THE NUTRITION OF PROTOZOA

I. A Simplified Medium for the Investigation of Unknown Factors in Blood Serum Essential for the Sustained Growth of Trichomonas vaginalis[1]

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Media devised for the cultivation of parasitic protozoa almost invariably require blood serum at the very least (Greene, 1945; Senekjie and Lewis, 1945; Ball *et al.*, 1945), if not also other blood constituents, as was supposed by earlier workers. Very little is known at present about the factors in serum necessary for the growth and maintenance of these organisms.

It has recently been demonstrated by Johnson and R. E. Trussell (1943) that the protozoan *Trichomonas vaginalis* can be grown and maintained in pure culture in a basal medium composed chiefly of Difco peptone, liver infusion, maltose, and cysteine, to which unheated blood serum must be added. Moreover, Johnson and M. H. Trussell (1945) have shown that liver infusion may be completely replaced by ascorbic acid, glutamic acid, choline chloride, folic acid, and xanthopterin. The luxuriant growth obtained with this medium in 36 to 48 hours, the low-grade pathogenicity of the organism, and the ease with which it can be handled *in vitro* are all factors which combine to render *Trichomonas vaginalis* admirably suitable as an assay organism for determining the chemical components of blood serum necessary for the growth and maintenance of a parasitic flagellate.

Before serum fractions could be tested with any degree of rapidity and assurance, however, it was necessary to devise a basal medium for *Trichomonas vaginalis* which could be made up in a minimum of time, which could be rendered complete with respect to serum or fractions prepared therefrom with a maximum of convenience, and whose composition was reasonably well defined from a chemical standpoint.

The present paper presents in detail the preparation of stock solutions and the compounding of a final medium from these stock solutions in order to obtain a culture fluid which will meet the requirements mentioned above. This medium has been used as a starting point in an attempt to devise a chemically defined medium for the sustained growth of *Trichomonas vaginalis*. Experiments dealing with fractionation of blood serum will be described in subsequent papers.

EXPERIMENTAL

Organism used. The organism used was a bacteria-free culture of *Trichomonas vaginalis* strain no. 2 which was isolated in March, 1945, from a patient with a

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severe vaginitis (Johnson, Trussell, and Jahn, 1945). Batteries of stock cultures were carried at 37 C in the revised medium described below and transferred every 48 to 72 hours. The inoculum used to assay serum fractions was prepared from a 48- to 72-hour culture which was centrifuged aseptically, washed twice, and resuspended in sterile Ringer's solution in a volume approximating three-fourths that of the original culture fluid. Then 0.03 to 0.05 ml (approximately 80,000 organisms) were introduced by pipette at the midlevel of the assay tube. Serial transfers of 0.03 to 0.05 ml of inoculum were made every 48 to 72 hours. Growth was measured by hemocytometer cell counts at the end of a 48- to 72-hour period.

Basal medium. The serum-free basal medium used was a modification of the original CPLM culture fluid devised by Johnson and Trussell (1943). In the revised medium described below, the Difco peptone was replaced by "trypticase" (Baltimore Biological Laboratories) and the liver infusion by a mixture of B vitamins, purines, and pyrimidines. In addition, acetate, asparagine, ascorbic acid, bicarbonate, and ribose were added.

Attempts to replace the Difco peptone by vitamin-free casein hydrolyzate (General Biochemicals, Inc.) fortified with cystine, tryptophane, and glycine were unsuccessful. Difco peptone, however, could be replaced by trypticase. The use of trypticase has the advantage of defining more clearly than Difco peptone the nature of the nitrogenous requirements of the organism, since trypticase is known to be a pancreatin digest of casein and contains no detectable carbohydrate.

No attempt has been made to limit the B vitamins, purines, pyrimidines, or the other pure chemical compounds added to a basis of essentiality. Rather, it was felt, the medium should be made as complete as possible with respect to *known* growth factors so as to rule these out in assays of serum fractions for the unknown substances necessary for the growth of *Trichomonas vaginalis*.

The constituents of the medium were prepared as follows:

(1) *Stock B vitamin solution.* The following B vitamins were suspended in 100 ml of 20 per cent alcohol: 20 mg of thiamine hydrochloride, 80 mg of pyridoxine hydrochloride, 20 mg of pyridoxamine hydrochloride, 20 mg of pyridoxal hydrochloride, 40 mg of calcium pantothenate, 100 mg of riboflavin, 40 mg of nicotinic acid, and 20 mg of *p*-aminobenzoic acid. The mixture was stored in the cold and shaken well before using.

(2) *TV stock solution I.* The following ingredients were dissolved in 1,000 ml of the modified Ringer's solution used in the CPLM medium (NaCl, 0.6 per cent; $NaHCO_3$, $CaCl_2$, KCl, 0.01 per cent) to give a double-strength stock solution: 2.5 g of maltose, 12.0 g of sodium acetate, 0.5 g of asparagine, 20 mg of choline chloride, 20 mg of inositol, 10 mg of ribose, 2 ml of *stock B vitamin solution*, 200 μg of biotin, and 200 μg of folic acid. This stock solution was kept in the cold under toluene.

(3) *TV stock solution II.* The following purines and pyrimidines were dissolved in 700 ml modified Ringer's solution: 50 mg of adenine SO_4, 50 mg of guanine HCl, 50 mg of xanthine, and 50 mg of uracil. One ml of 5 N NaOH was added, the suspension was shaken well, and 0.1 N NaOH was added drop by drop

until a clear solution resulted. The final volume was adjusted with Ringer's solution to 1,000 ml. This solution was likewise kept in the cold under toluene.

(4) *Sterile ascorbic acid sodium bicarbonate stock solution.* Two and five-tenths g of sodium bicarbonate were suspended in 40 ml of H_2O; 0.5 g of ascorbic acid dissolved in 10 ml of H_2O were added slowly with gentle stirring. This gave 50 ml of a 1 per cent ascorbic acid and 5 per cent sodium bicarbonate solution, which was then sterilized by Seitz filtration. This solution was prepared and added immediately before assay to prevent excessive exposure of ascorbic acid to an alkaline pH.

(5) *Methylene blue stock solution.* A stock solution of 0.5 per cent methylene blue in distilled water was used as described below to indicate the degree of anaerobiosis in the final medium. Its addition may be omitted.

TABLE 1

*Composition of complete trypticase nutrient medium for Trichomonas vaginalis**

(The amounts listed are the calculated values per 10 ml of final medium)

Component	Amount	Component	Amount
Trypticase (BBL)	200 mg	Riboflavin	8.0 μg
Sodium acetate $3H_2O$	48 mg	Thiamine HCl	1.6 μg
Cysteine HCl	15 mg	Pyridoxine HCl	6.4 μg
Maltose	10 mg	Pyridoxamine HCl	1.6 μg
Difco agar	10 mg	Pyridoxal HCl	1.6 μg
NaH CO_3†	5 mg	Ca pantothenate	3.2 μg
Asparagine	2 mg	Nicotinic acid	3.2 μg
Ascorbic acid†	1,000 μg	*p*-Aminobenzoic acid	1.6 μg
Choline chloride	80 μg	Biotin	0.8 μg
Inositol	80 μg	Folic acid	0.8 μg
Ribose	40 μg	Methylene blue (optional)	24.0 μg
Adenine SO_4	40 μg	Adjusted to pH 6.0, and Ringer's solution added to make:	8.0 ml
Guanine HCl	40 μg	Human blood serum diluted with equal volume of Ringer's solution†	2.0 ml
Xanthine	40 μg	Final volume	10.0 ml
Uracil	40 μg		

* To render the medium basal, serum is omitted.

† Added as sterile solutions after the medium was autoclaved.

To prepare 500 ml of the trypticase basal medium (so called in contrast to the CPLM medium), the following mixture was made: 250 ml of TV stock solution I, 50 ml of TV stock solution II, and 625 mg of Difco agar were brought to a boil in a 400-ml beaker heated over a steam bath. Heating was continued until the agar was completely dissolved. The solution was filtered while hot through porous Reeve-Angel filter paper. To the warm filtered mixture there were now added 0.3 ml of 0.5 per cent methylene blue, 12.5 g of trypticase (BBL), and 935 mg of cysteine hydrochloride. The mixture was stirred well, and mild heat was applied until a perfectly clear solution resulted. After being cooled to room temperature the mixture was adjusted to pH 6.0 with 1 N HCl or 1 N NaOH and checked by the glass electrode. Finally the solution was diluted to 500 ml with the modified Ringer's fluid, tubed in 8-ml lots, autoclaved, and allowed to cool.

Then 0.1 ml of the sterile ascorbic acid sodium bicarbonate solution was added aseptically to each tube. Tubes thus prepared may be stored at room temperature for a period of 2 weeks without any deterioration of the medium.

To render the medium complete, blood serum was diluted with an equal volume of Ringer's solution and passed through a sterile filter (Berkefeld or Seitz). Two ml of this sterile diluted serum were then added aseptically to each tube containing 8 ml of the basal Trypticase medium, giving a final volume of 10 ml.

The composition of the final complete trypticase nutrient medium for the cultivation of *Trichomonas vaginalis* is given in table 1.

Assay of serum fractions may now be accomplished by adding in place of the intact serum any one given fraction or combination of fractions to a tube containing 8 ml of the trypticase basal medium. As much as 2 ml of the material to be tested may be used. Whenever volumes less than 2 ml are employed, Ringer's solution must be added to compensate for the difference. The final volume of each tube should always be 10 ml to permit a correct cell count comparison with intact serum controls.

TABLE 2

Growth of Trichomonas vaginalis in trypticase medium compared with that in CPLM medium

NUMBER OF SERIAL TRANSFER	GROWTH OF TRICHOMONAS VAGINALIS EXPRESSED IN NUMBER OF CELLS PER MM³ AFTER 48 HOURS IN EACH SERIAL TRANSFER*	
	8 ml of trypticase basal + 2 ml of diluted serum†	8 ml of CPLM basal + 2 ml of diluted serum†
First culture	1,180	2,275
Serial transfer no. 1	1,190	2,500
2	1,550	2,185
3	1,170	1,055
4	915	1,170
5	1,565	2,450
6	1,040	2,200
7	1,135	2,500
8	1,565	3,360
9	1,655	2,275
10	1,580	1,450
11	1,585	1,140

* Each value is the average of duplicate determinations.

† The serum was diluted with an equal volume of Ringer's solution before assay.

RESULTS AND COMMENTS

The ability of this new trypticase medium to maintain sustained growth of *Trichomonas vaginalis* as compared with the original CPLM culture fluid is evident from table 2.

Growth through 11 successive subcultures is presented in table 2. To date, over 60 serial transfers have been made, and sustained growth has been well maintained. Although the number of cells present after 48 hours in the trypticase

medium was appreciably less than in the CPLM culture fluid, the organisms were more uniform in shape and larger in size, and possessed a greater degree of motility. The higher cell count obtained with the CPLM medium was due to the presence of stimulatory factors in the liver infusion used in that preparation. The same effect could be obtained with the trypticase medium if the B vitamins, purines, and pyrimidines were replaced by 0.15 per cent of Wilson's liver fraction L. In one experiment in which this was done, an average count of duplicate determinations came to 2,885 cells per mm^3 on the seventh serial transfer.

Attempts to omit agar from the basal medium have resulted in irregular growth. Whether the stimulatory effects of agar are mechanical or chemical is still being investigated.

The cell count method of measuring growth is time-consuming and tedious. Some attempts have been made to measure growth by a turbidimetric procedure involving the use of the Evelyn photoelectric colorimeter. It is possible that this technique may become practicable in future investigations.

SUMMARY

The original basal CPLM medium of Johnson and Trussell has been modified to make it suitable for the assay of unknown growth factors in blood serum essential for the sustained growth of *Trichomonas vaginalis* in pure culture. The Difco peptone was replaced by trypticase (BBL) and the liver infusion by a mixture of B vitamins, purines, and pyrimidines. In addition, acetate, asparagine, ascorbic acid, bicarbonate, and ribose were added. This medium has the advantage of being better defined chemically than the CPLM medium, and has been devised for convenient preparation from stock solutions in a minimum of time.

Although growth, measured by the number of cells per cubic millimeter, was appreciably less in 48 hours in the trypticase culture fluid than in the CPLM medium, nevertheless sustained growth in the trypticase medium has been maintained through 60 serial transfers. Furthermore, the organisms in the trypticase nutrient were more uniform in shape and larger in size, and possessed a greater degree of motility.

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