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Separation of Viable *Rickettsia typhi* from Yolk Sac and L Cell Host Components by Renografin Density Gradient Centrifugation

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Rickettsia typhi cultivated in the yolk sac of chicken embryos or in L cells irradiated 7 days previously was separated from host cell components by two cycles of Renografin density gradient centrifugation. Preliminary steps involved differential centrifugation and centrifugation over a layer of 10% bovine plasma albumin of infected yolk sac suspensions, or trypsinization and passage through filters of wide porosity of infected L cell suspensions. Rickettsial preparations obtained by these methods appeared to be free from host cell components while retaining high levels of hemolytic activity, egg infectivity, and capacity to catabolize glutamate. Average yields were 3.3 mg of rickettsial protein per yolk sac or 0.44 mg per 16-oz (ca. 475-ml) L cell culture. Extracts from these two preparations displayed malate dehydrogenase activity of electrophoretic mobility identical to each other but quite different in migration patterns from the corresponding host cell enzymes. This method of separation of rickettsiae from host cell constituents appears to be particularly well suited for the study of rickettsial enzymatic activity.

There is no single procedure of separation of rickettsiae from host components that can be applied to all species and for all purposes. Investigators interested primarily in the antigenic, chemical, or morphological properties of the rickettsiae often use procedures that are unlikely to yield viable microorganisms (18) or inactivate the starting material with a low concentration of formaldehyde (2, 4, 5, 11, 13, 14). Although these investigators have been highly successful in removing host cell material, the effect of formaldehyde on the purification procedure has not been adequately gauged. Those who wish to preserve the biological properties of rickettsiae generally follow the procedure outlined by Wisseman et al. (29) or introduce relatively minor modifications (1, 9, 15, 17, 26). The key steps in these procedures are treatment of the rickettsial suspension with Celite (Johns Manville product), bovine plasma albumin, and a proteolytic enzyme. Those who purify *Coxiella burnetii*, which retains its viability remarkably well under a great variety of environmental and experimental conditions, often introduce, as a final step, sucrose gradient isolation (22, 27). With the typhus and spotted fever rickettsiae there is steady deterioration of the physiological properties of the microorganisms during purification, and the essential Celite step is particularly detrimental. With the highly labile scrub typhus rickettsia Celite cannot be used, and purification with partial retention of infectivity was obtained by the substitution of Amberlite cation exchange resin (Rohm and Haas product) (31).

The purification of *Rickettsia typhi* and subsequent characterization by enzymatic analysis, metabolic stability, and complement fixation here presented establishes a procedure of separation of rickettsiae from host components that should have wide applicability. The use of Renografin was prompted by the findings of Friis (7) and Howard et al. (10) that *Chlamydia psittaci* and *C. trachomatis*, respectively, are successfully purified with Renografin density gradients without appreciable loss of infectivity.

MATERIALS AND METHODS

Preparation of pools and infected yolk sacs and L cell cultures. The seed consisted of ampoules of a yolk sac suspension of the Wilmington strain of *R. typhi* purified through step 2 (see below) and maintained at −70 C. Six-day chicken embryos were inoculated with 0.4-ml volumes of a 10^{-6} to 10^{-7} concentration of seed via the yolk sac and incubated at 35 C. After one-third to one-half of the embryos had died, 10 to 12 days later, the yolk sacs of the surviving embryos were harvested. Lots of 8 to 10 yolk sacs were mixed with equal volumes (30 to 50 ml) of brain heart infusion broth (BHI; Difco) in 250-ml glass thick-walled bottles containing glass beads, quickly frozen in an ethanol-CO_2 mixture, and maintained at −70 C. Infected cell cultures (LM_3 cell line derivative of NCTC L-929) were obtained as described pre-

viously (25) with the modifications in procedure described below. After irradiation (3,000 R with ^{60}Co) the cells were placed in 16-oz (ca. 475-ml) plastic flasks (5×10^6 cells/flask) and cultivated as monolayers for 7 days prior to infection. The medium was removed, and the cells were oscillated gently for 1 h at room temperature with 2-ml volumes of rickettsiae diluted in BHI, 15-ml volumes of buffered medium (25) were then added, and the cultures were incubated at 32 C. Generally 40 cultures were used for each experiment. The heavily infected cells were harvested and processed immediately as described below 136 to 140 h later.

Preliminary separation of rickettsiae. (i) From yolk sac. Step 1: The infected yolk sac pools were quickly thawed, shaken with glass beads, and further macerated by pipetting. They were diluted with additional BHI and centrifuged at 10,400 × *g* for 30 min. The fat adhering to the walls of the centrifuge tubes was carefully removed. The pellet was suspended in a volume of BHI approximately equivalent to the weight of the yolk sacs and centrifuged at 210 × *g* for 10 min. The pellet was resuspended in a similar volume of BHI and centrifuged as above. The two supernatant fluids were combined and centrifuged at 17,300 × *g* for 15 min. The supernatant layers and any fat adhering to the walls were discarded, and the pellet was suspended in about one-half the original volume in BHI. Step 2: Suspension volumes of 5 ml were layered over 20 ml of a mixture of a Pentex 35% aqueous solution of bovine albumin without preservative (Calbiochem) and concentrated diluent K36 (26) having a final concentration of 10% bovine plasma albumin in single strength K36. After centrifugation at 480 × *g* for 20 min, small sediments were discarded, and the BHI and bovine plasma albumin layers were combined and diluted with approximately 3 volumes of K36 and centrifuged at 10,400 × *g* for 30 min. The pellets were resuspended in K36 and centrifuged at 17,300 × *g* for 15 min, and the pellet was suspended in a total of 15 to 16 ml of K36. Diluent K36 consists of 0.1 M KCl, 0.015 M NaCl, 0.05 M potassium phosphate buffer, pH 7.0.

(ii) From cell culture. Step 1: The cells were released from the flask surface by brief treatment with 10 ml of 0.5% trypsin (GIBCO) in K36. The culture supernatants, the cells, and a wash of the flask with 5 ml of BHI were combined and centrifuged at 10,400 × *g* for 30 min. The pellets were suspended in K36 (approximately 2 ml/flask), and an amount of 5% trypsin (Difco) was added to a final concentration of 0.5% and shaken at 150 rpm in a 30 C water bath for 45 min. An equal volume of BHI was added, and the suspension was centrifuged at 210 × *g* for 10 min. The supernatant fluid was centrifuged at 17,300 × *g* for 15 min, and the pellet was suspended in K36, 1 ml/flask. Step 2: The suspension, in lots of 20 ml, was filtered through type AP 20, 47-mm, microfilter glass filters (pore size, 0.8 to 14 μm) in a Sterifil aseptic system assembly (Millipore Corp.). The filters were washed with 10-ml volumes of K36, and the combined filtrates and washes were filtered once again through an AP 20 filter. The resulting suspension was centrifuged at 17,300 × *g* for 15 min, and the pellets were suspended in a total of 15 to 16 ml of K36.

Renografin density gradients separation. Step 3: The procedure was identical for rickettsiae derived from yolk sac or cell culture. Linear density gradients of Renografin-76 (Squibb) were prepared with K36 as the diluent using a conical bore, triple outlet gradient marker connected to a multistaltic pump (Buchler Instruments). Six cellulose nitrate tubes (Beckman) (8.9 by 2.5 cm) containing 32 ml of 30 to 45% linear Renografin gradients were used for the first cycle, and three tubes (7.6 by 2.5 cm) containing 27 ml of 20 to 45% gradients were used for the second cycle. In both cases 2.5-ml volumes of rickettsial suspension were layered over the gradient and centrifuged in a Spinco L2-65 B ultracentrifuge (Beckman) at 25,000 rpm for 1 h. Rotor SW27 was used for the first cycle and rotor SW25.1 for the second cycle. After the first gradient centrifugation cycle, areas of the gradient above and below the rickettsial band were removed with a Pasteur pipette by suction. The rickettsial band was then drawn into a syringe through a 14-gauge cannula, diluted 10-fold with K36, and centrifuged at 17,300 × *g* for 10 min. The pellets were resuspended in a total of 7.5 ml of K36, and the suspension was passed through the second Renografin gradient; the rickettsiae were collected, centrifuged as above, and suspended in a volume of 6.5 ml of K36 and used as described below.

Test of biological activity. Hemolytic activity of the rickettsial suspensions was determined as outlined by Snyder et al. (21) by incubation of 0.3-ml samples of the appropriate dilutions of rickettsiae with 0.6-ml volumes of a 25% suspension of sheep erythrocytes for 2.5 h at 34 C. The reaction was terminated by the addition to each tube of 3 ml of saline containing 0.2% formalin. The tests were carried out in triplicate, and an additional set of rickettsiae heated at 56 C for 0.5 h served as hemolysis control. As in previous work (25), the hemolytic unit was defined as the rickettsial suspension that elicited an absorbance at 545 nm of 0.3 per milliliter after all additions.

Production of CO_2 from glutamate and glucose was determined as described previously (26) in 25-ml Erlenmeyer flasks fitted with plastic cups suspended from rubber stoppers. To each vessel were added 5 mM glutamate with 0.1 μCi of [U-^{14}C]glutamate or 0.5 mM glucose with 0.1 μCi of [U-^{14}C]glucose, 2 mM $MgCl_2$, and 0.2 to 0.3 ml of rickettsial suspension (replaced by K36 in control flasks) diluted to a total volume of 2 ml with K36. The flasks were incubated for 2.5 h at 34 C, and the reaction was terminated by the addition of 0.2 ml of Hyamine (Packard Instrument Co.) to the cups and 0.4 ml of 25% trichloroacetic acid to the reaction mixtures. The results were determined by the amount of $^{14}CO_2$ trapped by the Hyamine and were expressed in terms of nanomoles of CO_2 per milligram of protein, per embryo or per cell culture flask.

Chicken embryo infectivity was determined by the inoculation of two groups of 15 eggs with two concentrations of rickettsiae, usually 10^{-3} and 10^{-4}, and determination of mean survival times of the embryos. The numbers of infectious rickettsiae in a suspension were calculated as described by Weiss et

al. (24) on the basis of complete titrations with *R. typhi*. This method provided satisfactory comparisons of specimens inoculated into a single batch of embryos during the same day.

Polyacrylamide gel electrophoresis of rickettsial extracts. Rickettsial extracts were obtained by passing 5.5-ml samples from step 3 preparations, or from step 1 uninfected control yolk sac or L cell preparations, twice through a French pressure cell (AMINCO) at 20,000 lb/in^2. The suspensions were then centrifuged at 4,340 × *g* for 10 min, the sediments containing intact or imperfectly disrupted rickettsiae were discarded, and the supernatant fluids were stored at −70 C. Electrophoresis of samples was performed on an E-C vertical slab gel electrophoresis system (E-C Apparatus, St. Peterburg, Fla.). The buffer, pH 8.4, consisted of 0.089 M tris(hydroxymethyl)aminomethane (Tris), 0.0025 M disodium ethylenediaminetetraacetate, and 0.089 M boric acid (Tris-borate) (16). A 5% gel was prepared by the addition of 9.5 g of acrylamide and 0.5 g of *N,N'*-methylene bisacrylamide to 205 ml of Tris-borate, and, immediately before pouring, 0.2 ml of *N,N,N',N'*-tetramethylethylenediamine and 0.2 g of ammonium persulfate. The mixture was poured to a thickness of 6 mm and polymerization was complete in 30 min. Tris-borate buffer was then added to the electrode chambers, and a voltage of 300 V was applied for 1 h.

Samples of the extracts described above were diluted to a concentration of 400 μg of protein per ml with 0.05 M phosphate buffer, pH 7.4. To 0.25-ml samples were added crystalline sucrose to a final concentration of about 5% and approximately 0.01 ml of 0.05% bromophenol blue (tracking dye) in 5 mM NaOH. Duplicate specimens of four preparations were applied to the eight slots of the slab. A constant voltage of 300 V was applied for approximately 3 h, until the tracking dye had migrated off the bottom of the gel. The gel was then removed from the apparatus and stained for malate dehydrogenase activity by the method of El-Sharkawy and Huisingh (6). The staining reaction required approximately 4 h at 37 C in the dark and was terminated by immersing the gel in 5% acetic acid.

Miscellaneous procedures. Rickettsiae were stained by the method of Gimenez (8). Prior fixation of the slides with 1% buffered (pH 6.8) formalin greatly improved the reproducibility of differential staining.

All preparations were tested for contamination with cultivable bacteria by the inoculation of thioglycolate broth and blood plates, which were placed in a candle jar and incubated at 37 C. Tests for the presence of *Mycoplasma* were performed as described by Rothblat (19). No *Mycoplasma* were detected, and any preparation from which bacteria were cultivated was discarded.

Protein was determined by the method of Lowry et al. (12) with Dade Lab-trol (American Hospital Supply Corp., Miami, Fla.) as the standard.

Hyperimmune antinormal (uninfected) yolk sac serum was prepared by the subcutaneous injection of rabbits with 5-ml mixtures of equal volumes of yolk sac antigen and incomplete Freund adjuvant, 1 ml into each of five lymph node regions. The antigen consisted of 5 mg of step 1 yolk sac prepared in K36 instead of BHI. Three and 6 weeks later the rabbits received 1 mg of antigen plus adjuvant into the cervical lymph node region. They were bled 7 to 10 days after the last injection. A similar schedule of injections with L cell preparations did not yield satisfactory antisera. Complement fixation tests were performed by standard microtiter techniques (20, 23) using control and rickettsial preparations that had been heated at 56 C for 0.5 h to inactivate the rickettsiae. Serum dilutions of 1:160 and 1:320 were the most sensitive for the detection of antigen. The antigen titer was defined as the highest dilution eliciting at least 70% fixation. The antigen dilution was calculated on the assumption that 1 ml represented the undiluted contents of one yolk sac.

RESULTS AND DISCUSSION

Purification of rickettsiae. In step 1 from yolk sac the cumbersome procedure of disrupting the infected cells by shaking with glass beads and by repeated pipetting was retained in preference to the more expedient treatment of the yolk sacs with a blender or homogenizer. The former procedure disrupts primarily the highly infected entodermal cells, whereas the connective tissue is not fragmented and is readily removed by very low centrifugal forces. When mechanical means of disruption are used, somewhat higher centrifugal forces must be used and the supernatant material appears to have a higher concentration of host cell protein (H. R. Dressler, personal communication). Step 2 is a modification of a commonly used procedure, which is based on the precipitation, due to unknown reasons, of yolk sac material by bovine plasma albumin (3). It is not of particular value for the removal of L cell constituents. With disrupted L cells, passage through filters of large pore size is useful for the removal of a large fraction of the remaining L cell nuclei and membranes. Conversely, filtration is not useful for yolk sac material because remaining lipid covers the pores and interferes with the passage of the rickettsiae.

A number of Renografin density gradients were tried, and all effectively separated rickettsiae from host cell components. The two-cycle procedure finally adopted is illustrated in Fig. 1. In the first gradient (30 to 45%) the rickettsiae form a band (no. 2) near the top at a relative density of 1.19 to 1.20 g/ml, but are clearly separated from the bulk of the host material which remains above the gradient. In some cases, especially in suspensions derived from yolk sac, some of the host material forms a fibrous, funnel-shaped, vertical band that is removed easily. The rickettsiae either form a single broad band or two adjacent bands, barely

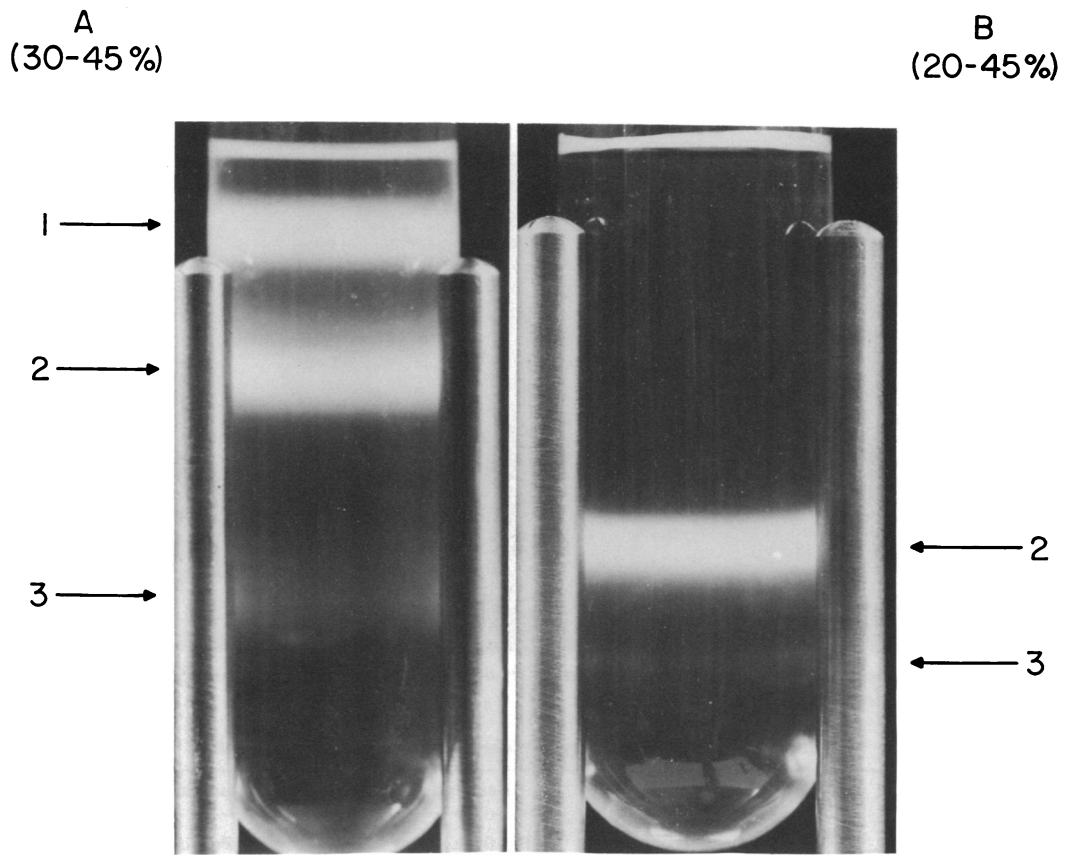

FIG. 1. *Bands obtained by Renografin density gradient sedimentation of rickettsiae derived from L cells. (A) First cycle. (B) Second cycle.*

distinguishable from each other. A third band (Fig. 1, no. 3) contains particles similar in size to rickettsiae, but, when examined by electron microscopy, these particles appear to be a mixture of rickettsiae and host cell mitochondria. In the second gradient (20 to 45%) the rickettsiae form a band again at a relative density of 1.19 to 1.20 g/ml at about the center of the tube (Fig. 1B), whereas extraneous material is barely visible. When the first gradient is overloaded, a faint band rich in mitochondria appears again (no. 3). The final rickettsial preparation, when stained by the Gimenez method (8) and examined by light microscopy, appears to contain no host cell material (Fig. 2). When examined by electron microscopy, only a rare disrupted mitochondrion is detected, whereas the appearance of the rickettsiae is typical.

Biological activity of rickettsial preparations. The protein content and biological activities of rickettsial preparations derived from L cells are shown in Table 1. Experiments 1 through 9, performed prior to standardization of the Renografin density gradient steps, involved some delays and imperfections as the procedure was being developed. Nevertheless, it is clear that the protein content declined more rapidly than biological activity measured by hemolytic activity and glutamate metabolism. Whereas hemolytic activity reflected only rickettsial activity, CO_2 production from step 1 rickettsiae probably included some host cell activity. On the basis of hemolytic activity, recovery of rickettsial activity at step 3 was 40% of step 1. Egg infectivity in individual experiments paralleled hemolytic activity, but these results are not shown, because averaging values from different experiments introduces a relatively large error due to egg variability. When the Renografin density gradient step was standardized and the procedure was executed more efficiently without attempting to evaluate steps 1 and 2 (experiments 10–12), recovery in terms of total and specific activity (per milligram of protein) was more satisfactory.

Table 2 displays the results obtained with

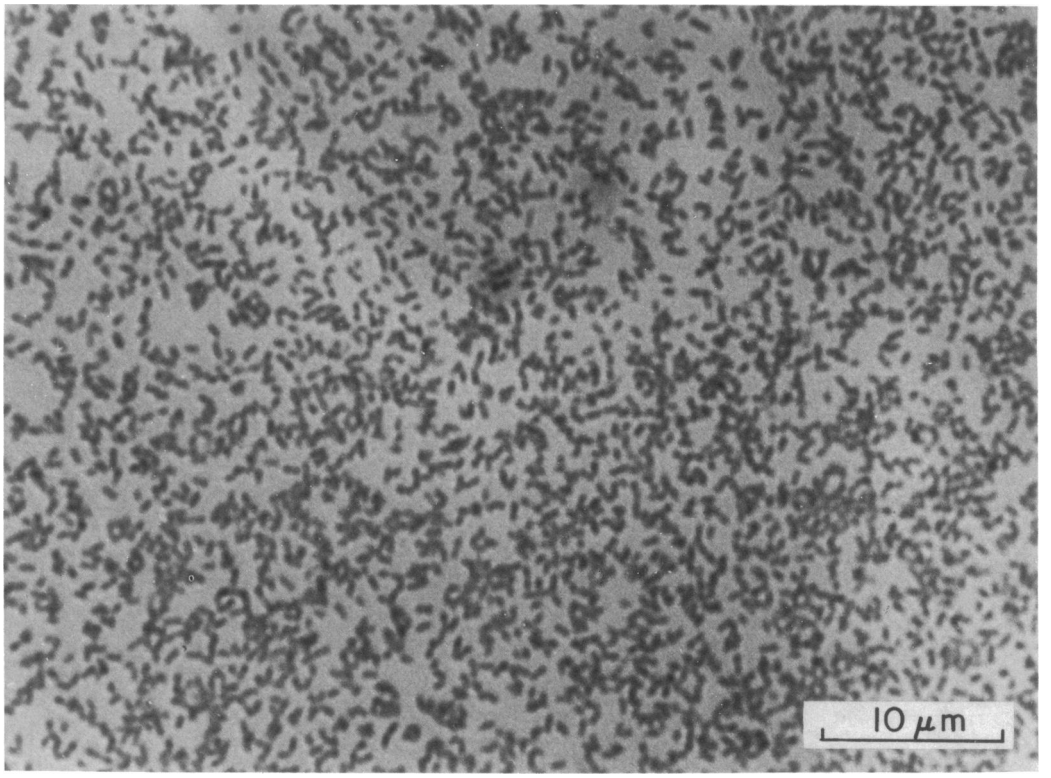

FIG. 2. *Photomicrograph of rickettsiae separated from yolk sac, step 3. Fixed in 1% buffered (pH 6.8) formalin and stained by Gimenez' method (8).*

TABLE 1. *Biological activity of rickettsiae separated from L cells*

Expt no.	Step	Protein/flask (mean ± SE[a] [mg])	Hemolytic activity[b]		CO_2 production from glutamate[c]	
			Per flask (mean ± SE)	Per mg of protein (mean)	Per flask (mean ± SE)	Per mg of protein (mean)
1–9	1	2.16 ± 0.16	989 ± 143	458	496 ± 96	230
	2	1.00 ± 0.14	728 ± 147	728	286 ± 60	286
	3	0.27 ± 0.04	388 ± 80	1,437	169 ± 28	625
10–12	3	0.44 ± 0.01	898 ± 81	2,041	902 ± 237	2,050

[a] SE, Standard error.
[b] Expressed in units as defined in Materials and Methods.
[c] Expressed in nanomoles for the 2.5-h period of incubation at 34 C.

TABLE 2. *Biological activity of rickettsiae separated from yolk sacs*[a]

Expt no.	Step	Mg of protein (mean [± SE])	Complement fixation units (anti-yolk sac serum)	Hemolytic activity (mean [± SE])	CO_2 production from glutamate (mean [± SE])	Egg infectivity
13[b]	1	42.2[c]	19,100	17,400	11,400	18[d]
	2	6.3	610	16,600	4,200	14
	3	2.7	<25	7,300	5,900	6
13–17[e]	3	3.3 ± 0.37	<25[f]	2,740 ± 207	2,106 ± 260	Not done

[a] See footnotes to Table 1.
[b] Activity per embryo.
[c] About one-half of this value can be attributed to the suspending BHI.
[d] × 10^7 infectious rickettsiae.
[e] Activity per milligram of protein.
[f] Three preparations only.

rickettsial suspensions obtained from yolk sac. In experiment 13 five determinations were performed with step 1, 2, and 3 rickettsiae. The results were confirmed in other experiments for which less complete data are available. The irregularity in the activity of step 2 rickettsiae, namely, high hemolytic activity and low production of CO_2 from glutamate, is not a consistent finding. If the overall trend is considered, there is a more rapid decline of protein content than of rickettsial activity. Host antigens, present in step 1 rickettsiae in very high concentration, as measured by the complement fixation test, were virtually absent in step 3 rickettsiae. Two other preparations were similarly tested, and in these cases also the antigen concentration was below the measurable level. Recovery of rickettsial activity as determined by hemolytic activity, glutamate metabolism, and egg infectivity was about 40%. Glutamate metabolism is a valid test of rickettsial activity in step 1 preparations, because the rate of CO_2 production from glutamate by uninfected yolk sac preparations is very low (H. R. Dressler, personal communication). When the results from five step 3 preparations from yolk sac were averaged and corrected for specific activity, they were very similar to those obtained with rickettsiae grown in L cells. Glucose was not utilized by purified rickettsial suspensions, as measured by CO_2 production.

Malate dehydrogenase activity of rickettsial extracts. Wisseman et al. (28) obtained excellent evidence that *R. typhi* catabolizes glutamate via the dicarboxylic acid pathway and thus is expected to have several dehydrogenases including malate dehydrogenase. In our studies, malate dehydrogenase activity was easily demonstrated with extracts from step 3 rickettsiae separated from yolk sac or from L cells. Figure 3 illustrates the electrophoretic migration pattern of this enzyme in acrylamide gel. Since no attempt was made to completely solubilize the enzyme or to separate it from other proteins, not all of the enzyme migrated through the acrylamide gel, and some of the bands are relatively broad. It is clear, however, that the migration patterns of the malate dehydrogenases of uninfected yolk sac and L cells are quite distinct from each other and from those of purified rickettsiae. The two preparations of rickettsiae, from yolk sac and from L cells, yielded enzymes with entirely compara-

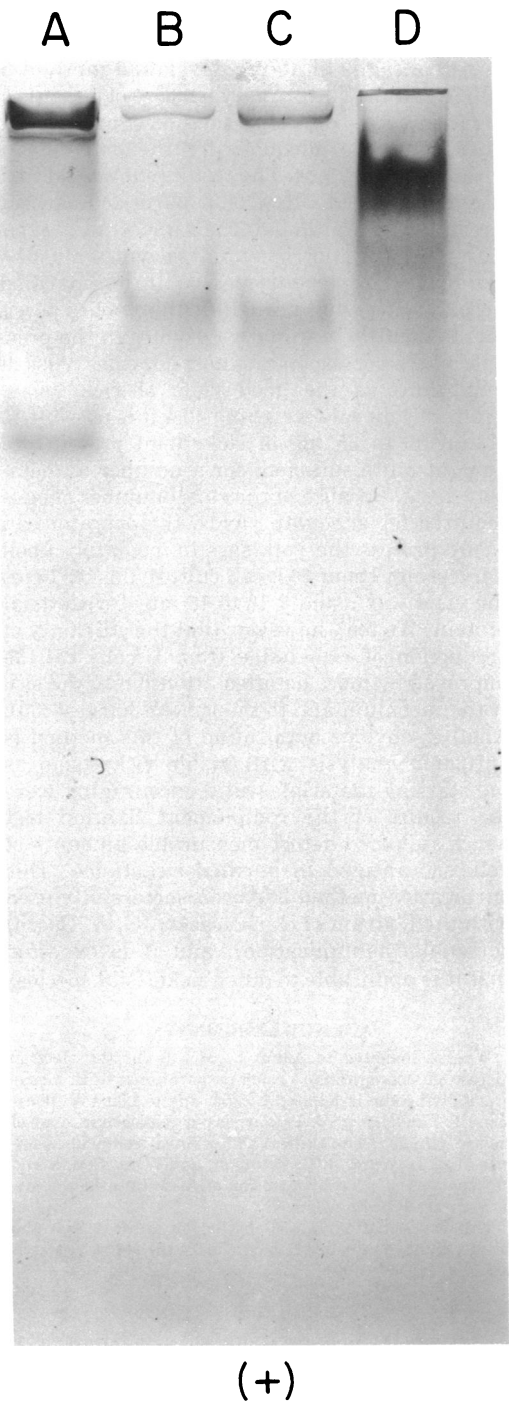

FIG. 3. *Acrylamide gel slab electrophoresis of host and rickettsial extracts. To each channel 0.25-ml samples containing approximately 100 μg of total protein were applied. The slab was subjected to a constant voltage of 300 V until the tracking dye (bromophenol blue) had migrated to the bottom of the gel (approximately 3 h). The slab was then stained for malate dehydrogenase (6). (A) L cell; (B) R. typhi purified from L cells; (C) R. typhi purified from yolk sac; (D) yolk sac.*

ble migration patterns and provided no evidence of contamination with host enzymes.

Advantages of above-described method of purification. The primary purpose of this investigation was to develop a method of purification that would be adequate for the detection of rickettsial enzymes. The results shown in Fig. 3 strongly indicate that this purpose has been accomplished, although the same kind of separation of host and rickettsial enzymes must be extended to other enzymes. With less purified preparations it has been difficult, if not impossible, to identify rickettsial enzymes in the presence of the corresponding host enzymes. Also of importance is the good yield of rickettsiae. From 10 yolk sacs weighing 40 g it is possible to obtain 30 to 35 mg of rickettsial protein, an amount quite sufficient for a number of metabolic tests. Because of the small number of eggs required for adequate yields, the operator can easily process the yolk sacs immediately upon harvesting. From 40 L cell culture flasks (16 oz) the yield was usually 16 to 18 mg of rickettsial protein. We feel, however, that the efficiency of production of rickettsiae from L cells can be improved by more detailed attention to the size of the inoculum and to the time of harvest (30). Another obvious application of this method is antigenic analysis with viable rickettsiae as the starting material. Quite encouraging were the results of the complement fixation test which failed to detect measurable amounts of yolk sac antigen in purified rickettsiae. This purification method has been successfully used with the E strain of *R. prowazeki* (G. A. Dasch, personal communication), and it is expected that it is applicable to other rickettsial species.

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