

# ISOLATION OF INTRACELLULAR SYMBIOTES BY IMMUNE LYSIS OF FLAGELLATE PROTOZOA AND CHARACTERIZATION OF THEIR DNA

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## ABSTRACT

A new method dependent on immune lysis is described for the isolation of intracellular symbiotes from two species of flagellate protozoa *Blastocrithidia culicis* and *Crithidia oncopelti*. The symbiote-containing flagellates are exposed to complement and antisera prepared in rabbits against symbiote-free organisms. The immune lysis seems to weaken the plasma membranes of the flagellates so that subsequent application of gentle shearing force liberates the intracellular entities in an undamaged condition. The symbiotes are then separated from other cellular components by DNase digestion and differential centrifugation. The average recovery of symbiotes isolated by this method is 20%. Light and electron microscopy establishes the structural integrity and numerical abundance of isolated symbiotes in the final fractions. Integrity of symbiotes is further indicated by the high activity of a marker enzyme, uroporphyrinogen I synthetase.

The DNA's of symbiote-containing and symbiote-free flagellates, and of isolated symbiotes were purified and compared after isopycnic centrifugation. The comparison establishes the presence of DNA's in symbiotes of both species. The guanine-cytosine (G-C) content of symbiote DNA differs from that of host DNA's in *C. oncopelti*, but resembles that of kinetoplast DNA in *B. culicis*. The latter observation was further shown by heat denaturation study. Renaturation kinetics indicate that the genome complexity of symbiote DNA in *B. culicis* is similar to that of bacteria.

Many invertebrate cells regularly harbor intracellular, self-reproducing entities referred to as Blochmann bodies (26), plasmids (27), or symbiotes (55). They possess certain bacterial characteristics, yet often defy *in vitro* cultivation (26). Unlike intracellular pathogens, they cause no adverse effects to the cells and frequently become closely integrated into the physiology of the hosts (60).

It has been suggested that mitochondria might be evolved from symbiotic bacteria, a hypothesis

supported mainly by their biochemical and molecular similarities (33, 43, 14). However, due to the difficulty of establishing the evolutionary continuity between these two entities, the endosymbiote model for the origin of mitochondria is subject to criticism (41, 64). The study of bacterial symbiotes which are presently associated with eukaryotic cells should have significant bearing on this hypothesis (39), and should also benefit our understanding of intracellular regulation in general (60).

Hosts of bacterial symbiotes include flagellate

protozoa, which are excellent organisms for such investigations because of the homogeneity of their cell population and relatively easy cultivability in axenic media. Two species of insect flagellates *Blastocrithidia culicis* and *Crithidia oncopelti* regularly possess "diplosomes" and "bipolar bodies," which have been recently shown to be bacterial symbiotes (6, 9). Electron microscope study showed that these symbiotes have no apparent bacterial-type cell walls and are enclosed each by two unit membranes, presumably representing a host-derived membrane and a plasma membrane of the symbiotes (7). It is thus suggested that they are derivatives of bacteria with defective cell walls (7). Both species of flagellates have been made symbiote-free by chloramphenicol treatment (6, 9, 10, 11). The symbiote-free strains are able to grow, but require additional nutrients including hemin (9). Subsequent biochemical studies showed adequate heme biosynthesis in symbiote-bearing flagellates, but not in those without symbiotes (8, 10). Particularly, only the former shows the activity of an enzyme in heme biosynthesis, uroporphyrinogen I synthetase, suggesting that it may be synthesized by and localized within symbiotes (8).

It is of special interest to demonstrate that symbiotes indeed possess DNA and that it has adequate informational potential for heme biosynthesis in the host-symbiote complex. For this study, intact symbiotes free from major host components are obtained here by a novel isolation procedure involving complement-mediated immune lysis.

## MATERIALS AND METHODS

### *Culture of Flagellates*

Normal symbiote-containing *Blastocrithidia culicis* (obtained from Dr. F. G. Wallace, University of Minnesota) and *Crithidia oncopelti* (American Type Culture Collection no. 12982) were cultured in media used before (9, 17, 58). Symbiote-free strains previously obtained by chloramphenicol treatment were cultured in semidefined media (9). Flagellates were harvested at late log phase of growth and counted in a Hausser hemacytometer (Hausser Scientific, Blue Bell, Pa.).

### *Preparation of Immune Sera for Immune Lysis*

Immune sera were prepared in rabbits by using symbiote-free strains and kept at  $-20^{\circ}\text{C}$  until use. The antisera agglutinated flagellates of corresponding species at  $10^{-4}$ - $10^{-6}$  dilutions. Complements for immune lysis were from guinea pig serum.

### *Procedures for Isolation of Symbiotes*

Flagellates were sequentially treated by hypotonic shock, immune lysis, and needle passage for the liberation of their symbiotes. In preliminary attempts, trypsinization was used after the liberation of symbiotes to remove host components, but appeared to lyse the symbiotes at trypsin concentration of as low as  $50\ \mu\text{g}/\text{ml}$  for 5 min. Sensitivity of the symbiotes to trypsin thus precluded its application in the present study. For the preparation of the final symbiote fractions, samples were treated with DNase followed by differential centrifugation. The whole procedure is schematically described in Table I. Isolated symbiotes were counted in a Petroff-Hausser bacteria counting chamber.

### *Microscopy*

At different stages during the isolation, samples were examined by light and electron microscopy according to methods described elsewhere (7, 9, 11).

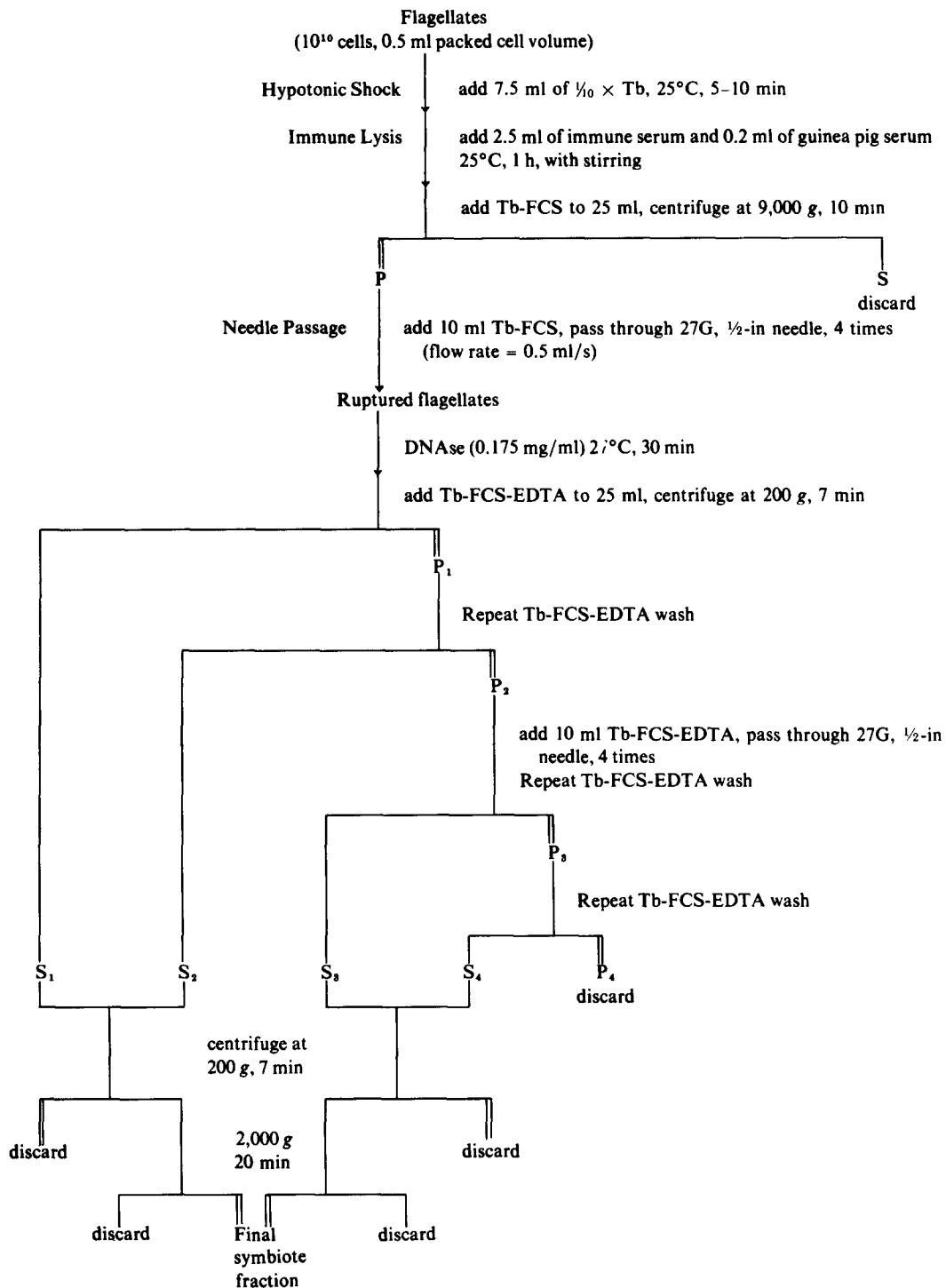
### *Biochemical Studies*

Samples were routinely examined for the absence of microbial contamination and were used for biochemical analysis either immediately or after storage at  $-20^{\circ}\text{C}$ . All chemicals used were of reagent grade.

**DNA:** For the isolation of DNA, samples were first disrupted in 7 vol of 50% sucrose in SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) by freeze-thaw and further lysed at  $37^{\circ}\text{C}$  for 15 min in 3% *N*-lauroyl sarcosine (Sigma Chemical Co., St. Louis, Mo., Na salt) in SSC. After fivefold dilution with SSC, samples were digested with RNase I and T<sub>1</sub> (Worthington Biochemical Corp., Freehold, N.J., preheated at  $100^{\circ}\text{C}$  for 10 min) at final concentrations of  $20\ \mu\text{g}/\text{ml}$  and 20 U/ml, respectively. Pronase (Calbiochem, San Diego, Calif., incubated for 18 h at  $37^{\circ}\text{C}$ ) at final concentration of  $200\ \mu\text{g}/\text{ml}$  was then incubated with the samples at  $37^{\circ}\text{C}$  for about 9 h. The samples were then dialyzed at least twice against 1 liter of  $1/10 \times$  SSC overnight. Deproteinization was performed with sodium perchlorate and chloroform-isoamyl alcohol (24:1). The DNA was finally collected by precipitation with ethanol, dissolved in  $1/10 \times$  SSC and stored over chloroform at  $4^{\circ}\text{C}$ . Isolated DNA's were routinely tested for the degree of denaturation by alkaline and formaldehyde treatment (20, 21), and of purity by spectroscopic and chemical analyses. Concentration of DNA was assayed by the diphenylamine method (16).

Buoyant densities of DNA were determined by cesium chloride equilibrium density gradient sedimentation at 40,000 rpm in a Spinco model E ultracentrifuge (Beckman Instruments, Inc. Spinco Div., Palo Alto, Calif.) fitted with an An-H rotor at  $25^{\circ}\text{C}$  (48). *Micrococcus lysodeikticus* DNA (buoyant density =  $1.731\ \text{g}/\text{ml}$ ) was used as reference DNA. Mole contents of guanine-cytosine (G-C) were calculated from the buoyant densities of the DNA samples using the empirical formula of Schildkraut et al. (48).

TABLE I  
*Preparation of Symbiote Fraction from Flagellates by Hypotonic Shock, Immune Lysis, and Needle Passage*



Flagellates used for isolation of symbiotes were washed free of culture medium and collected by centrifugation four times for 10 min at 6,000 g in PBS. All operations were at 4°C unless indicated otherwise. The buffers used include PBS, 10 mM phosphate-buffered physiological saline, pH 7.5; Tb, Trager's buffer (59) containing, in mM, NaCl 56, KCl 59, NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O 1, K<sub>2</sub>HPO<sub>4</sub> 90, NaHCO<sub>3</sub> 11, and glucose 14, pH 7.4; Tb-FCS, Tb with 2% inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.); and Tb-FCS-EDTA, Tb-FCS with 2 mM EDTA.

The ultraviolet absorbance-temperature profiles of DNA preparations were determined according to the procedure of Mandel and Marmur (31). The G-C contents of the DNA samples were calculated from the  $T_m$ 's according to the empirical formula of Marmur and Doty (35).

Renaturation studies of sheared and denatured DNA were performed according to the procedure of Wetmur and Davidson (67). The extent of DNA renaturation in 1 M NaCl, pH 7.0, was followed spectrophotometrically at 70°C (15–25° below  $T_m$ ) (63).

The sedimentation coefficients,  $s$ , of denatured DNA were determined by the alkaline band sedimentation method of Vinograd et al. (65) and Studier (57). Correction to  $s_{20,w}^{pH13}$  was made by a factor of 1.160 for the solution system (0.9 M NaCl-0.1 M NaOH, pH 13) used (57).

The kinetic genome complexity expressed in molecular weight units ( $N_D$ ), that is the number of daltons in the nonrepeating DNA complement, was calculated according to the equation of Wetmur and Davidson (67):

$$N_D = \frac{5.5 \times 10^8 (s_{20,w}^{pH13})^{1.25}}{k_2}$$

**ENZYME ASSAY:** Uroporphyrinogen I synthetase (URO-S) activity was assayed by a microspectrofluorometric method according to Sassa et al. (46) and expressed as picomoles of URO formed per second at 37°C. Protein concentration was determined by the Lowry procedure (29) using bovine serum albumin as standard.

## RESULTS

### *Morphological Observations*

Preliminary investigations indicated that disruption of flagellates for the liberation of symbiotes by mechanical methods was unsatisfactory. After these treatments, symbiotes were often damaged (Fig. 8) and recoveries were very low.

Satisfactory results were obtained with the isolation procedure of hypotonic shock-immune lysis-needle passage. During this procedure, light and electron microscopy showed an increasing abundance of symbiotes and a concomitant decrease of host-derived components, and the symbiotes maintained their structural integrity. Observations were made after each of the following steps.

**HYPOTONIC SHOCK:** Trager's buffer at 1/10 dilution caused swelling of the flagellates. The slender *B. culicis* swelled to a greater extent than the oval-shaped *C. oncopelti*. Light microscopy showed that flagellates remained intact and were

still motile. Experiments later indicated that this treatment was not essential for the liberation of symbiotes.

**IMMUNE LYSIS:** Immediately after the addition of immune sera, flagellates agglutinated into large clumps and only few remained motile after the treatment. Phase-contrast microscopy showed that some amorphous material adhered to the surface of the flagellates, probably products of the immune reaction. Although Giemsa-stained smears revealed that a small number of symbiotes were liberated, the majority of the agglutinated flagellates appeared intact (Fig. 2). Electron microscopy showed electron-dense depositions between plasma membranes of agglutinated flagellates, but no sign of physical disruption in their plasma membranes.

**NEEDLE PASSAGE:** Light microscope observations after this treatment showed a decrease in the number of flagellate clumps and the presence of numerous intact symbiotes along with cellular organelles and debris, indicating an extensive disruption of flagellates (Fig. 3). Very few symbiotes were liberated by hypotonic shock followed by needle passage in the absence of immune sera and/or guinea pig sera.

**DNASE DIGESTION:** After digestion for 30 min flagellate nuclei were almost completely absent, except very few empty ghosts; symbiotes and kinetoplasts remained intact (Fig. 4). The addition of EDTA to stop the reaction was found to reduce substantially the stickiness of the preparations.

**DIFFERENTIAL CENTRIFUGATION:** Repeated centrifugation at 200 g eliminated most of the unbroken flagellate clumps. The supernatant fractions consisted largely of symbiotes along with some kinetoplasts. The final centrifugation at 2,000 g for 20 min sedimented most of the symbiotes, while the majority of the kinetoplasts remained in the supernate.

**FINAL SYMBIOTE FRACTION:** Light microscopy of the final symbiote fractions showed the presence of abundant symbiotes, few kinetoplasts, and amorphous debris (Figs. 5 and 6). By electron microscopy, this debris was shown to be microsomes and membranous materials (Fig. 7). In Giemsa-stained preparations, the isolated symbiotes in the final fractions differed little from those in the flagellates (Fig. 1). Electron microscopy showed that symbiotes had granular cytoplasm and electron-lucid zones with DNA-like fibrillae, and were enclosed by two intact unit membranes (Fig. 9).

### Recovery of Symbiotes

Since each flagellate usually contains one symbiote (see Fig. 1), the total count of flagellates used for the isolation represents the initial number of symbiotes. Thus, the recovery of symbiotes ( $R$ ) in the final fractions can be determined as follows: number of symbiotes in the final fraction per total number of flagellates used for the isolation. On the basis of cell counts from six separately performed isolations,  $R$  ranged from 10 to 30% for the final fractions of both flagellate species (Table II).

The efficiency of the isolation procedure was also determined from the recovery of URO-S activity in the symbiote fraction. The enzyme activity was detected in symbiote fractions obtained from both species of flagellates. The average specific activities were  $97.4$  and  $94.4 \times 10^{-3}$  pmol/mg protein/s for the respective symbiote fraction of *B. culicis* and *C. oncopelti* (Table II). Recovery of URO-S activity in the symbiote fraction agreed well with that of symbiotes based on cell counts (Table II).

### DNA

The procedure used here for the isolation of DNA from flagellates and symbiotes produced native DNA essentially free of RNA and protein. The ratios of  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  of the DNA samples were  $0.55 \pm 0.05$  and  $1.50 \pm 0.15$ , respectively. These values were comparable to those reported for purified DNA from other sources (34). In the presence of formaldehyde, absorbance of the DNA increased at  $80^\circ\text{C}$ , but not at  $37^\circ\text{C}$ . The increase at  $80^\circ\text{C}$  corresponded to a hyperchromicity of at least 1.34, which was comparable to those for DNA samples denatured by heat or by  $0.1\text{ M NaOH}$  at  $25^\circ\text{C}$ . Table III shows the results of a typical run which indicated that the isolated DNA existed essentially in the form of native double-stranded helices.

**CRITHIDIA ONCOPELTI:** Isopycnic centrifugation in CsCl gradient of DNA's from symbiote-containing flagellates, symbiote-free flagellates, and isolated symbiotes indicated that they had different numbers of sedimentation bands (Fig. 10). The DNA of symbiote-containing flagellates showed three components with buoyant densities of 1.713, 1.702, and 1.693 g/ml, while that of symbiote-free flagellates showed only the first two components. The third component was present in the DNA of the isolated symbiotes which gave a

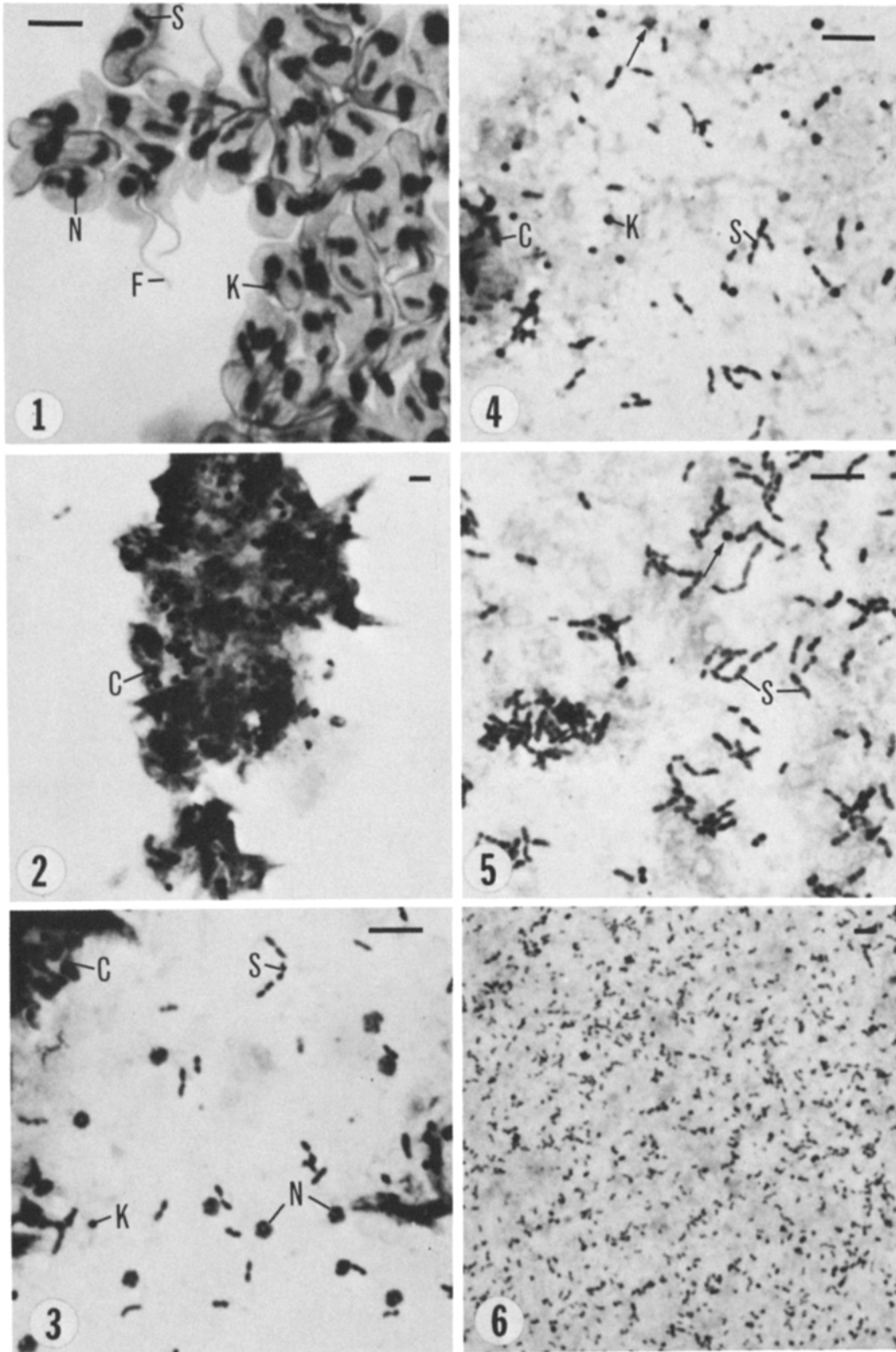
single sedimentation band in CsCl gradient, buoyant density = 1.693 g/ml (G-C = 33.7%).

**BLASTOCRITHIDIA CULICIS:** Initial study showed that the properties of DNA's isolated from symbiote-containing and symbiote-free flagellates were similar. Both strains produced identical sedimentation patterns in CsCl gradient with two bands of buoyant densities of 1.696 (kinetoplast) and 1.717 (nucleus) g/ml (Fig. 11 *a* and *b*), corresponding to 36.7 and 58.2% of G-C, respectively. The DNA's of both showed identical melting profiles with  $T_m$ 's of  $84.8$  and  $93.2^\circ\text{C}$  (Fig. 12 *a*) corresponding to G-C contents of 37.8 and 58.3%, respectively. Since these similarities did not permit a definite identification of a symbiote DNA in the flagellates, more detailed characterization of DNA's derived from these organisms was undertaken.

Renaturation of sheared and denatured DNA from both strains indicated biphasic kinetics. From the second order renaturation plots (Fig. 13 *a* and *b*), the renaturation constants were similar for the slow components (symbiote-containing,  $0.222$ ; symbiote-free,  $0.218\text{ liter}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ ), yet different for the fast components (symbiote-containing,  $1.950$ ; symbiote-free,  $0.427\text{ liter}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ ). This difference in the latter could theoretically be accounted for by the presence of a third fast-renaturing component with a second order rate constant of  $1.52\text{ liter}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ .

The DNA isolated from the symbiote fractions showed the following properties: (*a*) a single sedimentation band in CsCl gradient with a buoyant density of 1.695 (Fig. 11 *c*), corresponding to a G-C content of 35.7% (when symbiote-free flagellates were treated by the identical symbiote isolation procedure, no definite sedimentation bands were observed upon isopycnic centrifugation in CsCl gradient as shown in Fig. 11 *d*); (*b*) a monophasic melting profile with a  $T_m$  of  $82.1^\circ\text{C}$  (Fig. 12 *b*), i.e. 31.2% G-C; and (*c*) a linear renaturation kinetics with a second order rate constant of  $1.93\text{ liter}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$  (Fig. 13 *c*), a value comparable to that of the hypothetical third component estimated from the difference in renaturation kinetics between total DNA's of flagellates with and without symbiotes. It became clear that the DNA of isolated symbiotes is very similar to that of kinetoplasts in their G-C contents, as determined by heat denaturation and CsCl gradient centrifugation.

Genome complexity of the symbiote DNA of *B. culicis* was calculated from renaturation kinetics



and sedimentation velocities (Table IV). The band sedimentation experiments yielded an average  $s_{20,w}^{DH,13}$  of  $15.48 \pm 0.60$  S for the denatured fragments of DNA samples used in renaturation studies. With corrections for the G-C effect on the second order renaturation constant (67), the kinetic complexity of the symbiote DNA was determined to be  $6.72 \times 10^9$  daltons.

## DISCUSSION

### *Isolation of Symbiotes*

The present study has shown for the first time that immune lysis can be applied to the isolation of intracellular symbiotes of protozoa, and that this method is far more satisfactory than those used before. Bacterial symbiotes of ciliates and flagellates have been previously isolated by disrupting hosts with mechanical methods (36, 37, 44, 51, 52, 56, 66) normally used for mammalian cells (1, 12, 13). However, these methods rupture the rigid pellicle of protozoa with high shearing force which tends to damage the isolated symbiotes as well. This is clearly indicated by our preliminary study (see Fig. 8) and by the fact that the symbiotes so isolated invariably show certain signs of injury (53, 56), as compared with those *in situ* (4, 40).

The present method for the disruption of protozoa includes three successive treatments. Hypotonic treatment is dispensable, although it has been found to enhance the disruption of flagellates in the isolation of kinetoplasts (49). Immune lysis

proves to be essential for the eventual liberation of symbiotes. Previously, Trager (59, 61) successfully applied immune lysis to disrupt avian erythrocytes for the isolation of malaria parasites by using antiserum against uninfected red blood cells. Similarly, symbiote-free flagellates were used here to produce antisera, which would contain antibodies against host components and thus, would rupture host flagellates in the presence of complement with minimal damage to the liberated symbiotes. In the present work, although the flagellates were not disrupted, as mammalian cells are (19, 22, 42) after immune lysis, the plasma membranes of the protozoa must have been sufficiently weakened to facilitate their disruption by subsequent needle passage. These facts suggest that immune lysis may find a general application for the isolation of cell organelles.

The efficiency of the present isolation procedure is indicated by the numerical abundance and structural integrity of the symbiotes and the absence of major host components in the final fractions, as established by light and electron microscope observations. This is further substantiated by biochemical analyses on uroporphyrinogen I synthetase and DNA. Since previous studies have shown that heme-biosynthetic activity is associated with symbiote-containing, but not symbiote-free flagellates (8), it is assumed that uroporphyrinogen I synthetase, an enzyme in heme biosynthetic pathway, may be studied here as a special "marker" for symbiotes. This assumption is justi-

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FIGURES 1-6 Light micrographs of Giemsa-stained smear preparations showing the symbiotes, cell organelles, and flagellates at different stages of the isolation procedure. Magnifications: Figs. 1 and 3-5,  $\times 1,650$ ; Figs. 2 and 6,  $\times 650$ . Bars represent  $5 \mu\text{m}$ .

FIGURE 1 Flagellates of *Blastocrithidia culicis* cultured in Grace's complete medium for 2 days containing the rod-shaped symbiote (S). F, flagellum; K, kinetoplast; N, nucleus.

FIGURE 2 Flagellates of *B. culicis* agglutinated into a large clump (C) after immune lysis for 1 h at  $25^\circ\text{C}$ .

FIGURE 3 Flagellates of *B. culicis* after immune lysis-needle passage. Liberated from host cells are symbiotes (S), nuclei (N), and kinetoplasts (K). Some flagellate clumps (C) remain.

FIGURE 4 Flagellates of *B. culicis* after immune lysis-needle passage-DNAse. Symbiotes (S), kinetoplasts (K), and flagellate clumps (C) remain, while nuclei are largely removed, except few faintly stained ghosts (arrow).

FIGURE 5 Final symbiote fraction of *B. culicis* obtained by immune lysis-needle passage-DNAse followed by differential centrifugation. The fraction contains numerous intact symbiotes (S), very few kinetoplasts (arrow), and amorphous ground material. Unbroken flagellates are absent. Note the identical appearance of the isolated symbiotes and those *in situ* (Fig. 1).

FIGURE 6 Isolated fraction of *Crithidia oncopelti* containing abundant symbiotes.

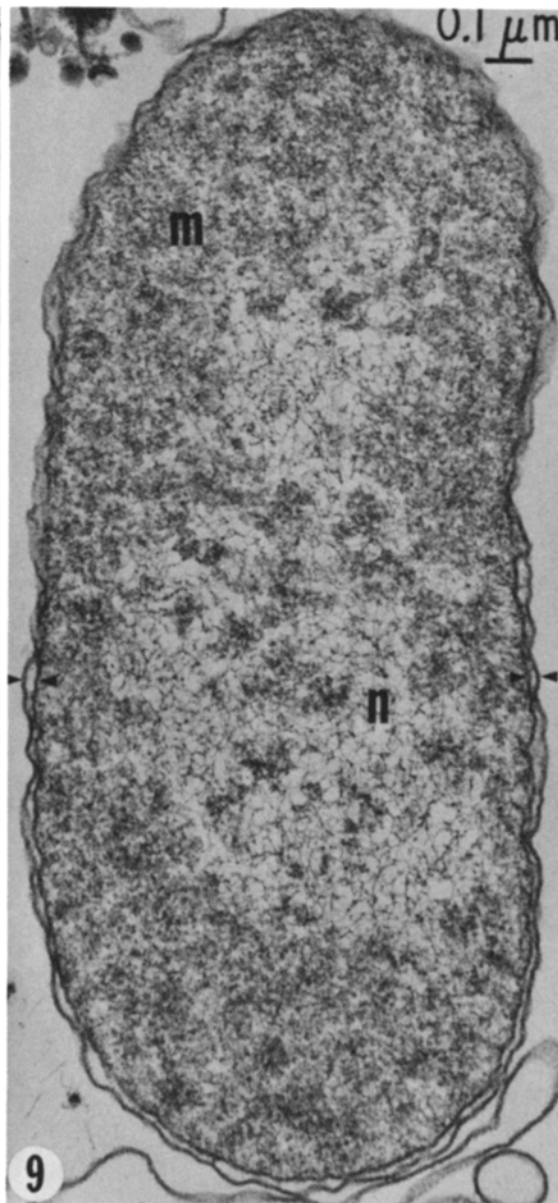
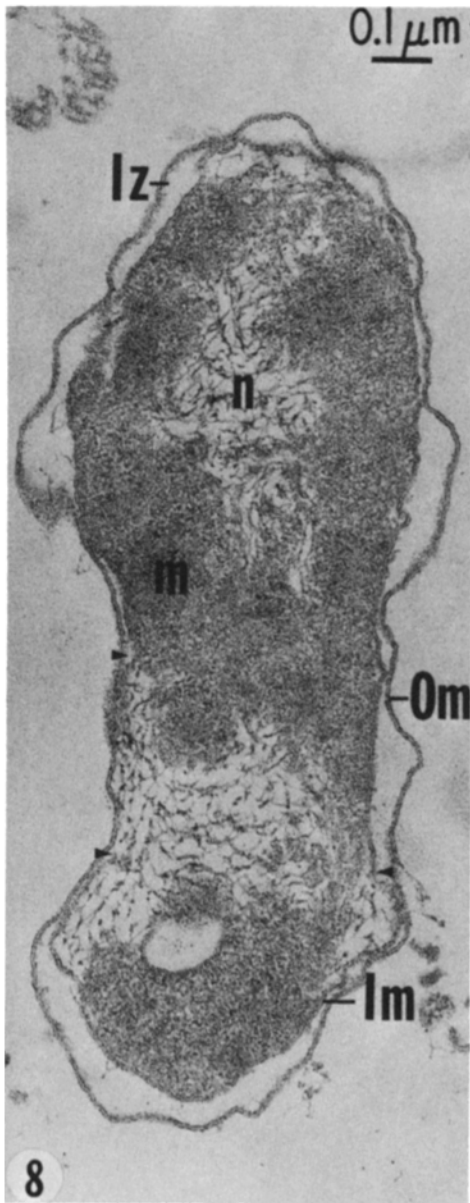
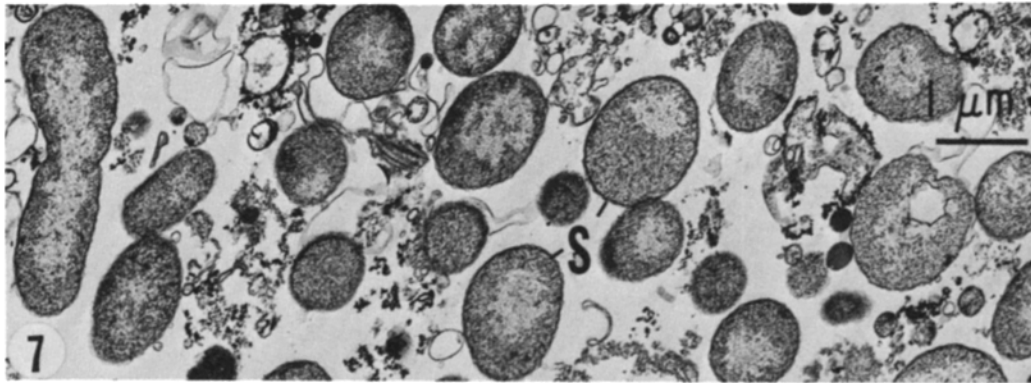




TABLE II  
Symbiote Recovery, DNA Buoyant Densities (g/ml) and URO-S Specific Activities ( $\times 10^{-3}$  pmol URO formed/mg protein/s) of Symbiote Fractions Isolated from *B. culicis* and *C. oncopelti*

Species	Isolation	No. of flagellates	No. of isolated symbiotes	Recovery of symbiotes		DNA buoyant density	Specific activity of URO-S
				Cell counts	URO-S activity		
		$\times 10^{10}$	$\times 10^6$	%			
<i>B. culicis</i>	1	1.2	2.2	20	—	1.695	—
	2	2.7	3.8	14	13	1.695	108
	3	2.7	6.0	22	—	1.695	—
	4	3.9	3.9	10	12	—	78.3
	5	3.1	8.5	28	30	—	106
<i>C. oncopelti</i>	6	0.45	0.67	15	26	1.693	94.4

fied by the finding in the symbiote fractions of a two- to threefold enhancement of enzyme specific activity compared to symbiote-containing flagellates (8). This result confirms not only the structural integrity of symbiotes but also their enrichment in the final fractions, as uroporphyrinogen I synthetase is known to be present in the cytosol of eukaryotic cells as a soluble enzyme (18) which otherwise would be lost during isolation. The agreement between recoveries determined by symbiote counts and by uroporphyrinogen I synthetase activity confirms that the enzyme detected in the symbiote-containing flagellates is totally associated with the symbiotes (8). The DNA's isolated from symbiote fractions appear to be homogeneous by isopycnic centrifugation in CsCl gradient. Although no parallel work is available for comparison with our study on the symbiote DNA of *B. culicis*, the buoyant density of DNA from symbiote fractions of *C. oncopelti* accords well with that

of a satellite DNA interpreted to be of symbiote origin (36) (see below). This indicates the absence of contaminating DNA from nuclei and kinetoplasts and thus confirms the absence or scarcity of these cell organelles in the symbiote fractions. Moreover, symbiote DNA's can be purified after DNase treatment of isolated symbiotes, indicating the structural integrity of their envelopes.

Previous ultrastructural studies of symbiotes *in situ* has led to speculation on the nature and origin of their peripheral membranes that can now be assessed with the present observation made on isolated symbiotes. Isolated symbiotes retain their outer envelopes, which have been considered to be host-derived membranes (7). When freshly freed from their host cells, parasitic protozoa also often preserve host-derived membranes, an observation which leads to the suggestion that they may be modified and become integral parts of the parasites (62). Such modifications seem even more

FIGURES 7-9 Electron micrographs of symbiotes liberated from host flagellates. Glutaraldehyde-osmium tetroxide fixation; uranyl acetate-lead citrate staining.

FIGURE 7 Symbiote fraction of *C. oncopelti* containing abundant intact symbiotes (S). Each symbiote consists of an electron-dense matrix and an electron-lucid nuclear area. Interspersed among symbiotes are dense granules, membrane fragments, microsomes, and some unidentifiable materials.  $\times 12,000$ .

FIGURE 8 A symbiote liberated from *B. culicis* by shaking with Ballotini beads (no. 12) for 4 min, in a Mickel disintegrator. The agranular appearance of the dense matrix (*m*) and clumping of DNA-like fibrillae in the nuclear zone (*n*) indicate degeneration of the symbiote. The irregular shape of the symbiote, discontinuation of the inner membrane (arrow), and the expanded intermediate zone (*Iz*) between the inner (*Im*) and outer membrane (*Om*) also suggest cell damage.  $\times 80,000$ .

FIGURE 9 A symbiote of *C. oncopelti* isolated by the procedure reported here. The integrity of the symbiote is indicated by the entirety of the double envelopes (arrows), the granular appearance of the matrix (*m*) and the network of DNA-like fibrillae in the nuclear zone (*n*).  $\times 63,000$ .

TABLE III  
Typical Reaction of DNA Preparations with  
Formaldehyde and NaOH

Treatment	$A_{260}$ of DNA	Hyperchromicity
None	0.573	1.00
37°C, 45 min, 1% (vol/vol) HCHO	0.581	1.01
80°C, 20 min, 1% (vol/vol) HCHO	0.767	1.34
100°C, 20 min, 1% (vol/vol) HCHO	0.767	1.34
25°C, 5 min, 0.1 M NaOH	0.745	1.30
100°C, 20 min	0.779	1.36

The reaction was carried out in glass-stoppered quartz cuvettes containing 1.0 ml of DNA in SSC, pH 7.0, at concentrations of 0.573 absorbance unit measured at 260 nm. Formaldehyde or NaOH was added at the concentrations indicated and the absorbance at 260 nm recorded with correction for volume changes at the temperatures and time intervals indicated.

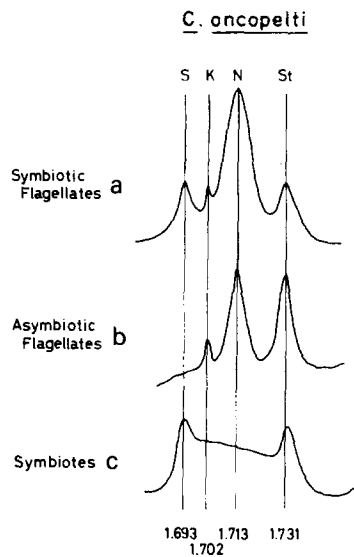


FIGURE 10 Microdensitometer tracings of UV absorption scan of *C. oncopelti* DNA preparations equilibrated in CsCl density gradients after 20 h at 40,000 rpm at 25°C. (a) Symbiote-containing flagellates; (b) symbiote-free flagellates; and (c) symbiotes isolated by immune lysis. In all cases, *M. lysodeikticus* DNA (buoyant density = 1.731 g/ml) was used as reference DNA.

likely for the host-derived membranes of symbiotes, which are associated more intimately and permanently than parasites with host cells. Indeed, sensitivity of the symbiotes to tryptic digestion suggests that their peripheral envelopes differ significantly from those of free-living bacteria

which are trypsin resistant unless stripped of their cell walls (30, 45). Perhaps, cell wall materials once possessed by these bacterial symbiotes have been lost or modified during the evolution of the symbiotic associations, a notion already suggested by ultrastructural study (7). It is noteworthy that mitochondria are also surrounded by two unit membranes of different biochemical nature (28). It would be of great interest to compare the chemical composition of symbiote envelopes with that of the two membranes of mitochondria.

#### DNA Studies

The DNA studies presented in this paper offer conclusive evidence for the presence of DNA in the symbiotes of *B. culicis* and *C. oncopelti*. This fact has not been clearly demonstrated previously, although it has been suggested by the ultrastructural findings of DNA-like fibrillae in the symbiotes of both species (7) and by a weak incorporation of [ $^3$ H]thymidine by those of *B. culicis* (23).

Previously, isopycnic sedimentation study has

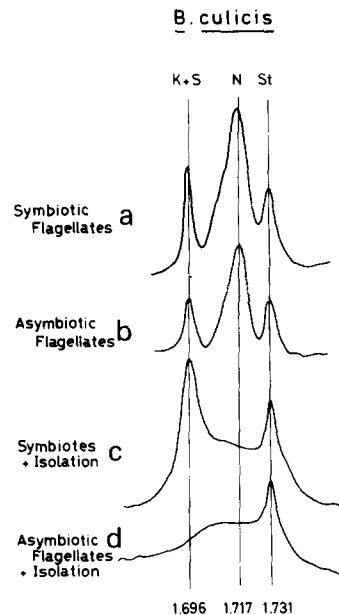


FIGURE 11 Microdensitometer tracings of UV absorption scan of *B. culicis* DNA preparations equilibrated in CsCl density gradients after 20 h at 40,000 rpm at 25°C. (a) symbiote-containing flagellates; (b) symbiote-free flagellates; (c) symbiotes isolated by immune lysis; and (d) symbiote-free flagellates treated by the identical procedure for the isolation of symbiotes. In all cases, *M. lysodeikticus* DNA (buoyant density = 1.731 g/ml) was used as reference DNA.

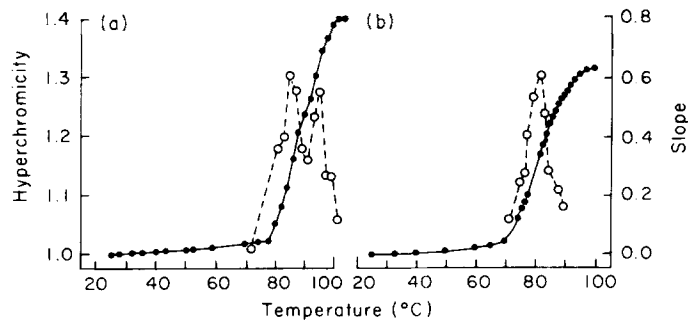


FIGURE 12 Thermal denaturation curves of DNA derived from *B. culicis* in SSC. The absorbance at each temperature ( $A_T$ ) was recorded with correction for the concentration dilution caused by solvent expansion relative to the volume at 25°C. Hyperchromicity ( $A_T/A_{25}$ ) was plotted vs. the temperature of the solution. The temperature corresponding to half the final increase in relative absorbance  $T_m$  was determined by plotting the differential  $(\Delta A/\Delta T)/(A_{100} - A_{25})$  vs. the temperature, where  $\Delta A/\Delta T$  was determined as  $(A_{T_2} - A_{T_1})/(T_2 - T_1)$  and  $A_{100} - A_{25}$  correspond to the maximum increase in absorbance. Maxima on the differential plot represent  $T_m$ 's. Hyperchromicity plot (—●—●—); differential plot (—○—○—). (a) Symbiote-containing or symbiote-free flagellates. (b) Isolated symbiotes.

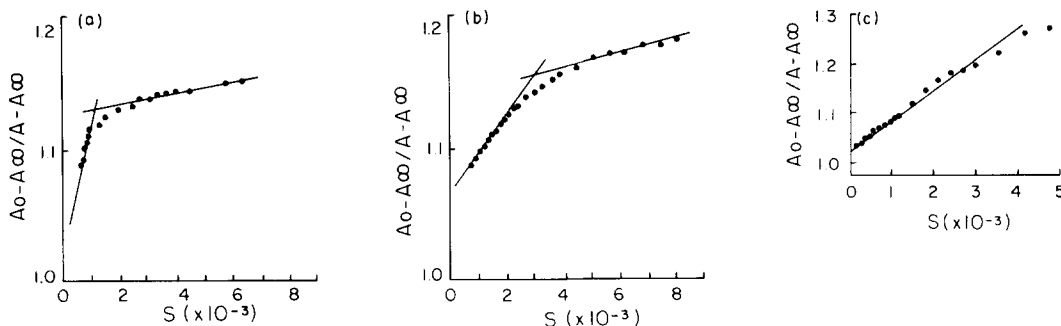


FIGURE 13 Second-order renaturation kinetics of sheared and denatured DNA's of *B. culicis* in 1 M NaCl, pH 7.0, at 70°C. The plots were normalized to an  $S_{20,w}^{H_2O}$  value of 15.5. Second order renaturation constants  $k_2$  were determined according to the equation of Wetmur and Davidson (67):  $[(A_0 - A_\infty)/(A - A_\infty)] = (k_2 P/2) t + 1$  where  $A_\infty$  = absorbance of native DNA,  $A_0$  = absorbance of denatured DNA,  $A$  = absorbance of DNA at time  $t$ ,  $t$  = time in seconds, and  $P$  = the total DNA phosphate concentration,  $1.47 \times A_\infty \text{ mol l}^{-1}$ . Thus,  $k_2$  = slope  $\times (2/P)$ . (a) Symbiote-containing flagellates. (b) Symbiote-free flagellates. (c) Isolated symbiotes.

shown that the DNA of *C. oncopelti* can be resolved into three different components including a "satellite" peak with a buoyant density of 1.691 (36), 1.693 (47), or 1.694 (38) g/ml. The satellite DNA was considered to be of symbiote origin, as it was predominant in a mechanically isolated fraction, presumably enriched in "bipolar bodies" or symbiotes (36). However, the enrichment of symbiotes was not verified in this fraction, which indeed contained nuclei and kinetoplasts, manifested as distinct DNA peaks after isopycnic centrifugation (36). Convincing evidence is provided here by showing that the satellite symbiote DNA (buoyant density = 1.693 g/ml) which is

absent in symbiote-free flagellates, is present in normal symbiote-containing flagellates and isolated symbiotes as a single homogeneous species.

The DNA of *B. culicis* has been previously studied only in the normal symbiote-containing strain by isopycnic centrifugation, which revealed two sedimentation bands, presumably derived from kinetoplasts and nuclei of the host organisms (38). Similar results were obtained in the present study for symbiote-containing as well as symbiote-free flagellates, both having identical banding patterns and, in addition, similar DNA melting profiles. The seeming absence of a third satellite DNA attributable to symbiotes raises the question

TABLE IV  
Comparison of Renaturation Kinetics and Genome Complexities of DNA

DNA source	Number of preparations	$s_{30, w}^{PH, 18}$	$k_2$ <i>liter · mol<sup>-1</sup> · s<sup>-1</sup></i>	Genome complexity <i>daltons</i>
Symbiote-containing <i>B. culicis</i>	3	16.5	0.222 (slow component) 1.950 (fast component)	$8.67 \times 10^{10}$
Symbiote-free <i>B. culicis</i>	2	14.6	0.218 (slow component) 0.427 (fast component)	$8.86 \times 10^{10}$
Isolated symbiotes of <i>B. culicis</i>	4	15.3	1.93	$6.72 \times 10^9$
<i>Paramecium</i> symbiotes:				
mu 540				$3.4 \times 10^9$
mu 551				$3.3 \times 10^9$
lambda 299				$0.71 \times 10^9$
<i>E. coli</i>				$2.5 \times 10^9$
<i>Mycoplasma fermentans</i> (PG 18)				$0.48 \times 10^9$

Second order renaturation rate constants ( $k_2$ ) were normalized to the average sedimentation velocity,  $s_{30, w}^{PH, 18}$  15.5. Average values for the genome complexity of DNA's of *B. culicis* were calculated from renaturation rate constants using the procedure and formulae of Wetmur and Davidson (67). Values for genome complexities of other DNA's were obtained from: *mu* 540 and *mu* 551 (15), *lambda* 299 (53), *E. coli* (67), and *M. fermentans* (PG 18) (2).

whether they contain DNA. However, CsCl centrifugation of isolated symbiote fractions produces a single homogeneous DNA band, which is absent in comparable fractions obtained from symbiote-free flagellates after identical treatment, thus indicating the symbiote origin of this DNA. Moreover, a significant difference was found between the DNA's of symbiote-containing and symbiote-free flagellates in renaturation kinetics that suggests the presence of an additional fast renaturing component in the DNA of the former, very probably associated with the symbiotes. The similarity of the second order rate constants between this fast component ( $1.52 \text{ liter} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ ) and the DNA of isolated symbiotes ( $1.93 \text{ liter} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ ) indicates the identity of the two DNA's. These results clearly demonstrate the presence of symbiote DNA in *B. culicis*. The absence of a distinct symbiote DNA band after isopycnic centrifugation of total cell DNA can be explained by the similarities between symbiote and kinetoplast DNA's in melting temperatures and buoyant densities, viz. G-C contents. An analogous finding has been previously reported by Stevenson (56) who found that the DNA's of *mu* endosymbiotes and macronuclei in *Paramecium aurelia* had a common

buoyant density and were thus not separable by isopycnic sedimentation.

The genome complexity of symbiotes in *B. culicis* was determined in the present study to be  $6.7 \times 10^9$  daltons. Parallel studies have been made recently in *Paramecium* endosymbiotes *lambda* and *mu*, whose genome complexities are of the order of  $10^8$  and  $10^9$  daltons, respectively (15, 53). A more recent report on endosymbiotic "xenosomes" of marine ciliates indicates a genome complexity similar to that of *lambda* (54). In comparison, symbiotes of *B. culicis* are more similar to *mu* than *lambda* and xenosomes. The genome complexities of conventional bacteria fall within the range of  $10^8$ - $10^{10}$  daltons (3, 24, 32), those of mycoplasma  $10^8$  daltons (2), and those of chloroplasts and mitochondria (including kinetoplasts)  $10^5$ - $10^8$  daltons (5, 25, 50) (see Table IV). As clearly indicated by the molecular size of their genomes, the symbiotes of *B. culicis* possess bacterium-type DNA. The same conclusion may well be applicable to the symbiotes of *C. oncopelti* in view of their overwhelming resemblance to those of *B. culicis* in many aspects (7-11).

Preliminary investigations (unpublished observations) show that the symbiote of *B. culicis*

possesses a genome with a multiplicity of about 10, a value close to that of lambda particles in *P. aurelia* (53), but less than that of mitochondria or chloroplasts (5, 25). This finding, if substantiated, would indicate that these intracellular bacterial symbiotes may well be "intermediates" between bacteria and mitochondria, thus lending support to our contention that study of intracellular symbiosis should benefit our understanding of the origin of cell organelles. However, Gibson (15) has found only a single-copy genome in the mu particle of *P. aurelia*. It is evident that further intensive study is needed to permit a generalization of the nature of intracellular bacterium-like entities.

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