lens. New glassware was washed with a good detergent, rinsed eight times, and air dried. All autoclaving was done at 15 pounds pressure for twenty minutes. All micropipettes used to obtain culture samples were used only once and then re-autoclaved. Rice particles equaling 29 particles to one-half gram were used. Adequate but not direct sunlight was used, and the room temperature was 22 degrees C.

OBSERVATION AND DATA

The pH curves were determined from each of the three-day pH reading during the 36-day experiment. Figures 1, 2, 3, 4, and 5 represent five of the 15 protozoan species used during the experiment.

¹ Accutint test paper, Anachemia Chemicals Ltd.

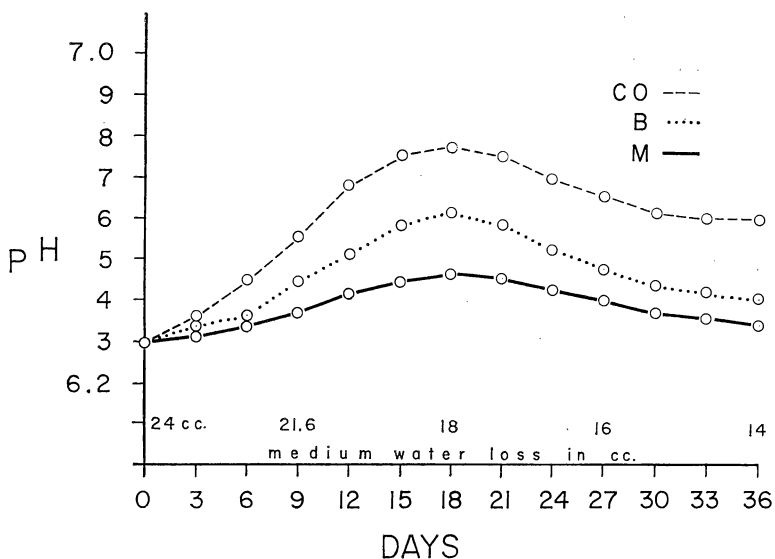


Figure 1. Correlation of days and water volume loss to the pH of the Alfalfa-distilled water-rice medium with the protozoan (CO) *Cryptomonas ovata*, (B) Bacteria, and (M) the medium.

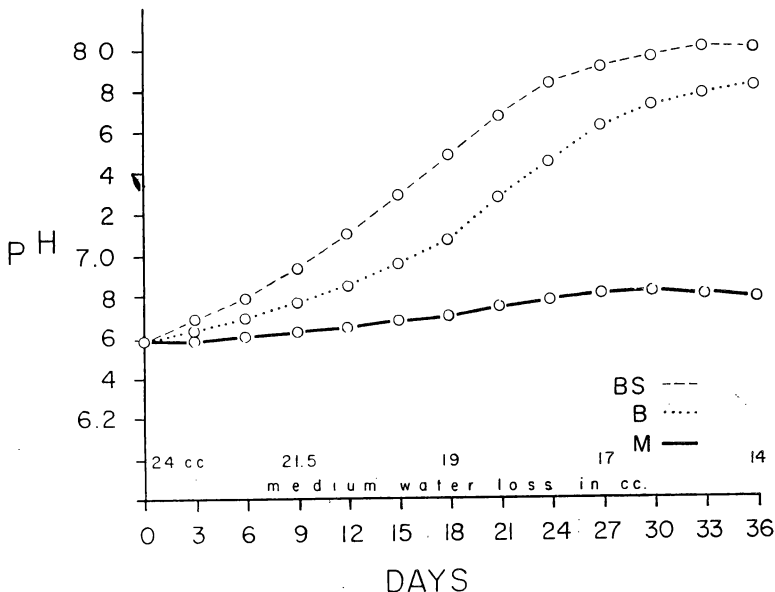


Figure 2. Correlation of days and water volume loss to the pH of the Hay filtrate-rice medium with the protozoan (BS) *Blepharisma undulans*, (B) Bacteria, and (M) the medium.

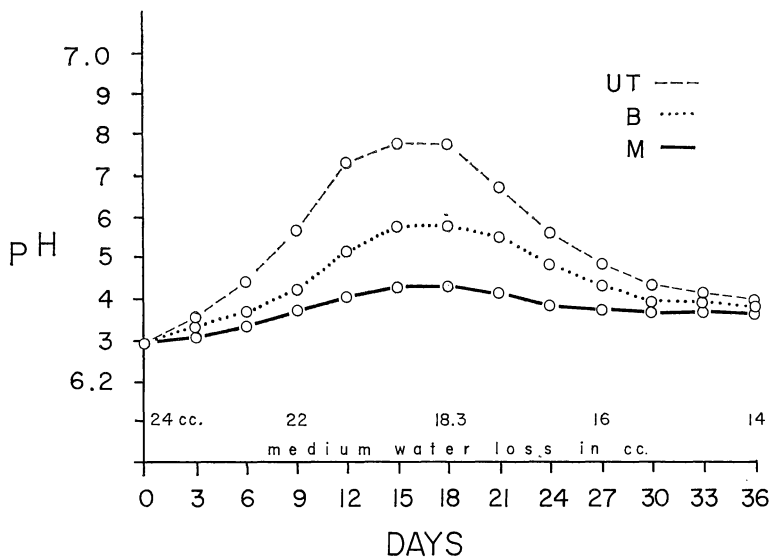


Figure 3. Correlation of days and water volume loss to the pH of the Soil filtrate-peptone tryptophane-rice medium with the protozoa (UT) *Urocentrum turbo*, (B) Bacteria, and (M) the medium.

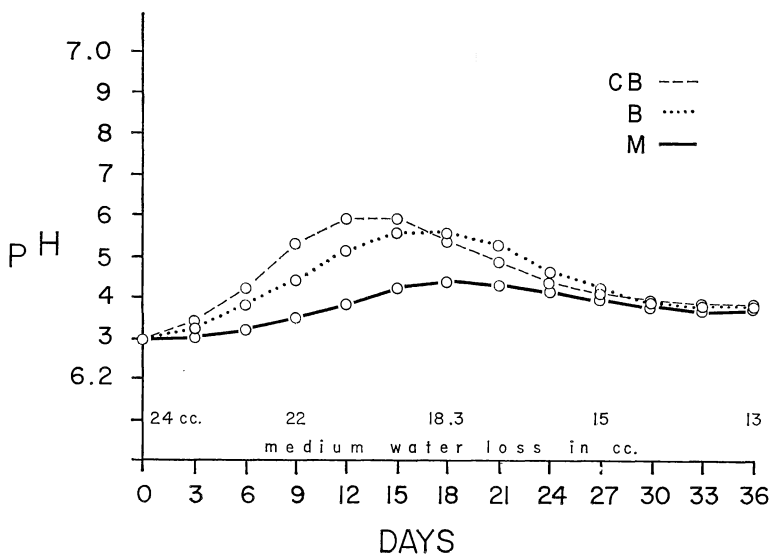


Figure 4. Correlation of days and water volume loss to the pH of the Soil filtrate-peptone tryptophane-rice medium with the protozoan (CB) *Coleps bicuspis*, (B) Bacteria, and (M) the medium.

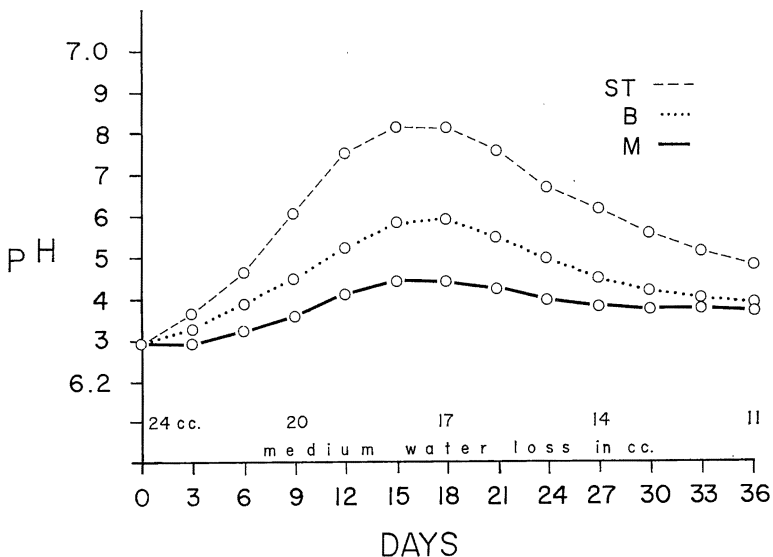


Figure 5. Correlation of days and water volume loss to the pH of the Starch agar-peptone tryptophane medium with the protozoan (ST) *Spirostomium teres*, (B) Bacteria, and (M) the medium.

Table 2 gives the results after 66 days from the beginning of the experiment. This represents data 30 days after termination of the 36-day experiment.

The component parts of the four media were tested for pH differences attributable to the nutrition. The distilled water had a pH value of 6.2 when tested separately. A rice particle boiled for 5 minutes and placed with 24 cc. of distilled water produced very little change in pH over a period of days. The alfalfa and distilled water did indicate a pH increase from 6.3 to 6.4 over a period of days. Peptone with distilled water indicated an increase above 6.3 pH but examination showed bacteria present.

DISCUSSION

The peptone tryptophane of the starch agar was adjusted to provide a medium that did not produce large numbers of protozoa, but on furnishing observable differences between the bacteria and the protozoa. In the other media the fluid content was regulated with respect to minimal nutritive substances. It was desired that excessive bacteria should not be produced as to interfere with the locomotion of the protozoa. A small number of protozoa were introduced into each of the different media at the beginning of the experiment. (See table 1.)

I will for convenience discuss each medium separately. The de-

Table 2. Results of the protozoa cultures after 68 days from the beginning of the experiment.

Protozoa	Medium water volume remaining in cc	Medium	Organisms present
<i>Spirostomum teres</i>	2.5/7*	Starch-agar peptone-tryptophane	Few <i>Spirostomum</i>
<i>Paramecium multimicronucleatum</i>	2.5/6*	" " "	Few <i>Paramecium</i>
<i>Leucophrys patella</i>	2.5/5*	" " "	Few <i>Leucophrys</i>
<i>Oikomonas termo</i>	1.7/6*	" " "	<i>Oikomonas good</i>
<i>Verticello microstoma</i>	2.5/3*	" " "	Few Teletroch and adult forms
<i>Chilomonas oblonga</i>	13	Alfalfa distilled-water rice	Good culture
<i>Colpoda steini</i>	13	" "	None
<i>Cryptomonas ovata</i>	13	" "	Few Organisms
<i>Blepharisma undulans</i>	13.5	Hay-filtrate rice	Organisms present
<i>Oxytricha fallax</i>	11.5	" "	Few organisms (large)
<i>Ochromonas mutabilis</i>	11.5	" "	Good culture
<i>Colpidium campyllum</i>	12.	" "	Organisms present

No experimental data at 68 days for the soil-filtrate peptone-tryptophane rice medium.

* The fluid of the medium above and below the agar of the medium.

scription will be based on the pH curve produced for the 36-day period at three-day intervals for the various cultures.

Alfalfa distilled-water rice medium. The following organisms were used with this particular medium: *Chilomonas oblonga*, *Cryptomonas ovata*, and *Colpoda steini*. Figure 1 illustrates the pH curve obtained for *Cryptomonas ovata* with the medium and bacteria inoculated into the medium. At the beginning of the experiment the medium had a 6.3 pH. Control tubes of this medium produced from 6.3 to 6.4 pH at the plateau. At the end of the experiment a small amount of bacteria was found in the control tubes. The bacteria inoculated into the medium produced a short lag phase and gradual acceleration of pH which reached a plateau on the eighteenth day. A pH of 6.4 was determined on the thirty-sixth day for the medium with bacteria inoculate. The protozoa *Colpoda*, *Chilomonas*, and *Cryptomonas* with the bacterially inoculated medium produced a higher plateau than did the bacterially inoculated medium alone. *Colpoda steini* and *Chilomonas oblonga* reached a peak pH at 6.7, and *Cryptomonas ovata* a peak of 6.8 at the plateau both at the eighteenth day. The lag phase of *Cryptomonas* was more abrupt and shorter than that of *Chilomonas* or *Colpoda*. *Colpoda steini* was unusual in that it had completely disappeared from the medium by the thirty-sixth day. This would suggest an early subculture with this medium.

Hay-filtrate rice medium was different from the others during the 36 days by not decreasing from the alkalinity peak or the plateau. The following organisms were tested with this medium: *Oxytricha fallax*, *Blepharisma undulans*, *Colpidium campylum*, and *Ochromonas mutabilis*. Figure 2 shows the pH curve for *Blepharisma* and bacteria with this particular medium. *Blepharisma* produced a pH curve characteristic of the other protozoa used with the hay but with slight differences. Control tubes of the medium passed through a long lag phase before air borne bacteria apparently contaminated the medium and produced a shallow curve. Bacteria inoculated into the hay-filtrate medium produced a lag phase from nine to twelve days, then accelerated to 7.8 pH at the end of the thirty-sixth day. The medium with inoculated bacteria and *Blepharisma* produced a shorter lag phase and a more pronounced acceleration curve than did the inoculated bacterial medium when tested separately. *Colpidium campylum* had a short lag phase of three days. The other protozoa with this medium compared to *Blepharisma* very favorably.

Soil-filtrate peptone-tryptophane rice medium the following protozoa were used: *Coleps bicuspis*, *Urocentrum turbo*, and *Halteria grandinella*. The control without bacteria or protozoa

had a beginning 6.3 pH with later values indicating a curve. The medium with the inoculated bacteria gave a higher curve than did the medium by itself, and had at the plateau a 6.6 pH.

The cultures of *Coleps* and *Halteria* resembled each other with a short lag phase, and reached a plateau on the twelfth to fifteenth day. *Urocentrum* had a longer lag phase with a peak at the plateau on the sixteenth day. Figure 4 illustrates the pH curve for *Coleps* with the bacterial medium. The inoculated bacteria, reached a pH peak on the eighteenth day while *Urocentrum* and bacteria reached a peak between the fifteenth and eighteenth days. The medium inoculated with bacteria and *Urocentrum* reached a plateau of almost 6.8 pH and *Coleps* and *Halteria* had a pH plateau of 6.7. Figure 3 illustrates the organism *Urocentrum* correlated to pH of the medium.

Starch-agar peptone-tryptophane medium. The following were used this medium; *Paramecium multimicronucleatum*, *Spirostomum teres*, *Oikomonas termo*, *Vorticello microstoma*, and *Leucophrys patella*. Figure 5 presents data for *Spirostomum teres*. The starting pH observed for the medium was 6.3. A slight curve was produced during the 36 days for the medium without inoculated bacteria or protozoa. Bacteria inoculated into the hay-filtrate medium produced a plateau of 6.6 pH between the fifteenth and eighteenth days. *Spirostomum*, *Paramecium*, *Leucophrys*, and *Oikomonas* indicated a pH plateau between the fifteenth and eighteenth days. *Vorticello* had a longer acceleration phase and indicated a plateau later than did the other protozoa with this medium. *Oikomonas* and *Vorticello* indicated a pH plateau of 6.9 with this medium and inoculated bacteria. The other protozoa indicated a 6.8 pH at the plateau. A pH of 6.4 or slightly higher was indicated at 36 days for *Spirostomum teres*.

SUMMARY

1. Three of the four media tested with bacteria and protozoa indicated a lag phase, acceleration, a plateau, and a decline in the pH of the media during the 36 days of this experiment. These three media were starch-agar peptone-tryptophane, soil-filtrate peptone-tryptophane rice and alfalfa distilled-water rice. The hay filtrate rice medium did not decrease from the plateau of 7.9 pH during the 36 days.

2. When tested separately: the control, the bacterial inoculated medium, and the protozoan media with bacteria showed pH differences over a period of 36 days.

3. In this experiment the pH sigmoid curve for a given medium was characteristic of that medium with different species of

protozoa tested, although variations existed in the lag phase, acceleration plateau, and the decline during 36 days.

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