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Protein Synthesis by *Treponema pallidum* Extracted from Infected Rabbit Tissue

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Virulent *Treponema pallidum* organisms, extracted from infected rabbit testes, incorporated amino acids into protein. A temperature of 34 C and a pH of 7.6 were optimal for protein synthesis, which was linear during in vitro incubation for 24 h. Selective inhibition of protein synthesis by erythromycin as judged by the incorporation of radiolabeled amino acids and radioautography demonstrated that treponemes were actively synthesizing proteins. Since addition of various sera and ultrafiltrates to the basal incubation mixture did not stimulate the level of protein synthesis, it was uncertain whether treponemes synthesized protein at a maximal or endogenous rate. Based upon the size of the unlabeled amino acid pool contained in infected testicular extract, it appeared that virulent treponemes utilized the majority of amino acids for protein synthesis but at varying efficiency.

The growth requirements and metabolism of virulent *Treponema pallidum* organisms are unknown, thus preventing their successful in vitro cultivation. Limited information derived from experiments examining in vitro survival of these spirochetes indicates that a slightly alkaline pH, strong electronegative Eh, and incubation temperature of 30 to 32 C prolong their viability and motility (4, 10). In addition, Nelson (5) and Weber (8) have reported the dependence of treponemal survival on pyruvate, albumin, and bovine serum ultrafiltrate.

Although nutritional studies on avirulent strains of *T. pallidum* have been performed (2, 9, 10), these data have not proved helpful in defining the physiologic needs of virulent *T. pallidum* organisms. Since experiments on virulent treponemes are restricted by the limited number of organisms extracted from infected rabbit testes, the inability of the treponemes to multiply, and their short survival time, we felt that the use of radiolabeled precursors of macromolecular synthesis might afford a satisfactory method for measuring the metabolic capabilities of these bacteria. Such studies would help to determine whether metabolic lesions exist in virulent treponemes, preventing their in vitro cultivation. In this paper, we describe an in vitro technique for monitoring the incorporation of radiolabeled amino acids into protein by virulent treponemes and establish experimental conditions for optimal protein synthesis.

MATERIALS AND METHODS

Bacteria. Virulent Nichols strain of *T. pallidum* was initially provided through the courtesy of Paul H. Hardy, Jr. (Johns Hopkins University).

Animals. New Zealand white male rabbits (Pel-Freeze) weighing approximately 7 pounds (ca. 3.2×10^3 g) were kept at 16 to 18 C in isolation cubicles prior to and during treponemal infection. Virulent *T. pallidum* organisms were injected intratesticularly at a cell density of 2×10^7 to 5×10^7 treponemes per testis.

Chemicals and radioisotopes. Bovine serum ultrafiltrate was purchased from Microbiological Associates and bovine serum albumin from Armour Pharmaceutical Company. The ^{3}H-labeled amino acid mixture (15 L-amino acids in concentrations similar to a typical algal protein hydrolysate) was supplied by ICN Radioisotope Division and the ^{14}C-labeled amino acid kits (50 μCi/μmol for each amino acid) by Schwarz-Mann.

Assay medium. Phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin, 0.5% bovine serum ultrafiltrate, 25 μg of pyruvate per ml, 1.2 mg of reduced glutathione per ml, and 10 μg of cycloheximide or erythromycin per ml was adjusted to pH 9.0 with NaOH prior to filter sterilization. The medium was then gassed in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2. Adjustment of the assay medium to an initial pH of 9.0 was necessary to obtain a final pH of 7.6 after the mixing of equal volumes of infected tissue extract and assay medium in individual assay vials.

Extraction of virulent T. pallidum. Testes were aseptically removed from infected rabbits 1 to 2 days after the early detection of an orchitis. The testicular

tissue from individual rabbits was minced and extracted by being shaken at room temperature in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 in 10 ml of PBS containing 6 mg of reduced glutathione (PBS-G). The fluid was removed and centrifuged twice at 500 × *g* to pellet host cellular components, including a majority of spermatozoa and blood cells. The supernatant fraction containing treponemes (0.2×10^8 to 1.0×10^8 organisms per ml) and some host tissue contaminants was added to vials containing an equal volume of assay medium. Attempts were made throughout the procedure to minimize oxygenation by gassing with the CO_2, H_2, and N_2 mixture, since these conditions significantly increased the length of in vitro survival of treponemes.

Assay for protein synthesis in infected tissue extract. Assay medium (0.45 ml) containing either cycloheximide or erythromycin was added to sterile 1-ml glass vaccine vials (Arthur H. Thomas Co.). All manipulations were performed under a constant stream of CO_2, H_2, and N_2 gases. An equal volume of pregassed tissue extract containing treponemes was introduced, and the vials were sealed tightly with rubber caps. Vials were incubated at 34 C for 3 h to allow the drugs to selectively inhibit protein synthesis in treponemes (erythromycin) or host cells (cycloheximide). Then 3 μCi of ^{3}H-labeled amino acid mixture or 0.15 μCi of the individual ^{14}C-labeled amino acid in a volume of 0.05 ml was added to individual vials. After incubation at 34 C for 18 h, 0.8 ml from each vial was transferred to a centrifuge tube containing 10 ml of cold PBS supplemented with 0.2% casein hydrolysate (PBS-C) to dilute out the radioisotope. After centrifugation at 20,000 × *g* for 15 min at 4 C, the pellet containing treponemes, some blood cells, and spermatozoa was resuspended in 5 ml of PBS-C and applied to a membrane filter (0.22 μm, Millipore Corporation) which retained treponemes and host tissue components. Filters were washed with 5% trichloroacetic acid and dried prior to counting in a scintillation spectrometer. The liquid remaining in the vaccine vials (approximately 0.15 ml) was examined by dark field microscopy to record the motility of treponemes and observe possible bacterial contamination. Also, portions of medium randomly selected from these bottles were inoculated onto rabbit or sheep blood agar plates and incubated under aerobic and anaerobic conditions to detect bacterial contamination.

Radioautography of ^{3}H-labeled T. pallidum organisms. After treponemes had been exposed to ^{3}H-labeled amino acids for 18 h at 34 C in medium containing cycloheximide or erythromycin, 0.8 ml of the medium was transferred to centrifuge tubes containing 10 ml of PBS-C (see above). After centrifugation at 20,000 × *g* for 15 min, the pellet was resuspended in 0.2 ml of PBS-C supplemented with 0.5% bovine serum albumin which increased the adherence of treponemes to the slide. A drop of the suspension was placed on clean slides, air dried, and fixed in absolute methanol for 5 min. Slides were then coated in the dark with Kodak NTB2 nuclear tract emulsion and stored in light-proof boxes at room temperature for 18 days. Slides were developed in Kodak D-19 for 6 min, rinsed in water for 30 s, fixed in undiluted Rapid Fixer (Kodak) for 2.5 min, and finally washed in water for 20 min. The temperature of all solutions was 18 C. After drying, slides were stained for 30 min in a modified Giemsa solution (1.5 ml of methanol, 3 ml of Giemsa stain, and 50 ml of water), rinsed in water, and air dried.

Amino acid pools in infected testes extract. Infected testes were minced in PBS-G, and the liquid extract was centrifuged as previously described. Trichloroacetic acid was added to the supernatant fraction to make a final concentration of 5%. After centrifugation, the pellet was washed a second time in 5% trichloroacetic acid. The combined supernatants, after trichloroacetic acid precipitation, were extracted five times with 10 ml changes of ether to remove trichloroacetic acid. The aqueous layer was evaporated to dryness in vacuo, and the amino acid content was determined with a Beckman amino acid analyzer.

RESULTS

Effect of incubation temperature on protein synthesis by treponemes extracted from infected tissue. There is considerable evidence suggesting that rabbits injected with *T. pallidum* organisms develop successful infections when housed at or below 21 C (3). Incubation above this temperature usually results in abortive infections. In addition, other information derived from in vitro studies suggests that virulent treponemes require temperatures below 37 C for continued survival (4, 5, 8). It was necessary, therefore, to establish the optimal in vitro incubation temperature for measuring protein synthesis in virulent treponemes. At the same time, protein synthesis by host cells had to be selectively inhibited since blood cells, spermatozoa, and other tissue components could not be completely removed from infected testicular extract and could compete with treponemes for radiolabeled precursor. Therefore, cycloheximide at a final concentration of 5 μg/ml was included in all assay media except as indicated. This concentration of drug inhibits protein synthesis in animal cells by greater than 80% within 2 to 3 h (M. V. Willis, J. B. Baseman, and H. Amos, Cell, in press) but has no inhibitory effect upon protein synthesis in procaryotic cells. An incubation temperature of 34 C was optimal for monitoring incorporation of radioactive amino acids into protein (Table 1). Incubation above or below this temperature caused substantial decreases in the level of protein synthesis, especially at the higher temperatures. Microscopic examination of treponemes exposed to temperatures of 38 and 40 C revealed that organisms lost motility within a few hours. At the other temperatures, 50 to 70%

of the treponemes remained actively motile after 21 h of incubation.

To demonstrate that the observed protein synthesis was of treponemal origin, two drugs known to inhibit protein synthesis in procaryotes were added to specific vials. Erythromycin (5 μg/ml) inhibited incorporation of ^{3}H-labeled amino acids in protein by 90%, whereas chloramphenicol (20 μg/ml) had no significant effect (Table 1). Treponemes remained actively motile after inhibition of protein synthesis by erythromycin.

Effect of pH on incorporation of ^{3}H-labeled amino acids into protein. It was important to determine the optimal pH of the medium at which protein synthesis in virulent treponemes occurred. Molar concentrations of phosphate were held constant in PBS, and variations in the final pH were accomplished by adjusting the initial pH of the assay medium with NaOH or HCl prior to mixing the medium with infected tissue extract. Maximal incorporation of amino acids into protein occurred in the pH range between 7.6 to 7.9 (Table 2). Measurements of pH at the termination of the experiment showed little variation from the initial pH value. At pH values of 7.2, 7.4, and 7.6, 90% of the organisms were actively motile; in contrast, at other pH values, the majority of treponemes were noticeably sluggish or nonmotile. Based upon these data, a pH of 7.6 was selected for additional studies.

Radioautography of T. pallidum. To further characterize and locate the cells synthesizing protein in the infected tissue extract, radioautography was performed. Testicular extract which was incubated in medium containing cycloheximide for 3 h prior to the addition of ^{3}H-labeled amino acid mixture contained heavy concentrations of developed silver grains over clumps of treponemes (Fig. 1a). In tissue extract incubated with erythromycin prior to radiolabeling, no grains were visible over similar areas (Fig. 1b). Although treponemes stained poorly with Giemsa, these results demonstrated that the metabolically active cells were indeed treponemes.

TABLE 1. *Influence of temperature on protein synthesis in virulent T. pallidum*

Temperature (C)	Counts/min[a]
28	787
32	2,697
34	3,474
36	3,053
38	82
40	92

[a] These numbers represent the average value of duplicate samples from three separate experiments. Samples, exposed to ^{3}H-labeled amino acid mixture, were prepared for counting as described. All media contained cycloheximide. When erythromycin or chloramphenicol replaced cycloheximide in vials incubated at 34 C, counts/min were 305 and 3,289, respectively.

TABLE 2. *pH requirement for protein synthesis*

pH[a]	Counts/min
9.0	130
7.9	3,885
7.6	3,672
7.4	2,780
7.2	2,265
6.9	1,075
6.7	694
6.5	105
6.2	69

[a] This value is the recorded pH at the start of the experiment after mixing assay medium with infected testicular extract. Details are as in footnote to Table 1; incubation was at 34 C. When erythromycin was added to the vials with a pH value of 7.6, counts/min was 340.

Effect of various sera and ultrafiltrates on protein synthesis. In an attempt to alter the level of protein synthesis in *T. pallidum* organisms, the assay medium as defined above was modified slightly so that it was deficient in bovine serum ultrafiltrate. Then various sera or ultrafiltrates or both were examined for their effect. Whole-egg, chicken embryo, or beef embryo ultrafiltrates (Grand Island Biological Company [GIBCO]), or bovine serum ultrafiltrate used originally in the assay medium were introduced to a final concentration of 0.5%. Calf serum (Pel-Freeze), rabbit serum (GIBCO) and fetal-calf serum (Flow Laboratories) were inactivated at 56 C for 30 minutes before they were added to the assay medium at a final concentration of 10%. The different sera with and without ultrafiltrate were not effective in stimulating protein synthesis above the level observed using the original assay medium (Table 3). Furthermore, fetal calf serum was slightly inhibitory.

Kinetics of incorporation of ^{3}H-labeled amino acids into protein. The kinetics of protein synthesis in infected testicular extracts was examined to measure the specific rate at which virulent treponemes incorporated amino acids into macromolecules during in vitro incubation. It appears from Fig. 2 that treponemes synthesized protein at a constant rate during the first 24 h of in vitro survival. However, between 24 and 48 h of incubation, no increase

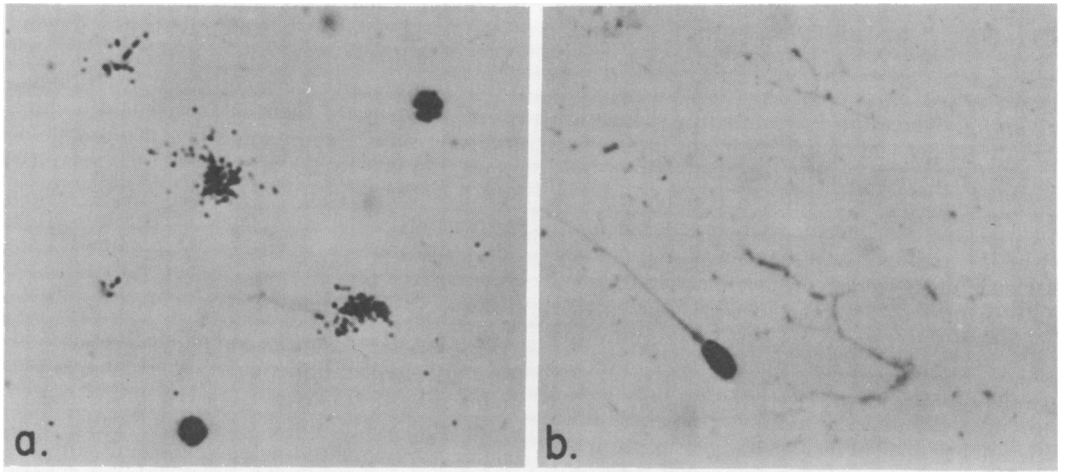

FIG. 1. *Radioautography of virulent T. pallidum organisms. Radioautographs were prepared by exposing treponemes in vitro with ^{3}H-labeled amino acid (3 μCi) for 18 h. Treponemes were incubated in medium containing cycloheximide or erythromycin for 3 h prior to addition of radioisotope. Slides were stained with modified Giemsa. ×880. (a) Treponemes exposed to cycloheximide (5 μg/ml); (b) treponemes exposed to erythromycin (5 μg/ml).*

TABLE 3. *Effect of different sera and ultrafiltrates on ^{3}H-labeled amino acid incorporation into protein*

Serum	Ultrafiltrate	Counts/min[a]
None	Bovine serum	1,856
	Whole egg	1,445
	Chicken embryo	1,751
	Beef embryo	1,375
Calf	Bovine serum	1,584
	Whole egg	1,450
	Chicken embryo	1,292
	Beef embryo	1,910
Fetal calf	Bovine serum	1,201
	Whole egg	1,236
	Chicken embryo	1,123
	Beef embryo	1,177
Rabbit	Bovine serum	1,668
	Whole egg	1,372
	Chicken embryo	1,472
	Beef embryo	1,469
None	Bovine serum	86[b]
Rabbit	Bovine serum	100[b]

[a] Average duplicate values from two separate experiments.

[b] Medium contained 5 μg of erythromycin per ml in place of cycloheximide.

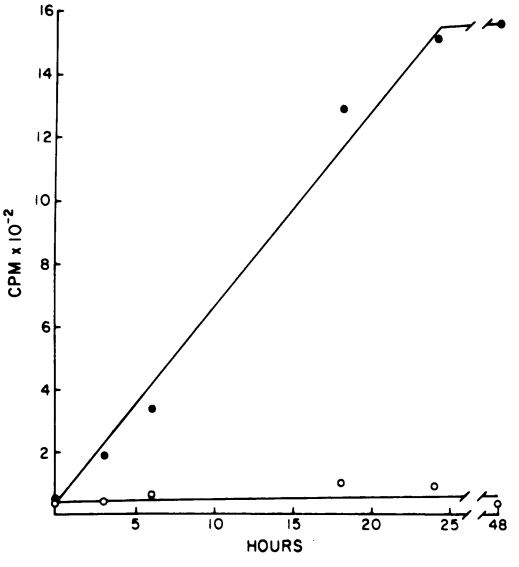

FIG. 2. *Kinetics of protein synthesis in virulent treponemes incubated in vitro. Treponemes were exposed to ^{3}H-labeled amino acids (3 μCi) for 3, 6, 18, 24, or 48 h prior to harvesting. Symbols: ●, Treponemes exposed to cycloheximide (5 μg/ml) for 3 h prior to radiolabeling; ○, treponemes exposed to erythromycin (5 μg/ml) for 3 h prior to radiolabeling. CPM, Counts per minute.*

in the level of radiolabeled protein was observed. Motility of treponemes also diminished or ceased after 24 h.

Incorporation of individual amino acids by T. pallidum organisms. The previous studies indicated that virulent treponemes actively incorporated amino acids into protein during in vitro incubation. It was important, however, to determine whether specific amino acids were preferentially incorporated or excluded by tre-

ponemes, since these data would help to clarify metabolic capabilities of the spirochetes. Individual ^{14}C-labeled amino acids having identical specific activities were incorporated into protein to varying degrees as determined by the counts per minute per vial (Table 4). It was interesting that aspartic acid and glutamic acid, the two amino acids most readily accessible to the tricarboxylic acid cycle, were least associated with the trichloroacetic acid-insoluble counts. However, since treponemes were added to assay vials as part of an infected testicular extract (see above), it was necessary to determine the concentration of unlabeled amino acids present in the extract prior to the addition of the individual ^{14}C-labeled amino acid. These data also appear in Table 4. The relationship between counts per minute per vial and micromoles of unlabeled amino acids contained in the extract indicates that the highest counts per minute are generally associated with the lowest concentration of unlabeled amino acid and, alternatively, the lowest counts per minute occur in vials containing the highest concentration of the particular amino acid. To correlate counts per minute with variations in the unlabeled amino acid pools, micromole values of the individual amino acids were divided by a common denominator (leucine, 0.034 μmol) and counts per minute were corrected accordingly. Amino acids were then categorized into three groups: group 1 (counts/min of 2,000 to 4,000), group 2 (1,000 to 2,000), and group 3 (counts/min less than 1,000). Considering these data, serine and valine were most actively incorporated into protein by virulent treponemes.

TABLE 4. *Relationship between incorporation of individual ^{14}C-labeled amino acids and amino acid pool size*

Amino acid	Counts/min per vial[a]	μmol/vial[b]	(Individual μmol/μmol of leucine) × individual counts/min[c]
Group 1			
Serine	724	.186	3,960
Valine	1,443	.060	2,540
Group 2			
Glycine	184	.334	1,807
Aspartic acid	95	.646	1,805
Threonine	762	.078	1,745
Leucine	1,575	.034	1,575
Isoleucine	1,413	.034	1,413
Alanine	480	.090	1,272
Group 3			
Arginine	1,107	.026	841
Phenylalanine	1,178	.020	695
Tyrosine	1,083	.020	639
Glutamic acid	87	.234	599
Lysine	195	.068	390
Histidine	234	.028	192

[a] Average duplicate values from four separate experiments.

[b] Average duplcate values from two infected testicular extracts.

[c] This calculation corrects for variations in individual amino acid pool sizes.

DISCUSSION

Virulent *T. pallidum* organisms, extracted from infected rabbit testes, were incubated under an atmosphere of CO_2, H_2, and N_2 gases in medium containing PBS supplemented with bovine serum albumin, bovine serum ultrafiltrate, pyruvate, and reduced glutathione. These components were reported to improve in vitro survival of virulent treponemes (5, 8) and in the current study prolonged their motility. Under these assay conditions, treponemes incorporated radioactive amino acids into protein. A temperature of 34 C and pH of 7.6 were optimal for protein synthesis which was linear during in vitro incubation for 24 h. After 24 h, treponemes became nonmotile, and the rate of protein synthesis declined.

Since the infected tissue extract contained both treponemes and host cell components, two criteria were used to demonstrate that treponemes were responsible for the observed protein synthesis. Erythromycin which selectively inhibits ribosomal function in procaryotes prevented incorporation of ^{3}H-labeled amino acids into protein by greater than 90%, whereas cycloheximide, an inhibitor of eucaryotic cells, did not significantly alter the rate or level of protein synthesis. In addition, radioautography showed localization of grains over clumps of treponemes incubated in cycloheximide-containing medium but not over host tissue components. No silver grains were associated with treponemes exposed to medium supplemented with erythromycin. It was interesting that chloramphenicol did not inhibit protein synthesis in virulent treponemes. These data are consistent with work by Abramson and Smibert (1) but are at variance with reports on the curative effects of chloramphenicol (6, 7).

Attempts to stimulate protein synthesis in treponemes by introducing different sera and ultrafiltrates to the medium were unsuccessful. Either testicular extract supplemented with the basal medium provides adequate conditions for maximal protein synthesis or virulent treponemes, incubated in vitro, synthesize protein at

an endogenous rate and are unaffected by the additions. Correlation of specific ^{14}C-labeled amino acid uptake with the size of the unlabeled amino acid pool contained in the testicular extract indicated that the majority of exogenous amino acids were utilized for protein synthesis to varying degrees by treponemes. It is not known whether particular amino acids function solely as precursors of protein synthesis or are degraded and serve as carbon and energy sources.

The assay for protein synthesis described here is a highly sensitive measure of metabolic activity in virulent treponemes extracted from infected tissue. The influence of temperature and pH as well as the effectiveness of several drugs were readily monitored with protein synthesis as the indicator of cell function and survival. It appears that virulent treponemes incorporate rabiolabeled amino acids into trichloroacetic acid-insoluble material and thus are not defective in their ability to synthesize protein during in vitro incubation. Studies are underway to further define the metabolic capabilities of virulent *T. pallidum* organisms.

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