THE NUTRITION OF PROTOZOA

II. The Separation of Human Blood Serum into Two Fractions, Both Essential for the Sustained Growth of Trichomonas vaginalis[1]

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In the preceding paper (Sprince and Kupferberg, 1946) it was pointed out that very little is known about the factors in blood serum necessary for the sustained growth of parasitic flagellates. The advantages of using *Trichomonas vaginalis* as an assay organism in investigating such factors were discussed. A detailed description of a basal medium, which was well defined chemically and was devised primarily for rapid and convenient assay of such factors in serum, was presented.

It is the purpose of this paper to report that human blood serum can be separated into two fractions— (1) an ether-soluble fraction, and (2) the remaining ether-insoluble residue—both of which are necessary for the sustained growth of *Trichomonas vaginalis*.

EXPERIMENTAL

Organism used. The organism used was a pure culture of *Trichomonas vaginalis* strain no. 2. The history of this organism, the manner in which it has been carried, and the preparation of the inoculum prior to assay have been described in the first paper of this series.

Basal medium. The trypticase basal medium used below and the assay procedure followed have been described in detail in the same publication.

Fractionation of serum. Serum fractions were prepared as follows: 100 ml of human blood serum[2] (pooled from negative Wassermann tests) were adjusted to pH 7.1 to 7.3 with several drops of either N HCl or N NaOH and checked with the glass electrode. The serum was then extracted with 200 ml of ethyl ether in a separatory funnel at room temperature. Eight to twelve extractions were made in this manner, the number being determined by lack of further color extractable by the ether. The pH was readjusted after each extraction to 7.1 to 7.3, and the volume of the aqueous phase was kept at approximately 100 ml to 125 ml.

[1] We wish to thank Dr. Jordi Folch-Pi of the Rockefeller Institute Hospital, New York City, for a sample of his plasma lipid fraction. For small quantities of human serum albumin, we are indebted to Dr. John Edsall of the Department of Physical Chemistry, Harvard Medical School. Human blood serum from negative Wassermann tests was made available to us through the kindness of Mr. J. H. Spooner of the State Department of Health, Trenton, New Jersey.

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[2] The serum was heated to 55 C for 15 minutes, as is usually done in routine Wassermann tests to destroy the natural complement.

The ether extracts were pooled, and one-fourth of the total volume (≈ 25 ml intact serum) was evaporated to dryness *in vacuo*. Five ml of distilled water were added and evaporated to dryness twice. Finally 22 ml of distilled water were added. The mixture was now adjusted to pH 7.1 to 7.3 and shaken vigorously for several hours. The emulsion formed was readjusted to pH 7.1 to 7.3, brought to 25-ml volume with distilled water (thereby equating it to serum in concentration), and sterilized by being filtered through a sterile Seitz or fritted glass filter (Corning GYVKU). Use of the Corning glass filter necessitated constant stirring with a rubber policeman. The remaining ether extract was kept in the cold room for future use.

The ether-insoluble phase was reduced by evaporation *in vacuo* at a temperature under 45 C to a volume at least 10 ml beyond the point of detectable ether odor. Distilled water was then added to give a volume of 100 ml (to bring to serum concentration), and the solution was sterilized by filtration as described above.

Several points in this separation procedure should be noted. Prolonged, vigorous shaking of the separatory funnel should be avoided, since a third layer may form between the ether and aqueous phases that consists of a viscous mixture of ether, water, and serum material. This often persisted even after standing overnight. In every case in which such a layer formed, it was regarded as belonging to the ether-insoluble phase and separations were made accordingly. Obviously, the addition of a sizable portion of third layer during the separation procedure to the ether-insoluble phase may introduce enough of the ether-soluble phase to render the former phase active per se when it is subjected to assay. Great care should be taken to avoid this.

At best, the sterile filtrations were long and tedious. Occasionally, a complete block of the filter would be encountered. In such cases, filtrations were effected by making up the fraction in question as a 5 per cent alcohol solution. For assay purposes, all serum fractions must be homogeneous, and must remain so when mixed with the basal medium.

Ether fractionation of serum below freezing temperatures has been reported to result in excellent extraction of lipoidal material (McFarlane, 1942). For our purposes, however, such attempts proved disappointing. Fractions were obtained which did not emulsify satisfactorily with water, and consequently did not lend themselves to subsequent sterile filtration. In this connection, see Cohn *et al.* (1946, footnotes 25 and 72). It is possible, however, that by the use of proper protective agents, stable emulsions may be obtained.

The preparation of serum fractions for assay. It will be noted that the sterile serum fractions were made up in volumes equivalent to their original concentration in serum. As indicated in the preceding paper (Sprince and Kupferberg, 1946), serum controls were always diluted with an equal volume of Ringer's solution and sterilized by filtration prior to assay. Consequently, all serum fractions equal to serum in concentration were likewise diluted with an equal volume of Ringer's solution and filtered sterilely before assay. This was done to permit the comparison of the potency of a given fraction in replacing serum.

Data for a typical experiment in which serum has been replaced by fractions prepared therefrom are given in table 1.

From table 1 it is evident that neither the aqueous emulsion of the ether extract nor the ether-insoluble phase *alone* supported growth. Even doubling the volume of the separate fractions added to the basal medium gave no growth response. Addition of the two components together resulted in sustained growth through 5 serial transfers in numbers comparable to 5 serial transfers with intact serum. The results presented above have been repeated with 5 different lots of pooled serum.

The ether-soluble phase. Attempts have been made to determine the nature of the active material in the ether-soluble phase.

TABLE 1

In vitro cultivation of Trichomonas vaginalis in fractions prepared from human blood serum

ML OF DILUTED SERUM CONTROL* OR DILUTED SERUM FRACTION* ADDED TO 8 ML OF TRYPTICASE BASAL MEDIUM TO GIVE A TOTAL VOLUME OF 10 ML PER TUBE	GROWTH OF TRICHOMONAS VAGINALIS EXPRESSED IN NUMBER OF CELLS PER MM³ AFTER 48 HOURS IN EACH SERIAL TRANSFER†					
	First culture	Serial transfer 1	Serial transfer 2	Serial transfer 3	Serial transfer 4	Serial transfer 5
Control (2 ml of diluted intact serum)	1,550	1,040	740	1,895	1,305	1,510
1.0 ml diluted aqueous emulsion of ether extract + 1.0 ml sterile Ringer's	0					
1.0 ml diluted ether-insoluble phase + 1.0 ml sterile Ringer's	990	540	0			
1.0 ml diluted aqueous emulsion of ether extract + 1.0 ml diluted ether-insoluble phase	1,080	685	1,205	2,335	1,755	1,145

* All samples of intact serum or serum fractions were diluted with an equal volume of Ringer's solution, as indicated in a preceding paragraph.

† Each value is the average of duplicate determinations.

(1) *The effect of heat.* One ml of diluted aqueous emulsion of ether extract (= 0.5 ml serum) was added to each of duplicate tubes containing trypticase basal medium before autoclaving. The tubes were then autoclaved and cooled, and 1.0 ml of the sterile diluted ether-insoluble phase was introduced. Sterile ascorbic acid sodium bicarbonate solution was now added as usual, and the tubes were assayed.

(2) *Dialysis of aqueous emulsion of ether extract.* Ten ml of undiluted aqueous emulsion of ether extract (= serum concentration, and pH adjusted to 5.8) was dialyzed against six 100-ml portions of distilled water over a period of 1 week at 4 C (to prevent microbial contamination). The dialyzate was then evaporated to dryness *in vacuo*, and the residue was made up to the original volume (10 ml) with distilled water and adjusted to pH 6.8. The homogeneous solution was

then diluted with 10 ml of Ringer's solution and sterilized by filtration. One-ml samples of this material were now introduced into duplicate tubes containing 8.0 ml sterile trypticase basal medium to which there had already been added 1.0 ml of sterile diluted ether-insoluble phase plus sterile ascorbic acid bicarbonate solution. Assays proceeded in the usual manner.

(3) *Replacement experiments.* The following amounts of representative fat-soluble compounds of physiological significance were dissolved in 50 to 100 ml of ether: 60 mg of lecithin, 30 mg of cholesterol, 30 mg of oleic acid, 30 mg of linoleic acid, 30 mg of ergosterol, 3 mg of α-estradiol, 300 μg of α-tocopherol, 60 μg of β-carotene, and 60 μg of vitamin A. The ether solution was evaporated to dryness, and the residue was made up in distilled water, adjusted to pH 7.2,

TABLE 2

The nature of the material in the ether-soluble phase essential for the sustained growth of Trichomonas vaginalis

FRACTION REPRESENTING ETHER-SOLUBLE PHASE ADDED*	GROWTH OF TRICHOMONAS VAGINALIS EXPRESSED IN NUMBER OF CELLS PER MM³ AFTER 48 HOURS IN EACH SERIAL TRANSFER†					
	First culture	Serial transfer 1	Serial transfer 2	Serial transfer 3	Serial transfer 4	Serial transfer 5
None (intact serum, control)..	1,040	1,140	1,565	1,655	1,580	1,585
None (aqueous ether-insoluble phase, control).....	990	0				
Autoclaved aqueous emulsion of ether extract.....	1,660	1,555	1,985	1,110	665	1,005
Dialyzate of aqueous emulsion of ether extract.....	2,075	1,340	1,895	1,695	1,600	1,640
Replacement mixture of pure compounds (described above).....	710	1,135	450	950	1,560	1,120

* All fractions equivalent to serum in concentration were diluted with an equal volume of Ringer's solution. One ml of the diluted material was then assayed in test tubes containing 8.0 ml of trypticase basal medium plus 1.0 ml of diluted ether-insoluble phase.

† Each value is the average of duplicate determinations.

and diluted to 30.0 ml. After dilution with Ringer's solution to 60 ml and sterile filtration, assays with 1.0-ml samples were undertaken as described previously.

The results obtained in these studies of the nature of the active material in the ether extract are summarized in table 2.

It is readily apparent that the active material in the ether-soluble phase is heat-stable and dialyzable, and can be replaced rather effectively by a homogenized aqueous emulsion of the mixture of compounds described above. A plasma lipid fraction prepared by Doctor Jordi Folch-Pi of the Rockefeller Institute Hospital, New York City, also proved to be quite active.

Preliminary experiments have indicated that the active constituent in the mixture is linoleic acid. In one instance in which linoleic acid (Eimer and Amend) was used in place of the ether-soluble phase, an average count of duplicate de-

terminations on the fifth serial transfer came to 955 cells per mm^3 as compared with 1,420 cells per mm^3 with intact serum controls. As will be seen below, present indications point to the passage of other stimulatory factors, in addition to linoleic acid or the mixture described above, into the ether extract during the separation procedure.

The ether-insoluble phase. Experiments were performed in which the ether-insoluble phase was replaced by a solution of human serum albumin.

A 5 per cent solution of human serum albumin (approximate concentration in human serum) was made up in distilled water and diluted with an equal volume of Ringer's solution for assay purposes. In each experiment described below, 1.0-ml samples of this preparation were assayed in tubes containing 8 ml of the usual trypticase medium to which 1 ml of a properly diluted fraction representing the ether-soluble phase was added.

TABLE 3

Replacement of ether-insoluble phase by human serum albumin

FRACTION REPRESENTING ETHER-SOLUBLE PHASE ADDED*	GROWTH OF TRICHOMONAS VAGINALIS EXPRESSED IN NUMBER OF CELLS PER MM^3 AFTER 48 HOURS IN EACH SERIAL TRANSFER†					
	First culture	Serial transfer 1	Serial transfer 2	Serial transfer 3	Serial transfer 4	Serial transfer 5
None (intact serum, control)..	1,180	1,170	1,170	915	1,565	1,655
None (human serum albumin, control).....	900	0				
Aqueous emulsion of ether extract.....	355	460	615	450	395	335
Replacement mixture of pure compounds (described above).....	570	0				

* All fractions equivalent to serum in concentration were diluted with an equal volume of Ringer's solution. One ml of the diluted material was then assayed in test tubes containing 8.0 ml of trypticase basal medium plus 1.0 ml of diluted human serum albumin.

† Each value is the average of duplicate determinations.

Table 3 presents data to demonstrate that the ether-insoluble phase can be replaced to some extent by human serum albumin if the ether extract component of serum is present in the medium. Sustained growth could be maintained under these conditions, but the resulting cell count was considerably less. Increasing the concentration of the ether extract and of the human serum albumin twofold did not effect an increased cell count. Evidently stimulatory factors normally present in the ether-insoluble fraction were lacking under these circumstances.

Attempts to replace the ether extract of serum with the mixture of pure compounds (lecithin, cholesterol, oleic acid, linoleic acid, ergosterol, α-estradiol, α-tocopherol, β-carotene, and vitamin A) in a medium containing human serum albumin in lieu of the aqueous ether-insoluble phase were unsuccessful. This would indicate that substances other than these compounds (including linoleic acid) were present in the ether extract of serum which contributed to its activity.

DISCUSSION

The separation of blood serum into two components, (1) ether-soluble and (2) ether-insoluble, both of which are necessary for the growth of a parasitic flagellate, is an observation which is of interest in light of previous investigations. In contrast to the findings of Cailleau for *Trichomonas columbae* (1936), cholesterol was not the active factor in the ether-soluble extract. Moreover, in confirmation of Kupferberg and Johnson (1941), estradiol was also found to be inactive. On the other hand, it should be noted that a number of fatty acids have been reported as growth accelerants for flagellates (Lwoff and Provasoli, 1937; Provasoli, 1937). In view of these findings and our own observation with linoleic acid, a reinvestigation of both saturated and unsaturated fatty acids as possible growth factors for parasitic flagellates might be in order.

The recent findings of Senekjie and Lewis (1945) are of particular interest. These investigators were able to demonstrate the existence of a factor in blood serum essential for the cultivation of leishmanias and trypanosomes. This active principle was dialyzable but not directly associated with albumin, euglobulin, pseudoglobulin, or any combination of these fractions. It is interesting to note that our factor in the ether extract of serum active for *Trichomonas vaginalis* was also dialyzable. It differed from the Senekjie-Lewis factor in being stable to autoclaving, whereas the latter factor survived heating at 70 C for 30 minutes only partially and was completely destroyed at 100 C. Nevertheless, the possibility that the active factor (or factors) present in the ether-soluble extract of our experiments is identical with that of Senekjie and Lewis should be considered.

SUMMARY

Human blood serum has been separated by ether extraction into two fractions, both of which are necessary for the sustained growth of *Trichomonas vaginalis* in trypticase basal medium. The fractions are (1) an ether-soluble fraction and (2) the aqueous ether-insoluble residue remaining after the extraction. Neither fraction alone supports growth, but addition of both to the basal culture fluid provides a complete nutrient medium. Preliminary experiments indicate that one of the active components of the ether-soluble fraction is linoleic acid (Eimer and Amend, pure). Serum albumin is one of the active components of the aqueous ether-insoluble phase. Factors necessary to effect optimal growth of *Trichomonas vaginalis* exist in both fractions, but the nature of these is, at present, unknown.

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