Influence of Temperature on Glutamate Catabolism and Glycogen Production by Organisms of the Genus *Chlamydia*[1] (37255)

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Previous work (1) indicated that multiplication rates of numerous strains of *Chlamydia psittaci* and *C. trachomatis* in chicken embryos were markedly influenced by the temperature at which the inoculated eggs were incubated. The rates were higher for each 2-degree increase above 35°, as reflected by a lowering of the average number of days the organisms required to multiply and kill chicken embryos. Certain strains multiplied most rapidly at 41°, but the growth of other *C. psittaci* strains and all *C. trachomatis* strains was slowed or completely inhibited at 41°.

Because of the positive growth responses of many chlamydial strains to temperature increases, tests were made to determine if two well-known biochemical activities of chlamydiae were similarly affected by increased incubation temperatures. These were: (a) the rate of glutamate catabolism by purified suspensions of chlamydiae (2) and (b) the time of appearance of glycogen-positive chlamydial microcolonies in cell cultures (3). This report concerns the amounts of $^{14}CO_2$ produced by purified suspensions of 3 strains of *C. psittaci* from randomly-labeled glutamic acid during incubation at 32°, 36°, 40°, and 44°. Also reported are the number of iodine-positive, *i.e.* glycogen-producing, intracellular microcolonies observed at intervals in cell cultures infected with a strain of *C. trachomatis* and incubated at 37° and 39°.

Materials and Methods. Chlamydiae. For the glutamate catabolism tests, the 3 strains of *C. psittaci* used were: NJ-1 from New Jersey turkeys (4), CP-3 from California pigeons (5), and IPA from arthritic Iowa lambs (6). The Nigg mouse pneumonitis (MoPn) strain (7) of *C. trachomatis* was used for glycogen tests.

Propagation of chlamydiae. The NJ-1, CP-3, and IPA strains were propagated in chicken embryos inoculated at the 6th day of incubation by the yolk sac (YS) route. Yolk sacs from embryos dying within 6–12 days postinoculation were pooled and frozen until suspensions of purified chlamydiae were prepared from them. The MoPn strain was propagated in McCoy mouse cell cultures overlayered with M199 medium fortified with 0.5% lactalbumin hydrolysate and 10% fetal calf serum. To promote a high rate of infection of cells, suspensions of MoPn chlamydiae were centrifuged for 30 min at 1000*g* in a swinging bucket rotor onto cell cultures previously grown on circular coverslips in flat-bottomed tubes, as suggested by Gordon and Quan (3).

Glutamate catabolism tests. Methods for purifiying suspensions of chlamydiae and reagents used for catabolism tests were those originally described by Weiss (8). Briefly, chlamydiae-laden YS were homogenized, treated with Pronase B (Calbiochem Co.), Celite (Johns-Mansville), and differentially centrifuged to remove extraneous protein, lipid, and debris. The number of viable chlamydiae in the suspension was determined by titration in chicken embryos, and "protein" content determined by the method of Lowry (9). The reference standard was a weighed sample of dried purified chlamydiae. Reaction mixtures consisted of 0.5 ml of the

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suspension of chlamydiae (containing 2–5 × 10^9 ELD_{50}), 0.4 ml of a solution containing 10 μCi U-^{14}C-gluatate (Calbiochem Co.) in Weiss's K36 buffer, 0.6 ml of 0.017 *M* nicotine adenine dinucleotide (NAD), 0.1 ml of a solution of 0.04 *M* $MgCl_2$ and 0.008 *M* $MnCl_2$, and 0.9 ml of K36 buffer. The chlamydiae suspension and solutions of substrate and cofactors were cooled in an ice bath and combined in 25-ml Erlenmeyer flasks stoppered by skirted rubber closures. Control flasks contained heat-inactivated chlamydial suspension plus substrate plus cofactors, or substrate alone, or cofactors alone, or substrate plus cofactors only. Combinations of reactants were tested in triplicate. All of the reaction flasks of any one test were simultaneously transferred from an ice bath to a Dubnof incubating shaker previously adjusted to the appropriate incubation temperature, gently shaken for 2 hr, and then replaced in an ice bath. Internal temperature of a control flask was monitored using a thermister wire inserted through the rubber stopper and immersed in the contents of a control flask in the shaker. The wire was connected to an electronic thermometer (Telethermometer, Yellow Springs Instrument Co.) and a recorder. Equilibration time between the ice bath and 32° was 15 min and 20 min to 44°. Temperature of the water bath at 1 in. below the surface was 1 degree above the temperature of the fluids in the flask.

Four tests of each strain, one at each incubation temperature, were conducted. A new set of reaction mixtures and flasks was prepared for each test. The chlamydial suspension was stable enough so that tests at all 4 incubation temperatures were completed using a single organism preparation.

A preliminary test to determine the rate of $^{14}CO_2$ evolution from U-^{14}C-glutamate over 2 hr at 44° by the CP-3 strain was performed. In this test, replicate reaction flasks were moved from the 44° water bath at 15-min intervals, and the results of $^{14}CO_2$ determinations for each interval were plotted.

In the principal tests, the reaction flasks were immediately cooled in an ice bath after each 2-hr run, and 0.2 ml of 10% Hyamine hydroxide (Packard Instrument Co.) was introduced by syringe and needle through the rubber closure into a plastic center well (Kontes Co.) suspended above the reaction mixture. Then 0.7 ml of 2.5% trichloracetic acid was introduced by syringe into the reaction mixture to release dissolved CO_2 to be trapped in the Hyamine. The reaction flasks were then placed back in the incubator and shaken for 1 hr. After cooling the flasks once again, the plastic cups containing Hyamine-trapped CO_2 were carefully removed and dropped into glass scintillation vials. Then 3.5 ml of absolute methanol was added to each vial followed by 15 ml of scintillant (Packard PPO, POPOP in toluene). Three successive 10-min counts of the radioactivity of each vial were made using a Packard 314EX scintillation counter. Counting efficiencies of 73 ± 2% were determined using ^{14}C-toluene as standard. The results in terms of disintegrations per minute for each flask in a triplicate set were averaged and from this the averages of triplicate control samples were subtracted. Division of this result by the specific activity per mole of the radioactive substrate and by the milligrams protein per flask gave the final figure of nmoles (n*M*) ^{14}C produced per milligram chlamydial protein per 2 hr. The amount of $^{14}CO_2$ produced by each strain at each incubation temperature in the absence of the essential cofactor, NAD, was compared with the amount produced in the presence of NAD.

Tests for glycogen. Coverslip cultures of McCoy cells infected with the MoPn strain of *C. trachomatis* were incubated at 37° and 39° and were removed from culture tubes at 0, 12, 24, 36, 48, and 72 hr postinoculation and fixed in cold methanol and stained with alcoholic I_2–KI solution according to the method of Gordon and Quan (6). Stained coverslips were placed on a drop of glycerol–iodine solution on a glass slide for microscopic examination at 500×. One hundred microscopic fields (total 4 mm^2) on each of 4 coverslips per time period per incubation temperature were examined. The number of iodine-positive microcolonies on each group of 4 slides was averaged.

Results. Glutamate catabolism tests. The preliminary test indicated that the rate of $^{14}CO_2$ evolution produced by CP-3 strain

chlamydiae at 44° from U-^{14}C-glutamate was relatively constant over the 2-hr test period. The principal tests indicated that the amount of $^{14}CO_2$ produced from U-^{14}C-glutamate by the NJ1, CP3, and IPA strains of *C. psittaci* in the presence of NAD was markedly increased as the incubation temperatures of the reaction mixtures were increased while the $^{14}CO_2$ production of the reaction mixtures without NAD remained relatively constant over the temperature range (Table I). The NJ1 strain increased $^{14}CO_2$ production 350% between 32° and 44°, the CP3 strain, 130%, and the IPA strain, which did not have any above-baseline activity at 32°, eventually had a 136% increase at 44°. An Arrhenius plot of the data from the NJ1 and CP3 experiments produced two virtually straight lines intersecting the 1/K equivalent at 36°, indicating that at that temperature the CO_2 evolution rates were identical for the two strains. Using portions of the line between the 1/K abcissal points represented by 34° and 44°, a 2.5-fold increase in activity occurred for the 10-degree rise in absolute temperature, thereby fitting the Arrhenius prediction.

Glycogen production tests. Coverslip preparations of McCoy cell cultures incubated at 39° examined at various intervals after infection with the MoPn strain of *C. trachomatis* contained 3 times as many iodine positive intracellular microcolonies at 24- and 36-hour examinations than those cultures incubated at 37°. However, by the 48th or 72nd hour after infection, the difference in number of positive microcolonies in the cultures incubated at the two temperatures was less significant although there always were more positives at 39° than at 37° (Table II).

Discussion. A temperature range of 32°–44° for the examination of enzymatic capabilities of *C. psittaci* strains was chosen because these temperatures corresponded to the range of normal body temperatures of natural hosts to chlamydiae: 34° ± 2° is the low diurnal temperature of mice, and 43° ± 1° is normal for pigeons and sparrows at 25° ambient temperature. Body temperatures increase 1° or 2° from these norms during the febrile stages of acute chlamydial infection. A high rate of glutamate catabolic activity at 44° by the CP3 strain chlamydiae was expected because it was consistent with the ability of these chlamydiae to survive and produce disease in pigeons and sparrows. But high glutamate catabolic activity at 44° was not expected of organisms of the NJ1 turkey strain because previous studies had shown that this strain had a higher thermal inactivation rate at 43° *in vitro* than the CP3 strain (5), and that it failed to multiply and cause disease in pigeons and sparrows (10). Thus, since glutamate catabolic activity remains fully operative at temperatures that are lethal to the microorganism, there is no necessary relationship between this activity and survival of the organisms in high-temperatured hosts.

As previously noted, however, increases in incubation temperature produced increased growth rates for various chlamydial strains (1). The IPA strain, for example, grew slowly at 35° but multiplied and killed embryos faster for each 2° increase in temperature through 41°. This corresponds to the present experiments, in which the IPA strain had no glutamate catabolic activity at 32° but had a high rate at 44°. The latter results suggest something else: studies of a variety of

TABLE I. Influence of Incubation Temperature on $^{14}CO_2$ Production from U-^{14}C-Glutamate by Various Strains of *C. psittaci*.

Strain	Flask temp. (°)	nM $^{14}CO_2$ produced/mg protein/2 hr		
		Without NAD	With NAD	% Increase
NJ-1	32	197[a]	390[a]	98
	36	259	686	164
	40	266	932	250
	44	253	1138	350
CP-3	32	346	535	55
	36	421	683	62
	40	352	838	138
	44	443	1021	130
IPA	32	279	214	−23
	44	269	637	136

[a] Each figure in this column represents an average of the determinations for 3 replicate reaction flasks.

TABLE II. Influence of Incubation Temperature on Glycogen Production by *C. trachomatis* (MOP strain) in McCoy Cell Cultures.

Test no.	Hours post-infection[a]	Average number iodine-positive microcolonies/100 fields/coverslip[b]		Inoculum/coverslip (ELD_{50})
		37°	39°	
1	0	—	—	15,500
	12	0	0	
	24	1.5	7.5	
	36	6.3	22.3	
	48	21.8	29.8	
	72	28.0	35.0	
2	0	—	—	83,000
	12	0	0	
	24	5	15	
	36	28	34	
	48	38	51	

[a] Cell cultures were infected by centrifugation of the organisms onto the coverslip preparations at 1000*g* for 30 min.

[b] Four coverslips per time period per temperature group were examined, and the microcolony counts were averaged.

chlamydial strains for glutamate activity should include incubating reactants at temperatures higher than 34°, as Weiss (8) had done, to avoid the possibility of falsely negative results.

A 2° increase in incubation temperature also increased the rate at which intracellular microcolonies of the MoPn strain of *C. trachomatis* are formed in cell culture. This fact may have relevance in experimental or diagnostic procedures where it is desirable or necessary to shorten the incubation periods for infected cell culture.

Summary. The amount of $^{14}CO_2$ produced enzymatically by purified suspensions of 3 strains of *Chlamydia psittaci* from randomly labeled glutamic acid was markedly increased by raising the incubation temperature of the reaction mixtures in 4 degree increments between 32° and 44°. The rate of $^{14}CO_2$ release during the NAD-dependent reaction was increased 350% over the 12-degree range by one chlamydial strain and 130 and 136%, respectively, for the other two strains. Temperature also influenced the rate of production of iodine-positive intracellular microcolonies of the Nigg mouse pneumonitis strain of *C. trachomatis* in cell cultures. Up to 3 times as many positive microcolonies appeared in infected cell cultures incubated at 39° for 24 hr than those at 37°. Differences in colony counts tended to even out after 48 hr however.

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