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Radiolabeling of *Treponema pallidum* (Nichols Virulent Strain) In Vitro with Precursors for Protein and RNA Biosynthesis

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We observed uptake of [*U*-^{14}C]serine, *U*-^{14}C-labeled amino acid hydrolysates, and [2-^{14}C]uracil by virulent *Treponema pallidum* in vitro for at least 96 h. No uptake of [2-^{14}C]thymine, [1-^{14}C]pyruvate, [*U*-^{14}C]pyruvate, and [2-^{14}C]uridine was detected. Treponemal protein and RNA biosynthetic activity was identified by erythromycin inhibition of amino acid and uracil uptake. Radioactivity due to uptake of radiolabeled amino acids by residual testicular cells in the cultures remained at background levels regardless of the presence or absence of cycloheximide. Accumulation of the radiolabeled substrates by *T. pallidum* proceeded at a linear rate for 48 to 96 h during incubation in vitro. The longevity of substrate uptake using the system of incubation described will facilitate future studies on the metabolism of the microorganism to help determine essential growth factors and environmental conditions for multiplication of *T. pallidum* in vitro.

Until recently, the most commonly accepted techniques to study the physiology of *Treponema pallidum* in vitro were dependent upon the use of light microscopy for direct counts and on the exploitation of the rabbit to evaluate retention of virulence by the microorganism (6, 26). Now, it is becoming increasingly apparent that these techniques are not completely satisfactory for physiological studies on *T. pallidum* in vitro. For example, Baseman and Hayes observed inhibition of radiolabeled amino acid uptake by erythromycin in cultures of *T. pallidum* without observing a coincident loss in treponemal motility (2, 3). Baseman et al. demonstrated that amino acid incorporation and alterations in carbohydrate metabolism appeared to be functions of the availability of oxygen in treponemal cultures (4). This finding conflicted with earlier evidence, based on motility and virulence studies, that the microorganism was an obligate anaerobe (26).

Baseman et al., Nichols and Baseman, and Schiller and Cox obtained information on the respiratory biochemistry of *T. pallidum* which could not be approached by using microscopy or in vivo tests for retention of virulence (4, 15, 20). Employing differentially labeled [^{14}C]glucose, these investigators showed that the microorganism exhibited catabolic activity characteristic of the hexose monophosphate shunt and the Embden-Meyerhoff-Parnas pathways (4, 15, 20). Incomplete hydrolysis of differentially labeled [^{14}C]pyruvate and lack of utilization of ^{14}C-labeled tricarboxylic acid cycle intermediates suggested that the microorganism does not possess a complete tricarboxylic acid cycle (4, 20). Substantiation for conclusions based on radiorespirometric data was obtained by analysis to identify characteristic enzymes associated with each pathway (20).

The information summarized above was obtained despite apparent difficulties in maintaining the motility of the microorganism in excess of 24 h, which suggested a rapid loss in viability. The metabolism of rapidly degenerating *T. pallidum* may be different than that of a treponemal population exhibiting better stability in the rate of isotope incorporation and in the number of motile microorganisms available during incubation. We had developed a serum-free medium in which up to 90% of the treponemes remained motile for 96 h (12). Our system of incubation held some promise to facilitate future studies on substrate utilization and turnover by *T. pallidum*. To this end, we extended our system of incubation for *T. pallidum* to monitor amino acid and uracil uptake.

MATERIALS AND METHODS

Preparation of assay medium. Pre-reduced new formula medium ($PRNF_{10}$) has been described elsewhere (19). To avoid dilution of radiolabeled substrates, the medium was modified for use in these experiments by deletion of serum as well as of those compounds to be included later as isotopically labeled substrates for *T. pallidum*. The medium was supple-

mented with the nutrients listed in Table 1. A portion of the unreduced mixture was set aside for extraction of *T. pallidum* from the testes of rabbits. To the remaining medium, concentrated solutions of the metabolic inhibitors erythromycin (Ilotycin; Dista Products Co., Indianapolis, Ind.) and/or cycloheximide (Acti-Dione; The Upjohn Co., Kalamazoo, Mich.). were added to obtain 10 μg/ml. After addition of the solution containing the reducing agents (12), to obtain a redox potential of −275 mV E_{cal} (pH 7.3), the medium was dispensed, 9.6 ml each, into (15 by 145 mm) anaerobe tubes (Bellco Glass, Inc., Vineland, N.J.) under a constant flow of 75% N_2 + 20% H_2 + 5% CO_2 (Matheson Gas Co., Elk Grove, Ill.) using a VPI anaerobic culture unit (Bellco). The tubes were stoppered, and the medium was incubated at 34°C for at

TABLE 1. *Supplemental nutrients for and modifications in medium PRNF*$_{10}$[a]

Component(s)[b]	Concn of stock solution (g/liter)	ml per 100 ml of medium
Mixture of:		
B_{12} (3)	0.125	1
α-Lipoate[c] (2)	0.1	
Folinic acid (5)	0.1	
Biotin (1)	0.01	
Thiamin pyrophosphate chloride (3)	0.5	1
Coenzyme A (3)	0.25	1
NADH[d] (2)	0.70	1
Mixture of:		
Putrescine-hydrochloride (2)	2.5	1
Isobutyric acid (4)	1.5	
Nicotinamide (1)	4.0	
Uracil[e] (2)	1.5	1
Adenine (2)	1.5	1
Bovine albumin Fr V (6)	100	4
$MnCl_2 \cdot 4H_2O$ (8)	0.0010	1
$CoCl_2 \cdot 6H_2O$ (8)	0.0005	1
Choline chloride (2)	10.0	1
Galactose (3)	190.8	1
$(NH_4)_2SO_4$ (9)	9.6	1
DL-Ornithine[f] (2)	2.0	1
Mixture of[g]:		
DL-Alanine (7)	1.78	2
L-Asparagine (1)	3.00	
L-Aspartic acid (1)	2.66	
L-Glutamic acid (1)	2.94	
Glycine (2)	1.50	
L-Proline (1)	2.30	
L-Serine[h] (1)	2.10	1

[a] $PRNF_{10}$ (pre-reduced new formula medium) was modified by the omission of newborn calf serum and nonessential amino acids listed in the original formulation (19). The volume of sterile glass distilled water used to dilute stock solutions was adjusted to obtain the same concentrations of components listed earlier for $PRNF_{10}$ to make allowance for the addition of the supplemental stock solutions listed in this table.

[b] All stock solutions were prepared with glass distilled water and sterilized with prerinsed 0.45-μm filters. The number in parentheses following each component corresponds to a vendor for each reagent listed below: (1) Nutritional Biochemicals Corp., Cleveland, Ohio; (2) Calbiochem, Los Angeles, Calif.; (3) Sigma Chemical Co., St. Louis, Mo.; (4) Nu Chek Prep, Inc., Elysian, Minn.; (5) ICN and K & K Laboratories, Inc., Plainview, N.J.; (6) Reheis Chemical Co., Phoenix, Ariz.; (7) Eastman Kodak Co., Rochester, N.Y.; (8) Fisher Scientific Co., Fairlawn, N.J.; and (9) Schwarz/Mann, Orangeburg, N.J.

[c] α-Lipoate (100 mg) was dissolved with 1 ml of 0.2 N NaOH, and the volume was adjusted to 10 ml with glass distilled water before incorporation into the concentrated stock solution of vitamins.

[d] NADH, Reduced nicotinamide adenine dinucleotide.

[e] Uracil was incorporated into all preparations of the medium except those to be used to monitor [2-^{14}C]uracil incorporation by *T. pallidum*.

[f] DL-Ornithine (200 mg) was dissolved in 10 ml of 1 N HCl, and the volume was adjusted to 100 ml with glass distilled water.

[g] This mixture of amino acids was used in place of the nonessential amino acid supplement listed in the original formulation for $PRNF_{10}$ (19).

[h] L-Serine was incorporated into all preparations of the medium except those to be used to monitor [^{14}C] serine incorporation by *T. pallidum*.

least 2 h before use.

Preparation of ^{14}C-labeled substrate solutions. The radiolabeled substrates used included [2-^{14}C]uracil (ICN Pharmaceuticals, Inc., Irvine, Calif., or New England Nuclear Corp., Boston, Mass.), [2-^{14}C]thymine (CEA IRE SORIN, Gif-Sur-Yvette, France), [2-^{14}C]uridine (Schwarz/Mann, Orangeburg, N.Y.), [1-^{14}C]pyruvate (New England Nuclear), L- or DL-[U-^{14}C]serine (Dhom Products Ltd., North Hollywood, Calif., or New England Nuclear), [*U*-^{14}C]pyruvate, and *U*-^{14}C-labeled L-amino acids (ICN Pharmaceuticals, Inc.), referred to as an L-amino acid hydrolysate in the text. The specific activity of single substrates ranged between 50 and 150 mCi/mmol, and was 1 mCi/mg for the L-amino acid hydrolysate. Before use all compounds were diluted by using glass distilled water to obtain 30 μCi/ml, sterilized by using a 0.45-μm disposable filter (Millipore Corp., Bedford, Mass.), and then diluted into the culture medium to obtain a stock solution containing 3 μCi/ml.

Preparation of testicular extracts. New Zealand rabbits (3.5 to 4 kg) exhibiting a well-developed orchitis 10 to 14 days after intratesticular inoculation with 5×10^7 *T. pallidum* were sacrificed, and their testes were surgically removed and aseptically minced and extracted (17). The first and second eluates, 3 to 5 ml each, withdrawn from the mincings were discarded. If not discarded, they quickly formed fibrin-like clots which served as a framework for intact cellular debris to remain in suspension even after low-speed centrifugation. The third and all subsequent eluates, never exceeding seven (3 ml each), were combined. The large particulate debris and spermatozoa were sedimented at $250 \times g$ for 10 min at room temperature; the supernatant fluids were withdrawn and diluted with an equal volume of pre-reduced medium, and the pellets were discarded. Contamination of the harvests by intact testicular cells (including spermatozoa) in the low-speed supernatant fluids did not exceed 10^3 cells per ml, based on observations using dark-field microscopy. The treponemal suspensions were diluted by using pre-reduced medium under 75% N_2 + 20% H_2 + 5% CO_2 to obtain ca. 5×10^7 *T. pallidum* per ml, estimated by a direct count by dark-field microscopy (19). The treponemes were maintained at 34°C for 30 to 90 min before use. Testicular extracts from rabbits not challenged with *T. pallidum* were prepared in the same manner to be used in control cultures. Low-speed sedimentation of the extracts from uninfected testes resulted in contaminating suspensions of testicular cells plus spermatozoa of about 10^4/ml.

Culture preparation and sampling procedures. The diluted testicular extracts, 1.2 ml, from either infected or uninfected rabbits, were inoculated into anaerobic tubes containing 9.6 ml of medium. A 1.2-ml portion of medium containing 3 μCi of radiolabeled substrate per ml was added to each tube. Each tube contained a final volume of 12 ml with 0.3 μCi of radiolabeled substrate per ml with or without 5×10^6 *T. pallidum* per ml. Tubes without *T. pallidum* but containing testicular extracts from uninfected rabbits served as controls to monitor nonspecific retention of radiolabeled substrate to filters and/or metabolic activity due to testicular cells. All tubes were prepared in duplicate.

Samples (2 ml) were withdrawn from each tube at the beginning of the experiment and every 24 h thereafter for 96 h. The samples were diluted with 8 ml of phosphate-buffered saline (pH 7.3) chilled on ice (13). The samples were suctioned through 0.22-μm filters by using a Millipore sampling manifold (Millipore Corp.). Before use, the filters were soaked 30 min at room temperature with phosphate-buffered saline + 10% bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) to decrease nonspecific attachment of the labeled substrates to the filters. The filters with retained testicular debris and/or treponemes were rinsed with four 10-ml volumes of cold phosphate-buffered saline, removed from the filtration unit, placed into scintillation vials, and immersed in 10 ml of Aquasol II (New England Nuclear). Radioactive counts were determined after the vials were cooled to 15°C in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) having a ^{14}C efficiency of 60 to 70%.

Direct counts. A separate set of duplicate cultures was prepared by using the same media and materials described above excluding the radiolabeled substrates. Instead, the medium was supplemented with cold substrates to obtain the same final concentration employed in cultures with their labeled counterparts. These cultures, rather than those containing ^{14}C, were used to estimate numbers of motile and nonmotile treponemes in suspension by direct counts with dark-field microscopy (19).

Contamination by exogenous microflora. All cultures, reagents, media, and harvests were checked for microbial contamination before, during, and after each experiment by using procedures outlined previously (19).

RESULTS

The data in Fig. 1 were obtained by incubating extracts from *T. pallidum* infected and uninfected rabbit testes in medium containing DL-[*U*-^{14}C]serine. Erythromycin, an inhibitor of procaryotic protein synthesis (2, 23), prevented incorporation of the radiotracer in the *T. pallidum*-containing extracts into particles which could be retained by the filters. Without erythromycin, retention of radioactivity on filters increased linearly with time when *T. pallidum*-containing extracts were used compared to no increase in radioactivity when uninfected testicular extracts were used. Background radioactivity due to exposure of filters to medium containing DL-[*U*-^{14}C]serine (medium control) was lower than that observed for media with testicular extracts. Medium containing cycloheximide (Fig. 1A), a potent inhibitor of eucaryotic protein synthesis (2, 5, 11, 22, 24), had no significant effect on isotopic incorporation compared to medium without cycloheximide (Fig. 1B).

Data obtained using *U*-^{14}C-labeled L-amino

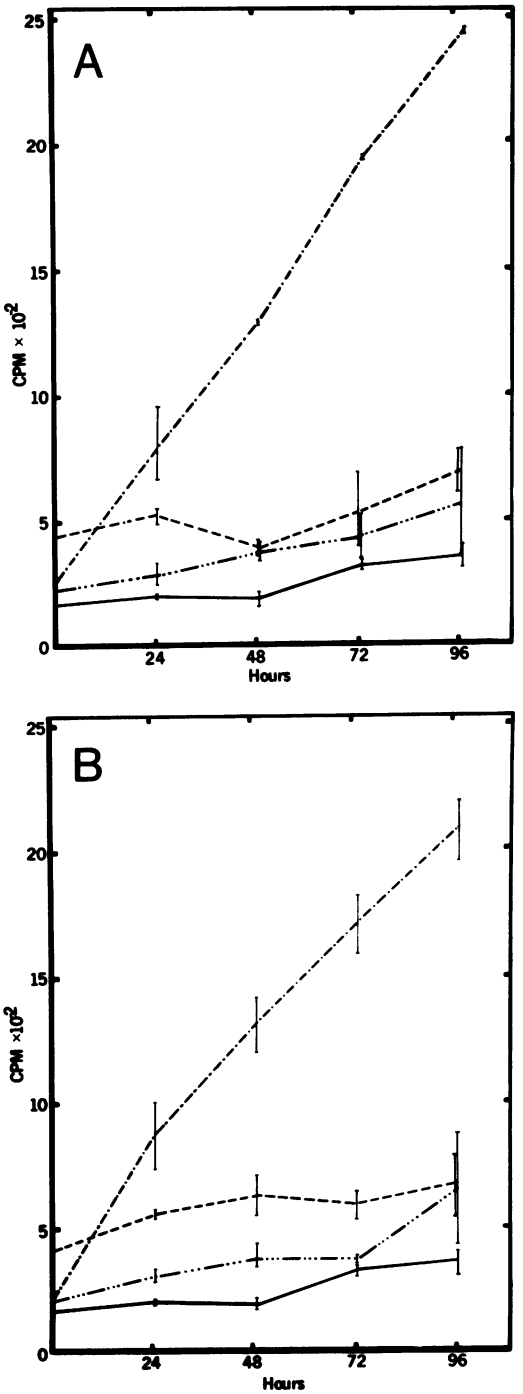

FIG. 1. *Retention of ^{14}C from DL[U-^{14}C]serine on filters using samples from cultures containing testicular extracts from a rabbit with a testicular syphiloma compared to a nonsyphilitic rabbit. The syphilitic testicular extracts were incubated with (···—···—···) and without (·—·—·) erythromycin. Erythromycin was not used with nonsyphilitic testicular extracts (·····). Medium without extracts (——) was incubated and sampled to monitor background counts. (A) and (B) illustrate data obtained by using medium with and without cycloheximide, respectively. The vertical line through each point on the graphs represents one standard deviation of the mean for two cultures. CPM = scintillation counts per minute.*

acid hydrolyates or L-[*U*-^{14}C]serine were not significantly different from data illustrated in Fig. 1 for DL-[*U*-^{14}C]serine (data not shown).

The data in Fig. 2 were obtained after incubation of testicular extracts from syphilitic rabbits in the presence of [2-^{14}C]uracil and cycloheximide. Uracil is not utilized by mammalian cells, although it is a precursor for procaryotic RNA synthesis (9). Uracil incorporation into particles retained by the filters was nearly linear between 0 and 48 h, regardless of the presence or absence of erythromycin. At every sampling time, however, uracil uptake was most pronounced in cultures without erythromycin. Estimates of numbers of treponemes in suspension were nearly identical, regardless of the presence or absence of erythromycin in the medium. Background radioactivity due to exposure of filters to medium containing [2-^{14}C]uracil remained at a low level throughout incubation.

We observed no significant increase in radioactivity above background levels with samples from treponemal cultures containing ^{14}C-radiolabeled thymine, pyruvate, and uridine.

DISCUSSION

Our data suggest that ^{14}C-labeled amino acids are incorporated into protein by *T. pallidum* in vitro. It was possible to distinguish procaryotic from eucaryotic metabolic activity because erythromycin selectively inhibits protein synthesis by procaryotes, and cycloheximide selectively inhibits protein synthesis by eucaryotes (22, 23). Erythromycin alone caused significant reductions in radioactivity retained by filters, whereas cycloheximide had no effect on retention of radioactivity. Therefore, retention of the radiotracer upon the filters was clearly a function of procaryotic metabolism.

Baseman and Hayes observed a similar phenomenon by using the same metabolic inhibitors and a larger number of individual amino acids (2, 3). However, the percentage of motile microorganisms dropped rapidly to nearly 0% after 48 h of incubation, and amino acid uptake ceased after 24 h in vitro (2). In contrast, we observed amino acid uptake continuing at a linear rate for at least 96 h, and a high percentage (85 to 90%) of the treponemes remained motile throughout

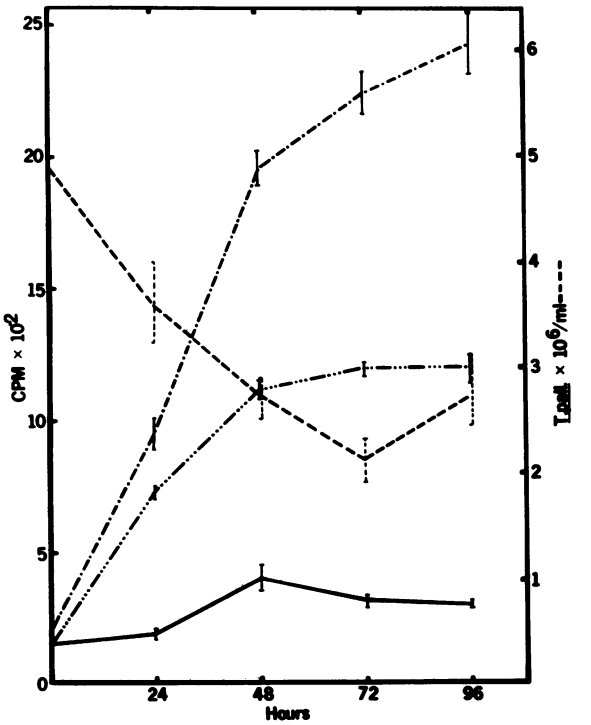

FIG. 2. *Retention of ^{14}C from [2-^{14}C]uracil on filters using samples from cultures containing a testicular extract from a rabbit with a testicular syphiloma incubated with* (···—···—···) *and without* (·—·—·) *erythromycin. Medium without an extract was incubated and sampled to monitor background counts* (——). *Estimates of numbers of motile treponemes in cultures incubated with or without erythromycin were nearly identical* (-----). *The vertical line through each point represents one standard deviation of the mean for two cultures. CPM = scintillation counts per minute.*

this period. Though possible, but not demonstrated here, improvements in treponemal motility retention and substrate incorporation in our system of incubation compared to observations reported earlier (2, 3) may be due to a more suitable medium, $PRNF_{10}$, and/or its redox potential (17-19).

We observed that the inhibition of [2-^{14}C]uracil uptake with erythromycin in the medium was not as pronounced compared to the inhibition of [*U*-^{14}C]serine uptake. Less pronounced inhibition of uracil uptake was expected since this compound is a precursor for RNA rather than protein synthesis. Erythromycin should not immediately affect uracil uptake into cellular components. Instead, cultures containing erythromycin would be expected to exhibit a decreasing rate of uracil incorporation into RNA, dependent upon the rate of turnover of enzymes for synthesis of RNA. Such a phenomenon was observed with the microorganism in the presence and absence of erythromycin (Fig. 2).

The potential contribution of testicular cellular debris to the total radioactivity was not directly assessed for studies with [2-^{14}C]uracil. However, it is possible that an inhibitor of eucaryotic RNA polymerase (α-amanitin) or inhibitors of procaryotic RNA polymerase (streptolidigin, rifampin, and streptovaricin) may be used to differentiate between the metabolic activities of the two components of the system (7, 8, 14, 21, 24, 25). In the present study, the progression from partial to complete inhibition of [2-^{14}C]-uracil uptake in the presence of erythromycin suggests that the procaryotic and not the eucaryotic component of the system was responsible for the differences in retention of radioactivity on filters. This contention is substantiated by observations that mammalian cells in general do not readily incorporate uracil (9).

T. pallidum remained motile in the presence of erythromycin for 96 h in vitro. Treponemal cultures with or without this antibiotic could not be identified on the basis of numbers or proportions of motile to nonmotile microorganisms in suspension. These observations confirm earlier observations and further substantiate the hypothesis that a functional dichotomy exists be-

tween treponemal biosynthetic activity and motility (2).

Our subjective observation should be noted that medium without erythromycin permitted treponemal elongation, after 96 h in vitro, two to four times the length of the microorganisms at the beginning of the experiment. Medium with erythromycin did not permit treponemal elongation. It would be very difficult to obtain a quantitative correlation between elongation and substrate uptake by using currently available techniques.

Treponemal elongation could account for the results illustrated in Fig. 2. Radioactivity of cultures incubated with radiolabeled uracil increased in the face of decreasing numbers of microorganisms. Similar results were obtained when we used the radiolabeled amino acids (data not shown). We postulate that the expected loss in radioactivity due to decreasing numbers of treponemes was overridden throughout incubation by substrate utilization for the elongation of *T. pallidum*. This hypothesis can be directly tested by using radioautographic techniques (2).

Recently, Nichols and Baseman observed uridine incorporation into treponemal RNA (16). Our attempts to label *T. pallidum* by using uridine were unsuccessful. However, uracil was a component in the formulation of our assay medium. Therefore, it is possible that uracil functioned as a competitive inhibitor against uridine in our experiments.

Baseman and Hayes observed that *T. pallidum* could not incorporate amino acids into protein and lost motility under anaerobic conditions (2, 3). However, isotope incorporation occurred when the treponeme was incubated in medium under a deoxygenated gaseous environment if the microorganism was exposed to oxygen during harvest from orchitic testes and/or the assay medium was prepared under conditions not rigorously controlled for anaerobiosis (2, 3). Our data were obtained with *T. pallidum* harvested under aerobic conditions and incubated with a serum-free medium having a low electronegative potential which was not deoxygenated before use. Further work with predetermined levels of oxygen in the gaseous phase of cultures can now be performed by using the information presented in this report as the basis for future studies. In fact, recently we observed unsustained multiplication of *T. pallidum* in vitro by using medium with serum and 6% oxygen (18). At this time, without further refining our experimental system of incubation in the presence of oxygen, it is not possible to suggest a role for oxygen in labeling *T. pallidum*.

The survival time of the microorganism, as judged from motility retention and isotopic uptake, was much longer in the system of incubation described here compared to other systems of incubation used for radiolabeling *T. pallidum* in vitro (2–4, 15, 20). As mentioned earlier, an increase in treponemal survival time for radiotracer studies could be very advantageous in several respects. For example, assimilation of carbohydrate carbon skeletons into cellular fractions may be observed (1, 10). Carbohydrate assimilation by *T. pallidum* has yet to be demonstrated, although carbohydrate catabolism has been documented (4, 20). Finally, it may be possible to use conditioned (spent) cell culture medium and/or supplemental nutrients to obtain limited multiplication of the microorganism during substrate incorporation (18, 19). If it is possible to observe increases in treponemal numbers while radiolabeling is in progress, then the data obtained may accurately reflect the metabolism of a growing microorganism rather than the occurrence of metabolic events associated with treponemal survival under "resting" state conditions.

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