Acta virol. **33** : 361—370, 1989

METHODS FOR PURIFICATION OF *RICKETTSIA PROWAZEKII* SEPARATED FROM THE HOST TISSUE: A STEP-BY-STEP COMPARISON

L. P. ANISKOVICH, M. E. EREMEEVA, N. M. BALAEVA, V. F. IGNATOVICH, M. I. ARTEMIEV, V. V. EMELYANOV, N. S. SMIRNOVA

The N. F. Gamaleya Research Institute of Epidemiology and Microbiology, the U.S.S.R. Academy of Medical Sciences, 123098 Moscow, U.S.S.R.

Received January 18, 1988

Summary. — Two methods for purification of *Rickettsia prowazekii* strains E, E Vir, and Breinl grown in chick embryo yolk sacs are described. These methods combine either differential centrifugation or sucrose mix, centrifugation through sucrose cushion, 10 mmol/l $MgCl_2$ treatment, filtration through a glass filter AP-20 and 2 cycles of verografin discontinuous density gradient centrifugation. The purification procedure including sucrose mix allowed to recover about 38—42 % biologically active rickettsiae, a yield which was by 10 % higher than that obtained by the method begining at differential centrifugation. The rickettsiae free of host cell components preserved their infectious activity. The obtained biomass was suitable for immunological and biological characterization of *Rickettsia prowazekii* and for isolation of its total DNA.

Key words: *Rickettsia prowazekii*; *purification*; *sucrose mix*; *verografin*

Introduction

Molecular biologic approach for the study of *Rickettsia prowazekii* was promoted by the development of new methods of preparing biologically active pathogens completely devoid of host tissue contaminants. Several methods were described meeting these requirements (Wisseman *et al.*, 1951; Balaeva *et al.*, 1968; Palmer *et al.*, 1974; Winkler, 1976; Dasch and Weiss, 1977; Smith and Winkler, 1977; Hanson *et al.*, 1981; Loginov *et al.*, 1982); they employ absorption to ecteola cellulose or celite, or BSA flocculation, and precipitation with antiserum against yolk sac antigens. The methods of *Rickettsia prowazekii* purification presented in this paper do not need these compounds and are economically highly efficient. In addition, we describe the yield and viability of purified microorganisms and their contamination with the host cell components.

Materials and Methods

Rickettsia prowazekii. Standard virulent strain Breinl (Wolbych *et al.*, 1922), virulent revertant of a weakly pathogenic strain E — strain E Vir (Balaeva, 1969) and strain E (Clavero and Perez-Gallardo, 1943) were used. The strains underwent in chick embryo yolk sacs 132—135, 8—10, and 278—282 passages, respectively. The yolk sacs just removed aseptically from the dead or dying embryos on days 6 or 7 post-infection (p.i.) contained large amounts of rickettsiae. The infected yolk sacs were used immediately or stored at −70 °C.

Purification. The purification of *Rickettsia prowazekii* completely devoid of host cell components falls into three stages.

Stage 1: *Preliminary preparation of the rickettsial biomass*; this was achieved by 2 different ways:

A. Differential centrifugation was carried out according to Weiss *et al.* (1975). The infected yolk sacs ($R^{100\%}$) were ground with glass beads, the homogenate was diluted in 4 vol of buffered K 36 solution (0.1 mol/l KCl, 0.015 mol/l NaCl, 0.05 mol/l phosphate buffer, pH 7.0) to prepare a suspension $R^{20\%}$ equivalent to 0.2 g of yolk sacs per 1 ml; the suspension was centrifuged at 10,400 × g for 30 min, the supernatant and the fat layer at the surface were removed and the pellet was resuspended in K 36 buffer in a vol equivalent to the yolk sacs weight. The centrifugation was carried out at 210 × g for 10 min. The pellet was resuspended in the identical volume of the same buffer and centrifuged as described above. The supernatants prepared by 2 low-speed centrifugations were combined and centrifuged at 17,300 × g for 15 min.

B. The infected yolk sac suspension was partially purified by the sucrose mix method according to the authors' original procedure based on recommendations of Hanson *et al.* (1981) and Palmer *et al.* (1974). The rickettsial suspension $R^{20\%}$ prepared as described above was mixed with an equal volume of 50 % sucrose solution in K 36 buffer and centrifuged at 27,500 × g for 1.5 hr.

Stage 2: *Purification by the sucrose cushion method.* This procedure had been designed for purification of *C. burnetii* (Williams *et al.*, 1981), here it is used for further purification of *Rickettsia prowazekii.* The rickettsial pellet prepared either by differential centrifugation or by sucrose mix was resuspended in K 36 buffer in a vol equivalent to the weight of yolk sacs. One vol of the resulting suspension was layered over 5 vol of K 36 buffer containing 30 % sucrose and 7.6 % Verografin (SPOFA, Czechoslovakia). The rickettsiae were sedimented by centrifugation at 25,000 × g for 60 min. After centrifugation, the supernatant was removed and the rickettsial pellet was resuspended in 1/2 of the original vol of yolk sac homogenate in buffer K 36. The rickettsiae were repurified by the centrifugation on sucrose cushion according to the above described schedule.

The pellet resuspended in buffer K 36 containing 0.218 mol/l sucrose and 10 mmol/l $MgCl_2$ (suspension $R^{80\%}$) was incubated at 0 °C for 10 min (Winkler, 1976) and centrifuged at 380 × g for 7 min. The supernatant was filtered through a glass filter AP-20 (47 mm, 50 μm, Millipore Corp., Bedford, Mass.) and centrifuged at 17,300 × g for 15 min. The pellet was resuspended in buffer K 36 (preparation $R^{580\%}$).

Stage 3: *The gradient centrifugation.* Ultracentrifugation of partially purified rickettsial suspension was carried out according to the procedure of Hanson *et al.* (1981) (the percentage of verografin in the discontinuous density gradient was modified).Verografin was diluted in buffer K 36. The 2 ml volumes of the suspension $R^{580\%}$ purified by sucrose cushion centrifugation was carefully layered over the verografin discontinuous gradient containing 1 ml of 45 % verografin solution (density d = 1.25 g/ml), 4.5 ml of 30 % solution (d = 1.215 g/ml), 2 ml of 30 % solution (d = 1.17 g/ml) and 4.5 ml of 20 % solution (d = 1.12 g/ml) (i.e. the equivalent of 3 to 5 infected yolk sacs per gradient; Dasch and Weiss, 1977), and centrifuged at 115,000 × g (L5-65 Beckman centrifuge, rotor SW 40) for 60 min. Then the parts of the gradient above to rickettsial bands were removed. The rickettsial material at the interphase 30—38 % was collected with a sterile syringe (needle 27 G I/2). The verografin-rickettsial mixture was diluted 10-fold with the buffer used for the verografin gradient. The rickettsiae pelleted by centrifugation at 17,300 × g for 10 min (Weiss *et al.*, 1975) were resuspended in the buffer K 36 (suspension $R^{1160\%}$) and reisolated by gradient ultracentrifugation according to the above described procedure.

To preserve the viability of rickettsiae they were resuspended in brain heart infusion broth (BHI, Difco). If the microorganisms were further used for the extraction of nucleic acids, SSC buffer (0.15 mol/l NaCl, 0.015 mol/l $Na_3C_6H_5O_7$, pH 7.0) ± sucrose or any other suitable buffer was used.

Preparation of antisera. Anti-rickettsial serum not reacting with the host cells was prepared by immunization of chinchilla rabbits weighing 2.5 kg with 2.5 ml of egg culture of *Rickettsia prowazekii* strain E containing 10^7-10^8 of ID_{50} per chick embryo per 1 ml. After 3 days a concentrated testicle suspension was prepared. It was injected intraperitoneally to an intact rabbit. The animal was bled on days 15 or 30 postinoculation (p.i.).

To prepare an antiserum to the intact chick embryo yolk sac antigens, the rabbits were immunized with the supernatant fluid from three-times-ether-extracted yolk sac suspension (1—2 mg of protein). For primary and secondary immunizations (day 44) the antigen was injected intramuscularly and into the footpads with an equal volume of complete Freund's adjuvant and intravenously without adjuvant. Subsequent doses were injected intramuscularly, subcutaneously and into the footpads with the adjuvent on days 51, 65, and 79. The animals were bled 3 months after the first immunization.

The antiserum to intact chick embryo yolk sac antigens and the anti-rickettsial antiserum showed in CF titres at least of 1 : 160 — 1 : 320 and 1 : 640 — 1 : 1280, respectively.

Differential content of the yolk sac protein and rickettsial protein in the samples was determined by complement fixation (CF) test in the presence of appropriate antisera (Zdrodovsky and Golinevich, 1972; Osterman, 1983). The samples were lyophilized and ether-treated at 4 °C for 24 hr. After extraction the samples were centrifuged at 1500 × g for 10 min. The traces of ether were then removed by short-term exposure in a lyophilizer. The pellet was suspended in the buffer K 36. For construction of calibration curves characterizing the dependence of the hemolysate absorption decrease (ΔA_{413}) in the concentration of yolk sac proteins or rickettsial proteins, suspension of uninfected yolk sacs or suspension of *Rickettsia prowazekii* strain E separated from host components by one of the methods described above were used. The efficiency of purification was expressed as the host cell component contamination index. This index is a percentage ratio of the proteins (yolk sac/rickettsial).

The rickettsial suspension with protein concentration of 6 μg/ml was purified in verografin density gradient. It was used as antigen in a modified enzyme immunoassay as described by Engwall and Perlmann (1972) For coating 100 μl of antigen at twofold dilutions in 0.1 mol/l carbonate buffer, pH 9.6, were given into the wells of 96-well plates (Linbro, Titertek) and incubated for 16 hr at 4 °C. The plates were rinsed 3 to 5 times, first with 0.05 mol/l sodium phosphate buffer, pH 7.2—7.4 containing 0.05 % Tween-20 (buffer II) and then with the same buffer without Tween (buffer I). Then 200 μl of 0.1 % of gelatin solution in buffer I were added into the wells and the plates were incubated for 40 min at 37 °C. After rinsing as described above, 100 μl of titrated rabbit antiserum to the intact chick embryos yolk sac antigen diluted 1 : 5000 in 0.1 % gelatin solution were added into the wells and kept for another 2 hr at 37 °C. After washing, a conjugate diluted in buffer II was added (goat antibodies against rabbit immunoglobulins labelled with horseradish peroxidase) and incubated for 2 hr at 37 °C. After rinsing the plates 100 μl of the substrate solution was given into the wells (1 % solution o-phenylene diamine in 0.05 mol/l citrate buffer pH 4.45 and 0.003 % H_2O_2). The reaction was stopped after 3 to 5 min by adding 100 μl of 0.1 N H_2SO_4 into each well and photometrically scanned by Titertek Multiscan at 492 nm.

Counting of rickettsiae. The rickettsiae were counted using a modified method of Silverman *et al.* (1979). Equal volume of rickettsial suspension and a reference suspension of *E. coli* stained with 0.1 % aqueous solution of methylene blue (3×10^8/ml) were rapidly mixed, sprayed by a syringe onto a hot slide, flame-fixed and stained by Chimenez. The microorganisms were counted in the visible light in the microscope (drawtube × 1.5, eye-piece × 7 and oil-immersion lens × 90). The concentration of rickettsiae was determined by the formula $C_R = R/E\ C_E$, where R and E are the counted quantities of rickettsiae and reference particles, respectively, C_E is the concentration of *E. coli* estimated by counting in a Goryaev chamber.

Biologic activity tests. The infectivity of rickettsiae in the material tested was determined by titration in chick embryos (Zdrodovsky and Golinevich, 1972) or white mice (Ignatovich and Rybkina, 1963).

Protein measurement. The protein was performed according to Lowry *et al.* (1951) in the presence of 5 % SDS using BSA as reference protein.

Electron microscopy. The rickettsial pellet was fixed by a mixture of glutaraldehyde, paraformaldehyde, and picric acid prepared in 0.2 mol/l cacodylate buffer pH 7.2 (Ito and Rikihisa, 1981) for 1 hr at room temperature. It was then fixed with 1 % OsO_4 solution in the same buffer, dehydrated in cool ethanol gradient and in absolute acetone and embedded in Araldit M according

Table 1. Step-by-step comparison of 2 methods for preparation of purified Rickettsia prowazekii (strain E) from infected yolk sacs (stored at —70 °C)[1]

Purification stage	Yield (%)						Contamination with host cell components (%)[5]	
	Rickettsiae[2]		Total protein[3]		Rickettsial protein[4]			
	1	2	1	2	1	2	1	2
Suspension of the infected yolk sacs (20 %)	100	100	100	100	100	100	1431	1431
Differential centrifugation	86		12.5		85.0		124	
Sucrose mix		95		15.7		93.4		157.8
Sucrose cushion: I			4.3	6.4	59.8	80.0	11.5	20.57
II	62	73	3.86	5.1	56.5	75.0	5.57	6.1
$MgCl_2$ treatment			3.3	4.1	49.6	59.4	4.04	2.26
Filtration through filter AP-20	51	60	2.9	3.7	43.0	56.0	3.3	1.79
Verografin density gradient centrifugation:								
I L-band			2.0	2.5	30.4	37.6	0.33	0.19
H-band			0.6	0.77	9.2	12.0	0.46	0.29
II L-band	25	34	1.5	1.9	23.3	29.5	0	0
H-band	1	2.5	0.06	0.08	0.93	1.3	0	0

[1] Average values of 3 tests
[2] Determined by the method of cell count with reference preparation; 5.7×10^{12} cells is taken as 100 %
[3] Measured according to Lowry
[4] Determined in the complement fixation reaction; 285.8 mg of rickettsial protein is taken as 100 %
[5] The ratio (percentage) of the proteins of yolk sacs / rickettsiae determined in CF
Notice: 1 and 2 indicate method 1 or 2, respectively.

to the standard procedure. Ultrathin sections obtained in Ultratome III LKB 8800 were contrasted for 30 min with aqueous solution of uranyl acetate and for 10 min by lead citrate according to Reynalds. The grids were examined in the microscope JEM 100 B by accelerating voltage 80 kV and aperture diaphragm of 50 nm diameter.

Results and Discussion

Two methods of production of preparative quantities of *Rickettsia prowazekii* of different strains from infected chick embryo yolk sacs freshly extracted or stored at —70 °C have been described, based on different first stage procedures: either a differential centrifugation or sucrose mix centrifugation. Further purification steps were performed on sucrose cushion followed by filtration through a glass filter AP-20 (stage 2) and finally, by 2 cycles of verografin discontinuous density gradient centrifugation (stage 3). Table 1 shows the results of stage-by-stage comparison of the two methods

of the preparation of *Rickettsia prowazekii* strain E from the yolk sacs kept at -70 °C. The efficiency of purification was evaluated using the following characteristics: 1. rickettsial yield determined by direct count or indirectly in CF in the presence of antiserum to *Rickettsia prowazekii* strain E; 2. contamination with hošt cell components. The latter parameter is the ratio of the proteins of the yolk sacs and rickettsiae expressed percentually. The differential protein content in the samples was determined in CF in the presence of appropriate antisera.

The comparison of the two separation methods indicated the advantages of sucrose mix over differential centrifugation. First, the replacement of four-stage differential centrifugation by the one-stage sucrose mix (unpurified yolk sac suspension was mixed with an equal vol of 50 % sucrose and centrifuged under specially chosen conditions) saves time. Second, as seen from experimental data presented in Table 1, the sucrose mix method yields 93.4—95 % of the starting amount of rickettsiae, which is by 10 % higher than the yield provided by differential centrifugation.

At the second stage, the sucrose cushion centrifugation employed by Williams *et al.* (1981) for *Coxiella burnetii* was first time used here for purification of *Rickettsia prowazekii* The primarily purified rickettsial biomass was layered onto the solution containing 30 % sucrose and 7.6 % verografin and centrifuged twice. The above indicated concentrations of sucrose and verografin appeared to be the most efficient as compared to other combinations tested. Two consecutive centrifugations through a sucrose cushion reduced the contamination index to a 1/22 of its value at the first stage. Thus, it may be concluded that this method is highly efficient.

Our purification procedure does not need celite adsorption of the yolk sac contaminants or the removal of the host cell material by BSA flocculation and precipitation with antiserum against yolk sac antigens (Dasch and Weiss, 1977; Smith and Winkler, 1977; Hanson *et al.*, 1981; Palmer *et al.*, 1974; Wisseman *et al.*, 1951). It is therefore, highly economically efficient. Moreover, celite does not seem appropriate for it worsens the physiological properitils of rickettsiae as reported by Weiss *et al.* (1975).

To achieve a higher purity the rickettsieae were further purified by $MgCl_2$ treatment (Winkler, 1976) and filtration through a glass filter AP-20 (Weiss *et al.*, 1975; Williams and Weiss, 1978). In the presence of Mg^{2+} ions at 0 °C the yolk sac mitochondria seem to aggregate (Winkler, 1976). After this treatment the contamination of the preparation with host cell components decreases in 2.7 and 1.3 times at the first and second purification methods, respectively. The use of a filter with a large pore diameter appreared as useful. It further reduced the contamination index by 1.2 times.

By the third final stage, the rickettsiae were purified twice by preparative discontinuous verografin density gradient ultracentrifugation according to a modified procedure of Hanson *et al.* (1981). Verografin was diluted in buffer K 36. An additional 20 % verografin layer was added to the gradient. The results of ultracentrifugation are presented in Fig. 1. About 93 % of the host cell material was retained on top of the discontinuous gradient (No. 1). They

form a hardly noticeable band in contrast to that reported by Weiss *et al.* (1975). This indicates that the purification method used in this paper before layering the verografin gradient was more efficient. The uppermost band was not observed at all in the 2nd gradient, which confirms the high degree of purification.

We achieved more efficient separation of rickettsial material than Hanson *et al.* (1981) by including an additional zone to the verografin gradient. The rickettsiae were well separated from the host cell material and formed 2 bands as seen in Fig. 1 (Nos. 2 and 3). The second major band formed in the 30—38 % interphase consisted of the so-called "light" rickettsiae (L-band). In addition to the major band, an additional H-band consisting of "heavy" rickettsiae and host cell mitochondria was seen in gradient I on the 45 % cushion (Weiss *et al.*, 1975). The purification of rickettsiae in the gradient I provides an almost complete separation of mitochondria from "light" rickettsiae (Smith and Winkler, 1977). Therefore, the H-band formed during centrifugation of the "light" rickettsiae in the gradient II consisted of only "heavy" rickettsiae. The absence of mitochondria in the H-band of the gradient II was confirmed by electron microscopy (Fig. 3).

The rickettsiae formed two bands, both in the 1st and 2nd gradients. The ratio of the H-band and L-band, however, characterizing the infectivity (Hanson *et al.*, 1981) was different in the two gradients. The infectivity index was found to be 0.15 in the 1st gradient and 0.04 in the 2nd on in use of feshly extracted infected yolk sacs. That the H : L ratio was different from zero in the 2nd gradient can probably be explained by the plasmolysis of some microorganisms in verografin solution. These verografin-damaged rickettsiae formed a band of higher-density area than the intact microorganisms. The data on the verografin-induced plasmolysis of rickettsiae leading to a decrease in infectivity were reported by Hanson *et al.* (1981). These authors, however, obtained a 0.2 infectivity index in the 2nd renografin gradient in PBS buffer (5.2 mmol/l KH_2PO_4, 8.1 mmol/l Na_2HPO_4, 116 mmol/l NaCl, pH 7.0). Indeed, it has been found that the substitution of K^+ ions for Na^+ ions increased the viability of rickettsiae (Weiss, 1965). Therefore, the use of buffer K 36 seemed to be more appropriate for the preparation of biologically active rickettsiae. The H : L value of 0.15 in the 1st gradient was apparently the sum of two components: 0.04 and 0.11. The former accounts for the share of rickettsiae that lost their infectivity owing to verografin-induced plasmolysis and the latter for the share of the pathogens that lost the infectivity in the course of purification.

When rickettsiae were prepared from the yolk sacs stored at —70 °C the infectivity index in the 1st gradient was twice as high as with the unfrozen material. In the 2nd gradient it was still equal to 0.04 (Table 1). This is in accordance with the data of Winkler (1976) that freezing and thawing leads to a lesion of cytoplasmic membrane of rickettsiae thereby causing inactivation. The decrease of infectivity resulting from freezing and thawing was confirmed by the method of titration of the egg culture of *Rickettsia prowazekii* strain E Vir in chick embryos. Thus, ID_{50} of this culture before and after

freezing and thawing were 8.45×10^7 and 1.09×10^6, respectively. This is the cause of the decrease of the final yield of biologically active rickettsiae. Whereas with the freshly extracted yolk sacs the 1st and 2 nd purification methods yield 28—32 % and 38—42 % of biologically active rickettsiae, respectively, with the infectious material stored at —70 ° the final yield appeared to be by about 10 % lower.

The rickettsiae from the L-band and H-band of verografin gradient were morphologically different which is confirmed by electron microscopic data. "Light" rickettsiae had a normal structure, the cellular wall was closely adjoined to the cytoplasmic membrane and a globular microcapsule was seen in all the microorganisms (Fig. 2). In contrast to the light rickettsiae, in the heavy ones the periplasmic space was expanded and not all the microorganisms had a microcapsule (Fig. 3).

The rickettsiae that underwent the 2nd verografin density gradient centrifugation apperaed to be uncontaminated by the host material (as shown by electron microscopic examination). No contamination with host components has been observed in a enzyme immunoassay after the treatment of a purified rickettsiae preparation having protein concentration 6 μg/ml with antiserum against intact chick embryo yolk sac antigens. The purified rickettsial preparation was biologically active as determined by the method of titration in white mice. Thus, for example, the infectivity in ID_{50} of the egg culture of *Rickettsia prowazekii* strain E Vir and of the preparation of these microorganisms purified by the first method were 3.78×10^8 and 1.27×10^5, respectively.

Thus, purification procedures presented in this paper are different from those described in literature: the purification is shorter and some reagents such as ecteola cellulose, celite, BSA and antiserum to yolk sac antigens were not needed. The described methods provided a high yield of biologically active *Rickettsia prowazekii* separated from the host cell components. These methods have been successfully used in our laboratory for the preparation of *Rickettsia prowazekii* of strains E, E Vir, and Breinl.

References

Balaeva, N. M. (1969): Enhancement of virulence of vaccine strain E of *Rickettsiae prowazekii* during passages in white mice lungs. *Vestn. Akad. med. Nauk SSSR* **1969** (**10**), 51—56 (in Russian).

Balaeva, N. M., Tikhonenko, T. I., and Zubok, L. P. (1968): Purification of *Rickettsia mooseri* cultivated in chick embryos by the method of column chromatography, pp. 193—199. In P. F. Zdrodovsky (Ed.): *Problems of Infectious Pathology and Immunology*, Meditsina, Moscow (in Russian).

Clavero, C. G., and Perez-Gallardo, P. F. (1943): Experimental study of a nonpathogenic immunizing strain of *Rickettsia prowazekii*. *Rev. Sanid. Hig. publ.* (*Madr.*) **17**. *Trop. Dis. Bull.* (1944), **41**, 24.

Dasch, G. A., and Weiss, E. (177): Characterization of the Madrid E strain of *Rickettsia prowazekii* purified by renografin density gradient centrifugation. *Infect. Immun.* **15**, 280—286.

Engwall, E., and Perlmann, P. (1972): Enzyme-linked immunosorbent assay. ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109**, 129—135.

Hanson, B. A., Wisseman, C. L., Jr., Waddel, A., and Silverman, D. J. (1981): Some characteristics of heavy and light bands of *Rickettsia prowazekii* on renografin gradients. *Infect. Immun.* **34**, 596–604.

Ignatovich, V. F., and Rybkina, N. N. (1963): Characterization of laboratory strains of *Rickettsia typhi*, pp. 155–121. In P. F. Zdrodovsky (Ed.): *Problems of Infectious Pathology and Immunology*, Medgiz, Moscow (in Russian).

Ito, S., and Rikihisa, Y. (1981): Techniques for electron microscopy of rickettsiae, pp. 213–240. In W. Burgdorfer and R. L. Anacker (Eds): *Rickettsiae and Rickettsial Diseases*, Academic Press, Inc., New York.

Loginov, V. S., Zezerov, E. G., and Ereskin, A. V. (1982): Studies on polypeptide composition and protective antigens of *Rickettsia prowazekii*. *Zh. Mikrob. Epidem. Immun.* **1982** (**12**), 78–83 (in Russian).

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Osterman, L. A. (1983): Studies on biological macromolecules by electrofocusing, immunoelectrophoresis, and radioisotope methods, pp. 121–124. Nauka, Moscow (in Russian).

Palmer, E. L., Mallavia, L. P., Tzianabos, T., and Obijeski, J. F. (1974): Electron microscopy of the cell wall of *Rickettsia prowazeki*. *J. Bacteriol.* **118**, 1158–1166.

Silverman, D. J., Fiset, P., and Wisseman, C. L., Jr. (1979): Simple differential staining technique for enumerating rickettsiae in yolk sac, tissue culture extracts, or purified suspensions. *J. clin. Microbiol.* **9**, 437–440.

Smith, D. K., and Winkler, H. H. (1977): Characterization of a lysine-specific active transport system in *Rickettsia prowazeki*. *J. Bacteriol.* **129**, 1349–1355.

Weiss, E. (1955): Adenosine triphosphate and other requirements for the utilization of glucose by agents of the Psittacosis-Trachome group. *J. Bacteriol.* **90**, 243–253.

Weiss, E., Coolbaugh, J. C., and Williams, J. C. (1975): Separation of viable *Rickettsia typhi* from yolk sac and L cell host components by renografin density gradient centrifugation. *Appl. Microbiol.* **30**, 456–463.

Williams, J. C., Peacock, M. G., and McCaul, T. E. (1981): Immunological and biological characterization of *Coxiella burnetii*, phases I and II separated from host components. *Infect. Immun.* **32**, 840–851.

Williams, J. C., and Weiss, E. (1978): Energy metabolism of *Rickettsia typhi*: pools of adenine nucleotides and energy charge in the presence and absence of glutamate. *J. Bacteriol.* **134**, 884–892.

Winkler, H. H. (1976): Rickettsial permeability. *J. biol. Chem.* **251**, 389–396.

Wisseman, C. L., Jr., Jackson, E. B., Hahn, F. E., Ley, A. C., and Smadel, J. E. (1951): Metabolic studies of rickettsiae. *J. Immunol.* **67**, 123–136.

Wolbach, S. B., Todd, J. Z., and Palfrey, F. W. (1922): *The Etiology and Pathology of Typhus*, Harvard UniversityPress, Cambridge, Mass.

Zdrodovsky, P. F., and Golinevich, E. M. (1972): *The Teaching of Rickettsiae and Rickettsioses*, pp. 92–94, 127–131, 143–144. Meditsina, Moscow (in Russian).

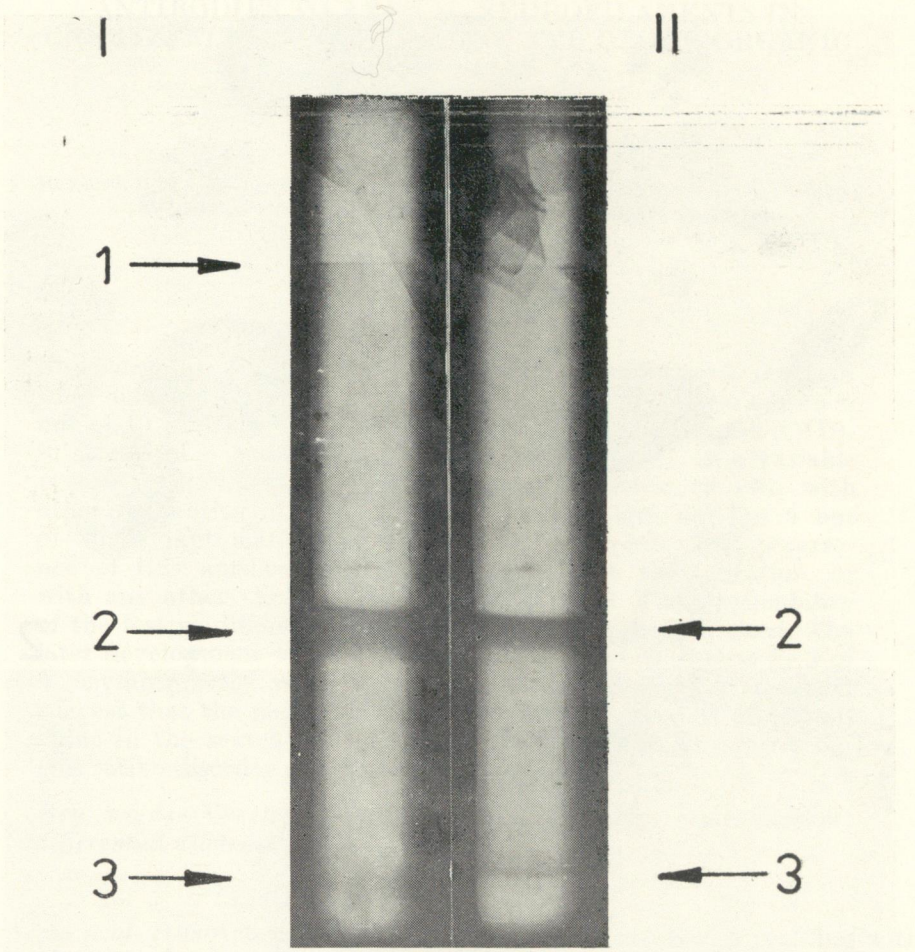
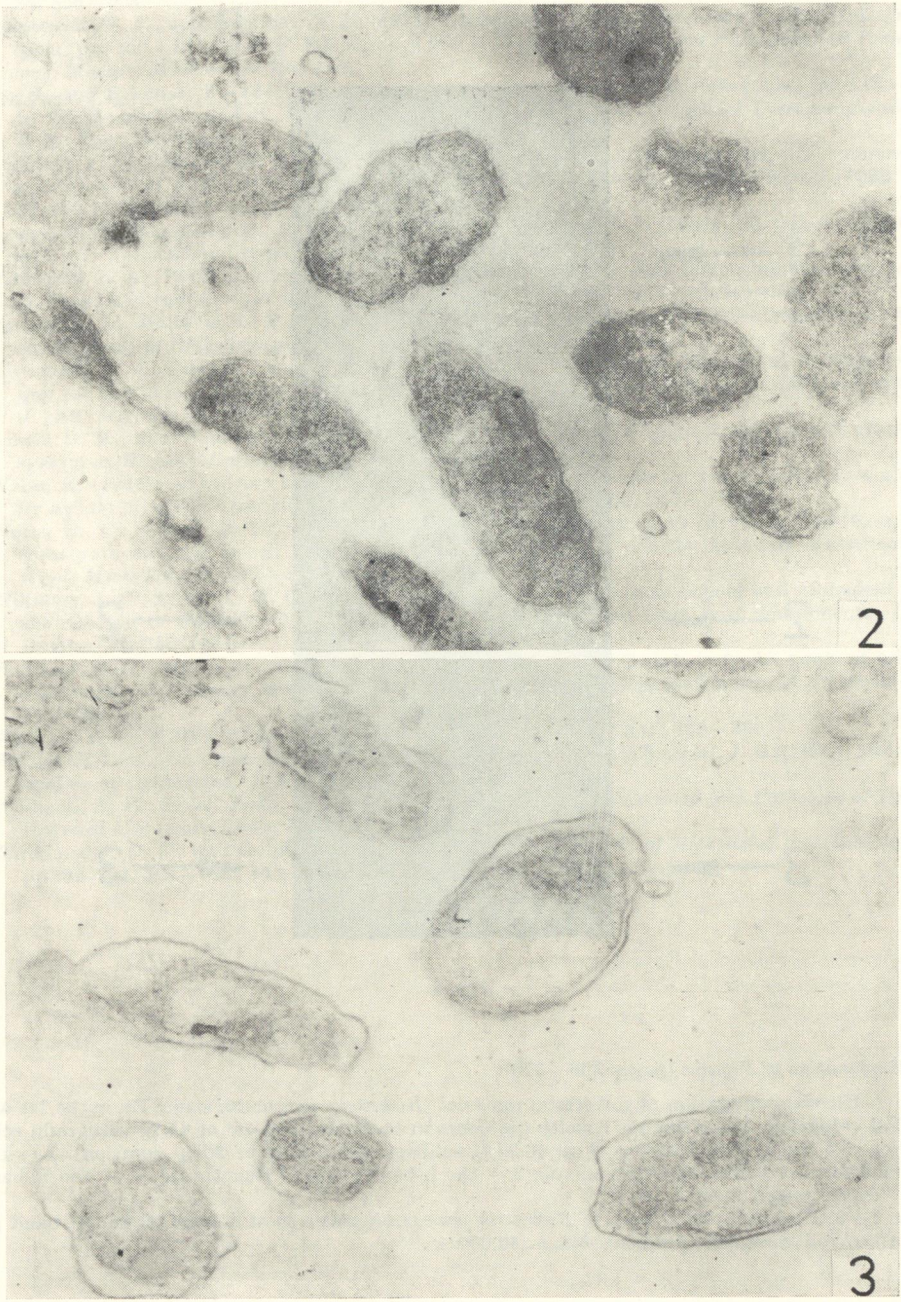


Fig. 1.

Explanation of Figures (pages 369—370):

Fig. 1. Ultracentrifugation of rickettsial material (*Rickettsia prowazekii* strain Et, in the 1st and 2nd verografin discontinuous density gradients in buffer K 36 (1 ml of 45 % verografin solution, 4.5 ml of 38 % solution, 2 ml of 30 % solution and 4.5 ml of 20 % solution). 1 — the band formed of host cell components, 2 — the L-band ("light" rickettsiae), 3 — the H-band ("heavy" rickettsiae).

Figs. 2—3. Electron micrographs of *Rickettsia prowazekii* strain E of L-band (2) and H-band (3) of the 2nd verografin gradient. Magn. 40,000 ×.



Figs. 2—3.

For legend see page 369.