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Nutritional Requirements of *Trichomonas vaginalis.**

ALFRED B. KUPFERBERG, GARTH JOHNSON, AND HERBERT SPRINCE.

From the Division of Microbiology, Ortho Research Foundation, Raritan, N.J.

Very little is known about the growth factor requirements of *Trichomonas vaginalis*. In an introduction to the study of growth factor effects Johnson[1] observed that ascorbic acid, glutamic acid and choline had a stimulating effect upon cell multiplication, and that where nicotinamide, folic acid and approximately 10% human serum were employed liver infusion was satisfactorily replaced. Sprince and Kupferberg[2] reported a medium which could be used as a starting point for the development of a chemically defined culture fluid for the sustained growth of *Trichomonas vaginalis*. A highly complex medium was devised with numerous growth factors included. In these two studies materials of natural origin were added to the media in such concentrations as to mask the essentiality of added *known* growth factors. It is the purpose of the present study to demonstrate that by reducing the serum and trypticase content of the medium of Sprince and Kupferberg a requirement for pantothenic acid and for phosphate was established for *Trichomonas vaginalis*. It was thought that an effort to simplify the above culture medium might reveal a requirement for certain of the component growth factors. Furthermore, the need for a medium to be used for routine maintenance of cultures as well as for testing the antitrichomonas activity of compounds made a cheaper medium desirable. The present study reports the effects of withdrawal of the above growth accessory substances upon cell multiplication of bacteria-free *Trichomonas vaginalis*.

Methods. Strain No. 2 of bacteria-free *Trichomonas vaginalis* was used as the test organism. The basal medium consisted of the *basal* trypticase medium previously reported by Sprince and Kupferberg.[2] The inoculum was prepared by washing cells grown for 48 hours in the trypticase medium. This was accomplished by centrifuging and resuspending in sterile Ringer's solution 4 times. The population was not adjusted. The inoculum consisted of 0.03 ml of the washed suspension. Five tubes were employed for each experimental series except where otherwise indicated. The contents of the tubes were agitated with the tip of the pipette in order to disperse the serum and inoculum. Subcultures were made at 48 hour intervals. Populations were determined by hemocytometer count in the second transplant following the first culture. This medium contained 2 ingredients of natural origin, serum and trypticase. An effort was first made to determine the minimal amounts of serum required for sustained culture in order that sensitivity to withdrawal of the known vitamins might be determined.

Effect of reduction of serum concentration. In order to increase the accuracy of measurement the serum was first diluted to various concentrations in 100 ml of modified Ringer's solution containing 0.6% NaCl and 0.01% $NaHCO_3$, KCl and $CaCl_2$. The diluted serum was then added in 2 ml volumes after Seitz filtration. The effects of graded amounts of human serum upon cell multiplication are shown in Table I. Similar results were obtained in a duplicate series. Since the cell counts in cultures containing 0.05 ml of serum were more uniform than those in which the minimal 0.03 ml was used, it was decided to employ 0.05 ml in the experiments to follow.

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1 Johnson, J. G., *J. Parasitol.*, 1947, **33**, 189.

2 Sprince, H., and Kupferberg, A. B., *J. Bact.*, 1947, **53**, 435.

TABLE I.
Effect of Graded Amounts of Serum on Cell Multiplication of *Trichomonas vaginalis.*

Ml undiluted serum per 10 ml final medium	Population in second transplant at 48 hr (cells/mm³)* Cultures				
	1	2	3	4	5
.01	0	0	0	0	0
.03	31	57	101	112	88
.05	470	410	240	450	440
.07	1310	1240	700	1190	760
.10	1910	1440	1860	2090	2190
1.0	2510	2470	2310	2270	2310

* Similar results were obtained in a duplicate series.

TABLE II.
Effect of Omission of Vitamins in Groups.

	Population of *Trichomonas vaginalis* in second transplant at 48 hr (cells/mm³)* Cultures				
	1	2	3	4	5
Control	350	190	280	360	280
" minus Group A	0	0	0	0	0
" " " B	390	460	190	400	400
" " " C	440	450	440	480	400

* Similar results were obtained in a duplicate series.

The effect of withdrawal of known growth factors. These materials were first deleted in groups. Where populations were affected by the withdrawal of a group of compounds, the effect of withdrawal of individual compounds was then explored.

Results. Preliminary studies showed that thiamine HCl, p-aminobenzoic acid, pyridoxine HCl, pyridoxamine HCl, pyridoxal HCl and inositol were not required for sustained growth in the presence of trypticase and serum. The withdrawal of sodium acetate, sodium bicarbonate, asparagine, ribose, adenine sulfate, guanine HCl, xanthine, and uracil was also without effect upon the growth of *Trichomonas vaginalis.* These materials were therefore deleted in the following study of essentiality of vitamins. In the experimental study upon withdrawal of 3 groups of vitamins, however, a specific vitamin requirement was uncovered. The compounds in this study were grouped as follows with the amount of each given in μg per 10 ml of final medium: A. Choline 80 μg, riboflavin 8.0 μg, calcium pantothenate 3.2 μg, nicotinic acid 3.2 μg; B. Biotin 0.8 μg, folic acid 0.8 μg; C. Ascorbic acid 1000 μg. The control medium contained groups A, B, and C plus trypticase 2% and serum 0.5%. Agar, maltose, cysteine HCl, and Ringer's were present in the same concentrations as in the original trypticase medium. These groups were then withdrawn from the medium separately. The results obtained are shown in Table II.

It is demonstrated in Table II that one or more vitamins in Group A are essential. Growth equal to that of the control was obtained where Groups B and C were omitted from the medium. The vitamins in Group A, namely: choline chloride, riboflavin, calcium pantothenate and nicotinic acid were then withdrawn singly. The effect of single omissions of these 4 vitamins may be noted in Table III. From these data we may conclude that pantothenic acid is an essential metabolite of *T. vaginalis.* In Table IV it is also shown that the effect of pantothenate is not dependent upon the presence of added vitamins.

It has been demonstrated by Denko *et al.*[3]

[3] Denko, W. D., Grundy, W. E., and Porter, J. W., *Arch. Biochem.*, 1947, **13**, 481.

TABLE III.
Effect of Omission of Single Vitamins.

Vitamins omitted from control	Population of *Trichomonas vaginalis* in second transplant at 48 hr (cells/mm³)* Cultures				
	1	2	3	4	5
None (control)†	710	570	540	950	720
Choline	620	620	940	550	660
Riboflavin	630	610	900	660	690
Nicotinic acid	630	880	540	670	870
Ca pantothenate	1	2	0	0	0

* Similar results were obtained in a duplicate series.
† Same as control in Table II except Groups B and C omitted.

TABLE IV.
Effect of Increase in Serum and Omission of All Known Vitamins.

Simplified medium†	Population of *Trichomonas vaginalis* in second transplant at 48 hr (cells/mm³)* Cultures				
	1	2	3	4	5
0.5% Serum	0	0	0	0	0
Ca Pant. and 0.5% Serum	780	530	640	620	580
Minus Ca Pant., plus 5% Serum	2150	1950	1890	1920	2040

* Similar results were obtained in a duplicate series.
† Unessential ingredients omitted from medium.

TABLE V.
Effect of Graded Amounts of Trypticase on Cell Multiplication of *Trichomonas vaginalis*.

% Trypticase in final medium	Population in second transplant at 48 hr (cells/mm³) Cultures			
	1	2	3	4
.008	10	9	7	14
.04	64	70	62	64
.08	151	108	111	165
.16	230	300	350	240
.24	450	420	410	420
.48	940	540	870	810
.72	1210	1300	1020	990
.96	1500	1600	1560	1500
1.20	1830	1640	1760	1620
1.36	1750	1810	1830	1460
1.60	1970	1750	1860	1680
2.00	2050	1920	1970	1750

that normal whole blood has on the average 33 μg of pantothenic acid per 100 ml. The effect of increased amounts of blood serum upon the requirement for added pantothenate was therefore explored. The medium was prepared minus the addition of all known vitamins and the serum content was increased from 0.5% to 5.0%. The results are indicated in Table IV.

As is shown in Table IV no need exists for Ca pantothenate when 5% human serum is employed in the medium in the presence of 2% trypticase (BBL).

Effect of reduction of trypticase concentration. Using the *basal* medium of Sprince and Kupferberg[2] the trypticase content was varied between 0.008 and 2.0% in the presence of 5% serum. The effect of these reductions is shown in Table V.

As can be seen from Table V, 0.24% of trypticase gave a uniform cell count. In several duplications of this experiment, trypticase concentrations below this value produced widely varying populations in the quadru-

TABLE VI.
Effect of Simultaneous Reduction of Serum and Trypticase Concentrations.

Conc. % final vol. Trypticase	Serum	Population cells per mm³ Culture 1	Transfer 1	Transfer 2	Transfer 3	Transfer 4
2	5	1980	2100	2300	1980	1870
2	0.5	680	700	610	690	690
0.24	5	560	440	480	440	470
0.24	0.5	0	0	0	0	0

TABLE VII.
Effect of Addition of Phosphate to Medium Containing Reduced Serum and Trypticase.

	Population cells per mm³ Culture 1	Transfer 1	Transfer 2	Transfer 3	Transfer 4
Serum 0.5%— Trypticase 2%	970	760	930	860	880
Serum 0.5%— Trypticase 0.24%	0	0	0	0	0
Serum 0.5%— Trypticase 0.24% KH_2PO_4 0.027%	276	304	315	260	270

plicate cultures with aberrations in shape and motility. For these reasons 0.24% trypticase was selected for use in further investigations.

Effect of simultaneous reduction of serum and trypticase concentrations. From the foregoing data independent reduction of serum to 0.5% and of trypticase to 0.24% is shown to produce low but consistent populations. The effect of simultaneous reduction of serum and trypticase to these low values is shown in Table VI.

The above data reveal that this medium which contains a full complement of added growth factors is deficient in some respects when serum is reduced to 0.5% and trypticase to 0.24%. It was then supplemented with a variety of mineral salts in order to determine whether this deficiency was due to a lack of an essential salt. Table VII shows the effect of the addition of phosphates.

The data in Table VII demonstrate a requirement for phosphate. Substitution of $NH_4H_2PO_4$ gave results comparable to those produced by the salts of potassium. The addition of $MgSO_4$, $FeSO_4$, $MnSO_4$ and $MnCl_2$ was without effect. The optimal concentration of phosphate was found at 0.027%. At this phosphate level the population of trichomonads was approximately 30% of that obtained with 2% trypticase.

Reduction of components in routine culture medium. From the data gathered in the foregoing study it is obvious that in a medium containing adequate serum and trypticase, added growth factors are not required. The trypticase also supplies sufficient phosphate. In view of this fact, the need for the salts included in the Ringer's solution was investigated. Deletion of the Ringer's solution produced no change in the ability of the medium to support optimal populations in serial cultures.

Composition and Population of STS medium. The elimination of all non-essential components left a Simplified Trypticase Serum medium having the following composition per 1000 ml of final medium: Trypticase (BBL) 20 g, cysteine HCl 1.5 g, maltose 1.0 g, Difco agar 1.0 g, distilled water to make 950 ml. The medium was adjusted to pH 6.0, with 1N HCl or 1N NaOH, heated in a boiling water bath until the agar was completely dissolved. The solution was filtered while hot through porous Reeve-Angel filter paper No. 845. To the warm filtered mixture was now added 0.6 ml of

0.5% methylene blue to serve as an indicator. The use of an indicator is optional. After being cooled to 46°C the mixture was readjusted, if necessary, to pH 6.0 using the Beckman pH meter. The solution was then brought back to 950 ml with distilled water, tubed in 9.5 ml volumes, autoclaved at 15 lb pressure for 15 minutes, and allowed to cool. To render the medium complete 0.5 ml of sterile, undiluted human serum was added to each tube giving a final volume of 10 ml.

Populations were counted through 11 serial cultures and compared with those in the original medium of Sprince and Kupferberg. It was found that the simplified medium supported populations equal to those in the original medium. Over 50 serial transfers have been maintained to date without visible differences in shape, size and degree of motility of the protozoa. Examination of living specimens by phase microscopy and stain techniques failed to show any difference in morphology. Three strains of *T. foetus*† and one strain of *T. gallinae* have been carried successfully in this medium at this laboratory. The former species requires adjustment of the medium to pH 7. It has recently been observed that all 3 species are satisfactorily supported by Baltimore Biological Laboratory Thioglycollate medium with dextrose and Eh indicator. This medium contained phytone and trypticase. It requires adjustment to pH 6 for *T. vaginalis* and *T. gallinae*. All 3 species require the addition of serum.

Conclusions. 1. Pantothenic acid has been demonstrated to be an essential metabolite for *Trichomonas vaginalis*. 2. A requirement for phosphate has been established. 3. A culture medium containing a greatly reduced number of components has been developed for routine use.

† Received from Dr. B. B. Morgan, University of Wisconsin.

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Turnover Rate of Phospholipid Phosphorus in the Liver of the White Rat.

JESSE L. BOLLMAN, EUNICE V. FLOCK, AND JOSEPH BERKSON.

From the Division of Experimental Medicine, Mayo Foundation, and the Division of Biometry and Medical Statistics, Mayo Clinic, Rochester, Minn.

The purpose of the experiments reported here is to provide a quantitative estimate of the turnover rate of phospholipid phosphorus in the liver of the white rat under normal conditions of equilibrium, that is, while the actual concentration of the phospholipid phosphorus in the liver is not changing.

The concept of "turnover" is one of dynamic equilibrium in which, during an interval of time *dt*, a number of phosphorus atoms of mass *dm'* "enter" the hepatic phospholipid molecules, and the same number "leave," so that the net mass of phospholipid phosphorus remains unchanged. The rates concerned are defined as follows:

$$r = \frac{dm'}{dt} \qquad (1)$$

$$R = \frac{r}{m} = \frac{dm'}{mdt} \qquad (2)$$

where r is the mass turnover rate; R is the proportional turnover rate; dm' is the mass of phospholipid P which is "turned over" in time dt; m is the mass of phospholipid phosphorus.

The method used for calculation of the rates is based on the formulation of Zilversmit, Entenman and Fishler.[1] These authors

[1] Zilversmit, D. B., Entenman, C., and Fishler, M. C., *J. Gen. Physiol.*, 1943, **26**, 325.