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Sedimentation of *Eperythrozoon coccoides.** (30779)

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Eperythrozoon coccoides is a minute organism of somewhat uncertain taxonomy, presently classified among the Bartonellaceae(1). It is indigenous to mice, inducing in them a mild disease characterized by splenomegaly and general lymphadenopathy(2). The organism is frequently present in transplantable tumors(3) and may, in part, be responsible for the splenomegaly which follows their transplantation. In association with certain other organisms of normally low pathogenicity, disorders of high morbidity and mortality may result(4).

Knowledge of the biology of this ubiquitous but relatively little known organism has advanced slowly since its discovery in 1928(5). Recently, the splenomegaly induced by *E. coccoides* was quantitatively related to certain extracellular annular structures in the blood of infected mice, and it was inferred that of the various structures observed in blood, these particular ones were the infectious particles(6). As an approach to their concentration, purification and characterization, the behavior of *E. coccoides* in moderate and high gravitational fields was investigated, using biological activity to determine the results.

Materials and methods. The preparation of infectious material and the determination of biological activity have been described(6). The latter depends upon the increase in spleen weight of mice 7 days after intraperitoneal inoculation of 0.1 ml of material. The average response of 5 mice determines a single experimental point. Justification for using spleen weight as a measure of activity of *E. coccoides* has already been discussed(6).

Differential and density gradient centrifugation were performed in the Spinco Model L ultracentrifuge at about 4°C. Gradient columns were prepared by consecutively layering 0.6 ml of aqueous sucrose solutions of increasing concentration. The tubes were allowed to stand 18 hours at 4°C before use.

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TABLE I. Effect of Freezing on Sedimentation of *E. coccoides* in a Spleen Extract.

Inoculum	Spleen wt/body wt,* mg/g	
	Fresh extract	Frozen-thawed† extract
Uninoculated	5.85 ± .37	
Uncentrifuged extract	19.89 ± 1.42	18.08 ± 1.41
Supernatant after 1 hr at		
5,000 × *g*	16.08 ± .62	6.35 ± .19
25,000 × *g*	14.35 ± 1.42	6.39 ± .45
50,000 × *g*	14.77 ± .38	6.92 ± .60
100,000 × *g*	13.96 ± .73	6.52 ± .34

* This is a measure of activity of *E. coccoides*. Figures represent average response of 5 mice ± standard error.

† An aliquot was immersed in methanol-dry ice for 5 minutes, then quickly thawed and maintained in an ice bath until inoculated.

The experimental sample (0.5 ml of undiluted plasma) was layered on the preformed gradient and centrifuged in the SW39 rotor without braking for 1 or 2 hours, and 0.5 ml fractions collected from the bottom of the tubes. Each fraction was assayed and its density determined by the falling drop method, using calibrated density gradients of bromobenzene-kerosene(7).

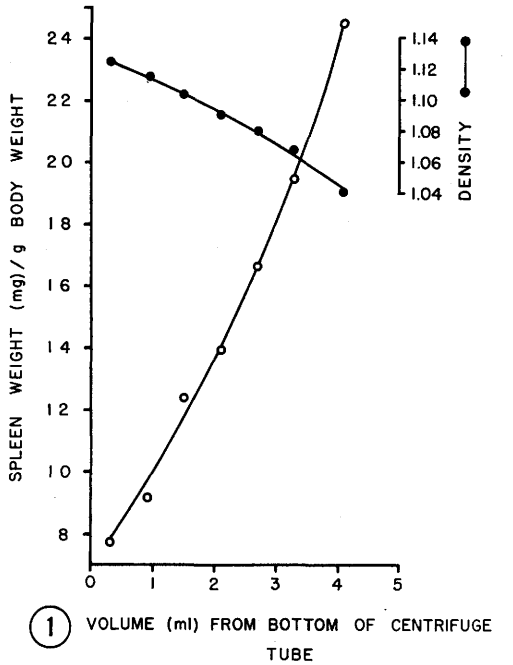
Results and discussion. Sedimentation of infectivity in freeze-thawed spleen extracts. Differential centrifugation had previously indicated(8) that the infectious activity resisted sedimentation at 50,000 × *g* for 1 hour, and was not completely sedimented even at 100,000 × *g* for 2 hours. On repeating this experiment, it was unexpectedly found that the activity was removed from the supernatant at only 5,000 × *g* for 1 hour. The two experiments differed in that a fresh spleen homogenate was used in the first instance, whereas a frozen homogenate was used in the second.

A direct comparison of fresh and frozen spleen homogenates resulted in the data summarized in Table I. They indicate a striking difference in the sedimentation behavior of the two materials. Apparently, on freezing and thawing, the infectious agent aggregated, and sedimented at relatively low gravitational forces either because of a change in size, density, or both. Additional investigation revealed that the activity of frozen material withstood sedimentation for 15 minutes at 5,000 × *g* but was virtually completely sedimented in 30 minutes. Though aggregated, frozen-thawed material was biologically active, suggesting that such homogenates might be useful as a preliminary step in the purification of the organism.

Sucrose density gradient centrifugation. Sucrose density gradient experiments, using fresh, infectious plasma indicated that after 1 to 8 hours at 125,000 × *g* (avg.) in a density gradient varying from 1.10 to 1.34, the greatest activity occurred in the fraction of lowest density, but activity was not confined to this fraction. In an effort to cause the peak of activity to approach the middle of the gradient, a preformed density gradient varying from 1.01 to 1.14 was prepared. The result (Fig. 1) indicated that the greatest activity was still in the fraction of lowest density but was spread throughout the gradient in a regularly descending order of activity. Recentrifugation of selected fractions obtained in the above experiments, to determine their hydrodynamic integrity, did not produce data which could be clearly interpreted, primarily because of losses in activity which occurred during these manipulations.

It was considered that the distribution of activity in the gradient, as shown in Fig. 1, could be due to heterogeneity in the density of the particles. Under these circumstances activity should also occur throughout the gradient if the infectious material were initially placed at the bottom of the density gradient, *i.e.*, in the position of greatest density. On the other hand, if the density were homogeneous but the infectious particles varied in size, then the activity should remain at the bottom of the gradient. Fig. 2 shows the result of such an experiment. The activity was again spread through the tube, indicating that the infectious agent was heterogeneous in density, although not ruling out the possibility that it was also heterogeneous in size. The possibility that aggregation of particles could be a contributing factor in these results must also be considered.

The activity recovered from the inoculation of mice with a single fraction from a density gradient (lowest density, Fig. 1) gave a distribution pattern similar to the original



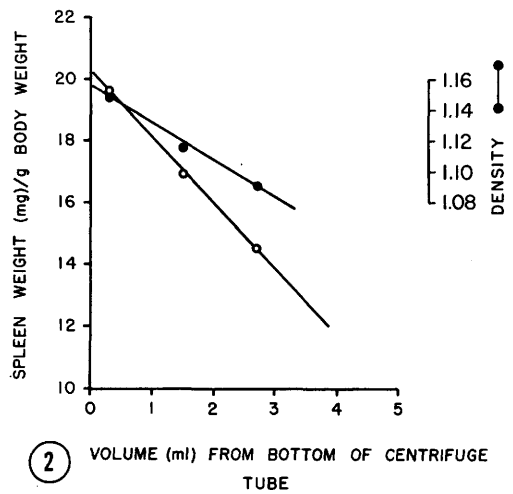


FIG. 1. Distribution of activity of plasma in a sucrose gradient after one hour at 93,850 × *g* (av.).

Fig. 2. Distribution of activity when infectious material was initially placed at the bottom (most dense fraction) of a density gradient. The plasma was mixed with sucrose of the highest density, followed by consecutive layers of uninfected, less dense sucrose. Centrifugation was two hours at 93,850 × *g* (av.).

material, indicating that individual fractions were capable of producing a complete spectrum of infectious particles.

The low density of *E. coccoides*, together with its destruction by ethyl ether or the fluorocarbon, trifluorotrichloroethane (Neilson and Stansly, unpublished data), suggest that a significant part of its structure consists of lipid. This was substantiated by the sensitivity of *E. coccoides* to freezing, which resulted in its aggregation, an effect characteristic of certain lipoproteins(9) and lipid-containing viruses(5). Boundary spreading in high gravitational fields, such as has been observed with *E. coccoides*, is also characteristic of lipoproteins(9) and some lipid containing viruses(10), and may result from varying amounts of lipid in the individual particles. The extreme particle heterogeneity of *E. coccoides* would appear to render hydrodynamic methods unsuitable for its characterization, but possibly of some limited value for its purification if advantage can be taken of its aggregation when frozen and thawed.

Summary. Frozen and thawed *Eperythrozoon coccoides* in spleen homogenates was biologically active but aggregated. As a result, it was completely sedimented after 30 minutes at 5,000 × *g*, whereas from freshly prepared homogenates it was not completely sedimented even after one hour at 100,000 × *g*. In fresh infectious plasma, *E. coccoides* exhibited extreme particle heterogeneity as determined in a sucrose density gradient following centrifugation for 1 to 2 hours at 93,850 × *g*. Possible reasons for this behavior are discussed.

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