THE ISOLATION FROM BEEF SERUM OF A SURVIVAL FACTOR FOR TREPONEMA PALLIDUM*

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The problem of the cultivation in vitro of virulent Treponema pallidum has been approached in this laboratory by investigations of the physical and chemical factors which influence the survival of this organism in artificial media.

A previous report (1) described techniques which permitted the isolation of T. pallidum in a relatively tissue-free state from rabbit testicular syphilomas. These organisms survived in vitro and retained virulence for a period of several days. As a medium consisting of known constituents was used for the extraction of the testes, the only unknown substances available to the organisms were those derived from the testicular tissue. It was found that this tissue extract was necessary for the survival of T. pallidum, since the organisms when centrifuged and resuspended in the basal medium would survive for only 12 to 24 hours. After the addition of either normal rabbit or bull testicular tissue extracts or ultrafiltrate of beef serum2 to the medium used for resuspending T. pallidum survival was supported for 5 to 8 days. Similar effects of serum ultrafiltrate have been reported by Eagle and Steinman (2).

It was found that mixtures of the known vitamins, glucose and other sugars, amino acids, and various purines and pyrimidines would not substitute for the testicular extract or the serum ultrafiltrate and hence it appeared likely that the effect of the ultrafiltrate of serum or the testicular extract was due to a hitherto unrecognized component of blood or tissue extracts.

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1 The basal medium contained 0.00035 M crystalline bovine albumin or fraction V bovine plasma, 0.015 M Na₂HPO₄-12H₂O, 0.00475 M KH₂PO₄, 0.0020 M sodium thioglycolate, 0.0015 M L-cysteine hydrochloride, 0.0015 M glutathione, 0.0015 M sodium pyruvate, 0.0085 M sodium bicarbonate, and 0.15 M NaCl. For complete details see Nelson (1).
2 Either a commercial preparation obtained from the Microbiological Laboratories, Coral Gables, Florida, or a preparation made in this laboratory by filtering whole beef serum through cellophane.
3 Unpublished results.

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The present report is concerned with the isolation in crystalline form of this survival-promoting factor. This material will be referred to as the TPS factor, indicating *T. pallidum* survival factor.

**EXPERIMENTAL**

*Biological Assay Technique*—One rabbit testicular syphiloma was removed aseptically, cut into ten transverse slices with a specially designed instrument (3), washed with 10 ml. of chilled 0.85 per cent aqueous NaCl solution, and placed in 16 ml. of a basal medium. The *T. pallidum* were extracted with gentle shaking at 30° for approximately 2 hours in an atmosphere of 5 per cent CO₂ and 95 per cent nitrogen. The suspension was then centrifuged at 1000 r.p.m. at room temperature for 10 minutes to remove erythrocytes, spermatozoa, and tissue debris. The supernatant fluid containing the *T. pallidum* was decanted and two 0.8 ml. portions removed, mixed with 0.2 ml. of saline, placed in a Brewer jar as described below, and incubated in order to gage the ability of the *T. pallidum* to survive in the presence of the naturally occurring tissue extract. The remainder of the solution was placed in a 100 ml. centrifuge tube, covered with a sterile cotton plug and a rubber cap, and spun at 5000 r.p.m. in the angle head of an International PR-8 refrigerated centrifuge at 5° for 50 minutes. The supernatant fluid was discarded and the sedimented *T. pallidum* resuspended in 16 ml. of basal medium. 0.8 ml. portions of the resuspended *T. pallidum* were pipetted into 13 X 100 mm. tubes containing 0.2 ml. of an isotonic saline solution of the sample to be examined for TPS activity. An aliquot of 0.8 ml. of the resuspended organisms was also added to 0.2 ml. of either phosphate buffer or saline. Failure of *T. pallidum* to survive in this tube showed that the "survival-promoting" tissue extract had been removed by the recentrifugation. In an additional set of controls 0.8 ml. of the resuspended organisms was added to 0.2 ml. portions of a series of 5-fold falling dilutions of ultrafiltrate of beef serum. A comparison of the fraction under assay with this series gave a rough index of its degree of activity. In order to furnish a zero hour control reading, samples were taken from one or two of the tubes at the beginning of each experiment and the number of organisms motile out of a total of 100 was determined by dark-field microscopy. All the tubes were then placed in a Brewer anaerobic jar and the air thrice evacuated and replaced by a gas mixture of 95 per cent nitrogen and 5 per cent carbon dioxide. The tubes were incubated at 30° and motility determinations were performed at 24 hour intervals.

Fractions to be examined for activity were dissolved in a volume of isotonic 0.85 per cent aqueous NaCl solution equal to the original volume.
of the serum or ultrafiltrate of serum from which the fraction had been separated. In order to conserve material this dilution was made on an aliquot basis.

Preliminary Investigations—The ultrafiltrate of beef serum retained its TPS activity after heating for $\frac{1}{2}$ hour either at 55° or at the temperature of the boiling water bath. The material obtained by ashing the ultrafiltrate showed no activity when assayed in the form of soluble chlorides, thus making it extremely unlikely that the TPS activity of the ultrafiltrate was due to an inorganic substance. Samples of the solid material obtained from ultrafiltrate of beef serum by sublimation from the frozen state were extracted with a variety of solvents. The TPS factor was found to be soluble in absolute ethanol and acetone, but insoluble in ether, chloroform, or benzene. Activity could be removed from ultrafiltrate of beef serum by treatment with charcoal (Darco G-60) or Magnesol and recovered from the charcoal by extraction with water, ethanol, or acetone. The TPS factor could not be recovered from Magnesol. After treatment with anhydrous acetone in the presence of mineral acid the factor formed a condensation product with the acetone which was ether-soluble. On the basis of these preliminary findings the following isolation procedure was adopted.

Fractions of serum that were discarded during the isolation procedure showed no TPS activity when assayed in 10-fold and 100-fold concentrations.

Isolation of Active Component of Beef Serum in Crystalline Form—10 liters of 10 per cent aqueous sodium tungstate solution were added to 10 liters of beef serum. 10 liters of $\frac{2}{3} N$ H$_2$SO$_4$ were then slowly added to precipitate the protein (4) which was removed by filtration through Celite on a Büchner funnel. The filtrate was stirred with 2 kilos of charcoal (Darco G-60) for 3 hours at room temperature. The charcoal was removed by filtration and extracted by stirring with 2.5 liters of acetone (Merck, c.p.) for 2 hours at room temperature. The mixture was filtered and the charcoal was again extracted with 2.5 liters of acetone. The combined extracts were stored overnight at 4°. An inactive crystalline precipitate formed which was removed by filtration and the filtrate concentrated to dryness in an atmosphere of nitrogen under reduced pressure at 30–40°. The solid material was further dried by triturating it with benzene and removing the benzene by distillation under reduced pressure. The residue was extracted three times at room temperature with

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6 Obtained from the Darco Corporation, 60 East 42nd Street, New York.
6 A hydrated form of magnesium acid silicate prepared by the Westvaco Chlorine Products Company, South Charleston, West Virginia.
7 No. 535 obtained from Johns-Manville, New York.
200 ml. portions of ether (Merck, U. S. P.). The residue was then dried under reduced pressure (3 to 5 μ for 2 hours at 30–40°) over P2O5 and then stirred for 48 hours at room temperature with 500 ml. of anhydrous acetone9 containing 100 gm. of anhydrous Na2SO4 and 5 ml. of concentrated H2SO4 in order to form an isopropylidene condensation product with acetone (5). The mixture was then filtered through a fritted disk and the filtrate was neutralized with an excess of solid Ba(OH)2·8H2O (10 gm.) and again filtered. The filtrate was concentrated to dryness in an atmosphere of nitrogen under reduced pressure at 30–40°. The syrupy residue was extracted with 200 ml. of ether (Merck, U. S. P.) by shaking with 50 ml. portions and decanting the ether solution. The ether extracts containing the active material were combined and filtered with suction through a fritted disk funnel and concentrated to about 10 ml. 20 ml. of petroleum ether (b.p. 30–60°) were added and a granular precipitate formed in the solution. The precipitate was removed by filtration and dissolved in 2 ml. of benzene. Petroleum ether was added to slight turbidity and the solution was allowed to stand several days at 4°, at the end of which time crystallization had taken place. The material was recrystallized from hot ethyl acetate. M.p. 147–148°; [α]D20 = −12° (Me&CO, C 2.0); yield 50 mg. Several recrystallizations are necessary in order to remove an inactive crystalline material, which is apparently diisopropylidene D-glucose. The inactive compound has a melting point of 108°; [α]D20 = −18° (H2O). The recorded constants for diisopropylidene D-glucose are melting point 110–111° and [α]D20 = −18.5° (H2O) (6).

The Molisch test for carbohydrate gave a green ring. Tests for phosphorus, nitrogen, and sulfur were negative. The iodoform test was negative, but became positive after the compound was treated with 1 N HCl for 5 minutes on the steam bath. The compound was soluble in ethanol, acetone, chloroform, benzene, ethyl acetate, and ether, but was insoluble in petroleum ether and only slightly soluble in water. A tentative formula for the compound based on the condensation of one isopropylidene group is C15H26O10.11 Calculated, C 49.16, H 7.15, Me&JO 15.85; found, C 49.07, H 7.20, Me&CO 15.85 (7). At this stage an assay showed the compound to have very slight TPS activity.

9 It is interesting to note that the initial acetone eluate of the charcoal showed no TPS activity until it had been concentrated to dryness and all ether-soluble material removed. This suggests that the inhibitory properties (1) of whole serum may be due to an ether-soluble material; the question is under investigation.

9 Prepared by distilling acetone (Merck, c.p.) from anhydrous K2CO3.

10 All melting points were taken on a Fisher-Johns melting point block and are uncorrected.

11 Combustion analyses were performed by the Clark Microanalytical Laboratory, Urbana, Illinois.
Removal of Isopropylidene Residue with Consequent Regeneration of Full TPS Activity—50 mg. of the isopropylidene compound were dissolved in 3 ml. of 1.5 N HCl and the solution was allowed to stand overnight at room temperature. The mineral acid was then removed by treatment with excess Ag₂CO₃ and the solution was filtered. The solution was treated with H₂S gas and again filtered. 50 ml. of absolute ethanol were added and the solution was concentrated to dryness in an atmosphere of nitrogen at 30–40°. The solid residue was crystallized from 2 ml. of absolute ethanol by adding an equal volume of ether and allowing the solution to stand several days at ice box temperature. Yield 25 mg.

<table>
<thead>
<tr>
<th>Material added to medium</th>
<th>Per cent of T. pallidum motile during incubation at 30°</th>
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<tbody>
<tr>
<td></td>
<td>1 day</td>
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<tr>
<td>Serum ultrafiltrate</td>
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<td>20.0 vol. per cent</td>
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<tr>
<td>4.0</td>
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<tr>
<td>0.8</td>
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<td>0.16</td>
<td>98</td>
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<td>0.03</td>
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<td>0.02</td>
<td>14</td>
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<tr>
<td>T. pallidum survival factor</td>
<td></td>
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<tr>
<td>375.0 γ per ml.</td>
<td>92</td>
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<tr>
<td>75.0</td>
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<td>18</td>
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<td>Phosphate control</td>
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</table>

The compound was hygroscopic and after drying at 100° under reduced pressure was analyzed for C₁₂H₂₂O₁₀·H₂O. Calculated, C 41.85, H 7.03; found, C 41.95, H 6.92. M.p. 95–98°; [α]₂₅ = +18° (H₂O, C 2.0).

TPS Activity of Isolated Compound—To determine the activity of the TPS factor on a weight basis, a 3 mg. per ml. solution of TPS was made in isotonic saline buffered with phosphate buffer (0.01 M Na₂HPO₄·12H₂O and 0.0038 M KH₂PO₄). 5-Fold falling dilutions of this solution were made in the same buffer, and these were compared with 5-fold falling dilutions of whole serum ultrafiltrate. The organisms were incubated at 30° and motility counts were made at daily intervals. The results are shown in Table I.

Preliminary Chemical Investigation on TPS—Since the removal of the isopropylidene residue resulted in a considerable loss of material, chemical
investigations were carried out on the isopropylidene compound. Although this compound gave an atypical Molisch test, the anthrone test (8) for carbohydrate was positive. The isopropylidene condensation product of TPS did not reduce Fehling's solution either before or after hydrolysis under a reflux with 5 per cent HCl for 8 hours. The hydrolyzed material did not form an osazone after treatment with phenylhydrazino hydrochloride-sodium acetate under the usual conditions and was no longer biologically active. Treatment of the isopropylidene derivative of TPS with acetic anhydride in pyridine yielded a sirup which could not be crystallized. The isopropylidene derivative of TPS had a neutral reaction in aqueous solution; however, 12 mg. of the compound consumed 3.1 ml. of 0.01 N NaOH when treated with alkali at room temperature. This is equivalent to 0.95 mole of alkali per mole of compound on the basis of the formula C_{16}H_{26}O_{10}, thus indicating the presence of one ester or lactone group in the molecule.

DISCUSSION

The beneficial effect of beef serum or an ultrafiltrate of beef serum in promoting the survival of virulent T. pallidum is apparently due in large part to a specific compound (the TPS factor) which has been isolated as described above. While it is recognized that other components of serum may be important in the metabolism of T. pallidum, or more specifically may be essential growth requirements, the activity of such materials was not detected with the present assay techniques.

The TPS factor could be removed from deproteinized beef serum by adsorption on charcoal. It was also found that after eluting the factor from the charcoal into acetone an isopropylidene condensation product could be formed. This isopropylidene condensation product, after repeated recrystallization from ether-petroleum ether, benzene, and finally ethyl acetate, had a sharp melting point (147–148°) and corresponded to C_{16}H_{26}O_{10} on analysis. The compound resulting from the removal of the acetone residue with aqueous mineral acid was crystallized from ethanol-ether and after recrystallization melted at 95–98° and yielded C_{12}H_{20}O_{10}·H_{2}O. The latter compound would promote the survival of T. pallidum to the same extent as the ultrafiltrate of beef serum (Table I).

The isopropylidene derivative of TPS was non-reducing before and after hydrolysis. The compound gave an atypical Molisch test but a positive test for carbohydrate with the anthrone reagent (8). The consumption of 1 mole of alkali per mole indicates the presence of an ester or lactone group.

A search of the literature has failed to identify the compound or its
isopropylidene derivative on the basis of the elemental analysis, melting point, and optical rotation.

Since the effect of TPS on only one organism (T. pallidum) is known, it is impossible to say whether or not the isolated factor is identical with any compounds which have not yet been isolated in a crystalline condition but are known to be present in a natural product and to affect the growth or survival of other microorganisms.

Further chemical and biological studies are in progress on the TPS factor.

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SUMMARY

1. A crystalline compound which is necessary for the prolonged survival in vitro of virulent Treponema pallidum has been isolated from beef serum.

2. The compound is isolated in the form of its isopropylidene derivative, which gives analysis for \( C_{15}H_{26}O_{10} \) and has a melting point of 147–148°; \([\alpha]_D^{28} = -12°\) (Me\(_2\)CO, C 2.0). The compound after removal of the isopropylidene group yields \( C_{12}H_{22}O_6 \) and has a melting point of 95–98°; \([\alpha]_D^{28} = +18°\) (H\(_2\)O, C 2.0).

3. The isopropylidene compound gives a negative test with the Molisch reagent and is non-reducing before and after hydrolysis with 5 per cent aqueous HCl. The compound, which gives a neutral reaction in aqueous solution, takes up approximately 1 mole of alkali per mole at room temperature, indicating the presence of an ester or lactone grouping.

4. At a concentration of 3 to 375 γ per ml. the compound will prolong the survival of T. pallidum in vitro to the same extent as do an ultrafiltrate of beef serum or normal tissue extracts.

BIBLIOGRAPHY

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