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Separation of *Treponema pallidum* from Tissue Substances by Continuous-Flow Zonal Centrifugation

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The zonal ultracentrifuge was used for separation of *Treponema pallidum* from large volumes of rabbit testicular syphiloma extracts by continuous-flow centrifugation in a cesium chloride density gradient. The gradient was linear with radius from a density of 1.05 to 1.36 g/ml. Operating speeds were 15,000 rev/min for the continuous-flow phase and 25,000 rev/min for a 30-min banding period. A total of 9×10^9 (24.3%) treponemes were recovered from the original extract. Of the treponemes recovered, 88% formed a band at a density of 1.170 to 1.211 g/ml. Within the limits of present methods of assay, these fractions were relatively free from testicular particulates and protein when compared with treponemes recovered after differential centrifugation. Observations of the isolated fractions by dark-field and electron microscopy indicated a lack of gross morphological damage to *T. pallidum*. Their antigenic characteristics were also retained, as evidenced by their ability to react with syphilitic sera in the indirect fluorescent-antibody procedure.

At present, *Treponema pallidum* can be propagated only in vivo. Therefore, harvesting of the organism must involve its extraction from the tissue in which it is grown, usually rabbit testes. It is obvious that large quantities of spirochetes free from host contaminants are required for use in analytical studies and to produce effective vaccines.

The zonal centrifuge has proven useful for separating particles ranging from whole cells to large protein molecules (1, 2, 4–6). Since the organism falls within this particle size, these techniques should be applicable to separating *T. pallidum* from tissue debris. This paper describes the application of continuous-flow gradient centrifugation to the separation of *T. pallidum* from tissue debris.

MATERIALS AND METHODS

Rabbit testes infected with *T. pallidum*, Nichol's virulent strain, were finely minced and agitated in 0.85% sodium chloride (50 ml/testis) on a rotator at 190 cycles/min for 30 min. The supernantant fluid was centrifuged at $300 \times g$ for 10 min to remove gross tissue particles and most of the red blood cells. This procedure was repeated three times on the same minced tissue with fresh 0.85% NaCl, and the four extractions were pooled. Normal rabbit testes were extracted and centrifuged in the same manner as the infected testes.

Gradient centrifugation was performed in a model L-4 preparative ultracentrifuge equipped with a Beckman ω^2t digital integrator and either an Anderson B-XXVI (a latter modification of B-XVI; 6) or a Beckman B-XVI continuous-flow rotor.

A cesium chloride density gradient was formed by pumping 450 ml of CsCl (density, 1.40 g/ml) into the rotor previously filled with 0.85% NaCl. The rotor was then slowly accelerated to 15,000 rev/min for the continuous-flow phase, and the *T. pallidum* suspension was pumped through the rotor inboard to the gradient at a flow rate of 4.75 liters/hr. The flow rate was modified until an acceptable level of "clean-out" was obtained. After completion of the continuous-flow phase, the rotor velocity was increased to 25,000 rev/min and run until a digital integrated ω^2t force of $1,500 \times 10^7$ rad^2/sec had been reached. The rotor velocity was then slowed to 3,000 rev/min, and the contents were displaced through the center core by pumping CsCl solution with a density of 1.45 g/ml into the rotor edge. The gradient was collected in 32 fractions of 25 ml each. Ultraviolet absorption (at 280 nm) of each fraction was measured with a Beckman DU spectrophotometer, density was deter-

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mined from the refractive index by use of a Bausch and Lomb Abbe refractometer, and protein concentration was measured by the method of Lowry et al. (12).

Treponemes were enumerated (3) in the combined extracts before centrifugation and in each fraction collected from the zonal procedure.

Immunodiffusion was performed by Wadsworth's gel chamber microplates technique (15). Plates were stained with Amidoschwarz 10B, and the stained plates were used as negatives for photographs. Goat anti-rabbit whole serum was obtained from Hoechst Pharmaceutical Co.

Specimens for electron microscopy were made by floating a grid coated with Formvar and carbon on a drop of *T. pallidum* suspension. After drying, the grids were shadowed with a carbon-platinum pellet and examined with a Philips 200 electron microscope.

The indirect fluorescent-antibody technique (8), with buffered saline used in the first step and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit globulin in the second step, was employed to detect the presence of rabbit globulin on *T. pallidum*.

The fluorescent treponemal antibody absorption (FTA-ABS) test (10, 14) was used to detect alteration in antigenicity of *T. pallidum* after zonal centrifugation.

RESULTS

Figure 1A illustrates the ultracentrifugal pattern obtained from 2 liters of testicular extract containing 37.0×10^9 treponemes. A total of 9.0×10^9 (24.3%) treponemes were recovered in 26 fractions of 25 ml each. Fractions 1 through 10 contained 4.4% of the recovered treponemes and 79.8% of the soluble protein. Small particles of testicular tissue were also present. Most of the recovered organisms (88%) were sedimented in the density range of 1.170 to 1.211 g/ml in fractions 11 through 13, which contained only 11.6% of the soluble protein and less microscopic particulate matter. At a density of 1.221 to 1.271 g/ml, fractions 14 through 17 contained fewer treponemes (6.5%) and less particulate matter. As density increased to 1.333 g/ml in fractions 18 through 26, concentrations of treponemes and of extraneous particles decreased.

In comparison, 2 liters of normal rabbit testicular extract (Fig. 1B) formed a band of particulate matter in the density range of 1.164 to 1.190 g/ml. This is significant because this particulate band persistently overlaps the density zone in which most of the treponemes band. Efforts to resolve further the particles therein have thus far been unsuccessful.

Representative electron micrographs of the suspension after zonal centrifugation (Fig. 2) revealed that significant reduction in particulate debris had occurred in contrast to the amount present before centrifugation (Fig. 3).

Figure 4 shows immunodiffusion patterns obtained with goat anti-rabbit serum (Hoechst Pharmaceutical Co.) and goat anti-rabbit testicular homogenate (11) in the center wells, and concentrated *T. pallidum* sonic extract from the zonal procedure, concentrated *T. pallidum* sonic extract before zonal centrifugation, effluent from the flow-through stream,

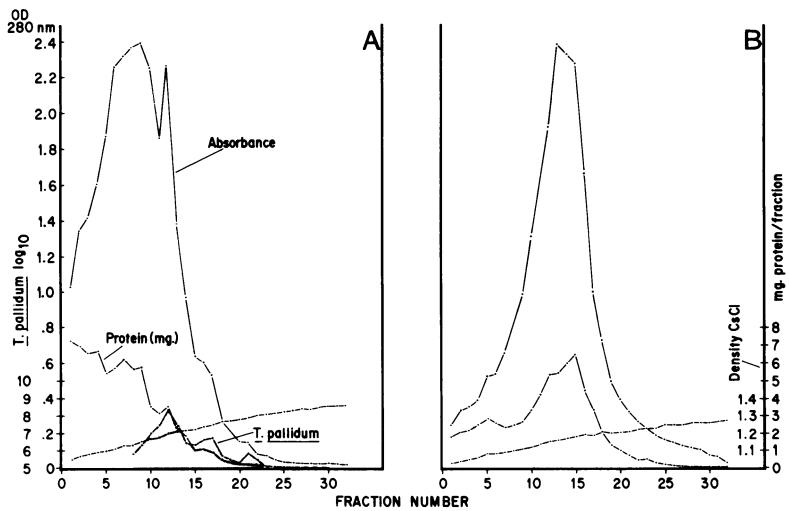

FIG. 1. *Zonal fractionation of (A) 2 liters of rabbit testicular syphiloma extract and (B) 2 liters of normal rabbit testicular extract in a cesium chloride density gradient. Protein concentrations are given in milligrams per fraction. Treponemal counts are given as appropriate powers of 10. Centrifugation was to* $\omega^2 t = 1{,}500 \times 10^7$ *rad²/sec.*

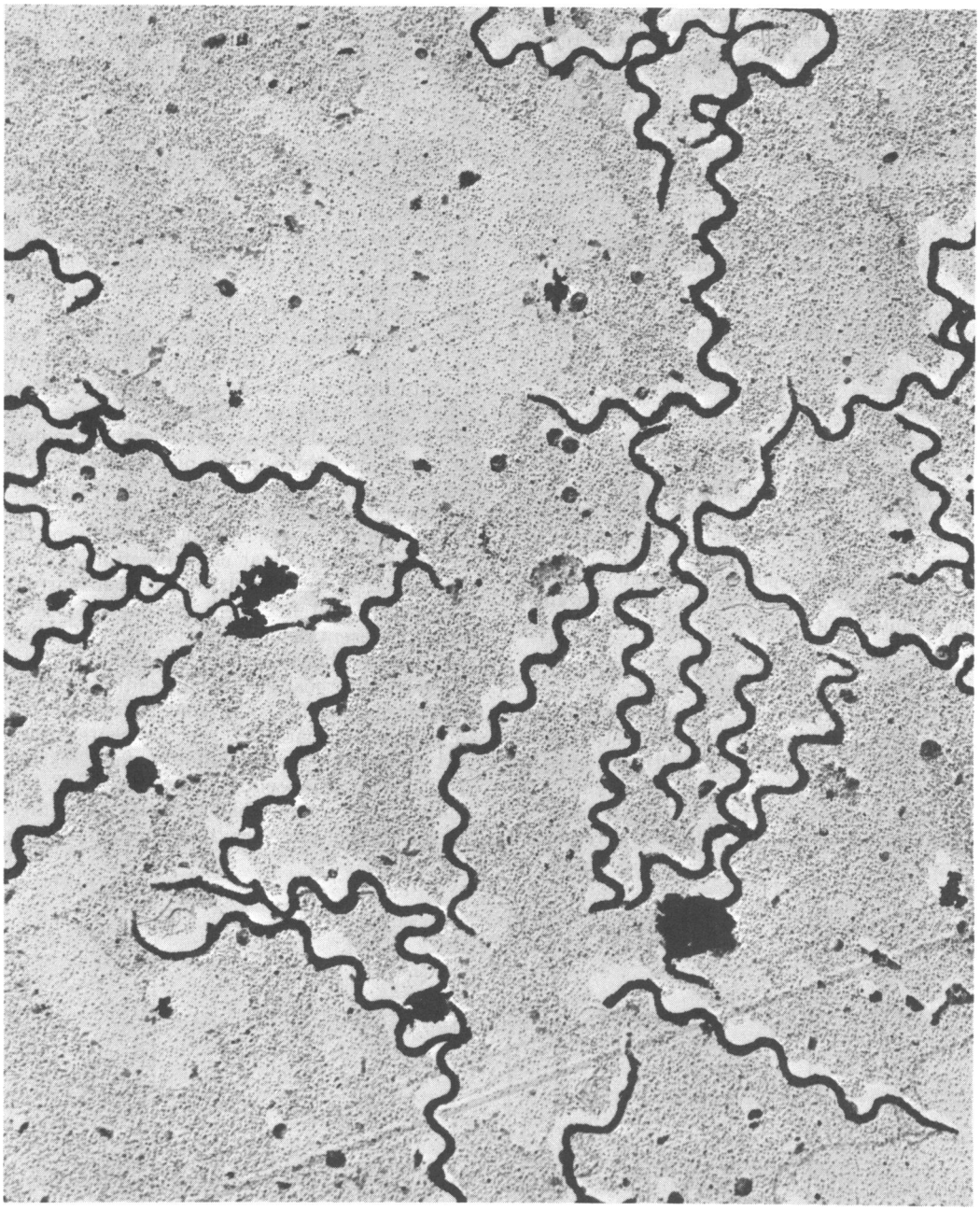

FIG. 2. *Electron micrograph of T. pallidum after zonal centrifuge run.* ×*13,750.*

and normal rabbit testicular sonic extract in the peripheral wells. No reaction occurred between the zonal centrifuged *T. pallidum* and either antisera, whereas distinct precipitin bands were evident between the antisera, starting suspension, effluent, and normal testicular homogenate.

The uncentrifuged testicular extract gave a 1+ level of reactivity when overlaid with anti-rabbit globulin in the fluorescent-antibody procedure, but after zonal centrifugation the recovered organisms were nonreactive, indicating removal of rabbit globulin. Also, the organisms appeared to retain their antigenic characteristics by reacting with syphilitic sera in the indirect fluorescent-antibody procedure.

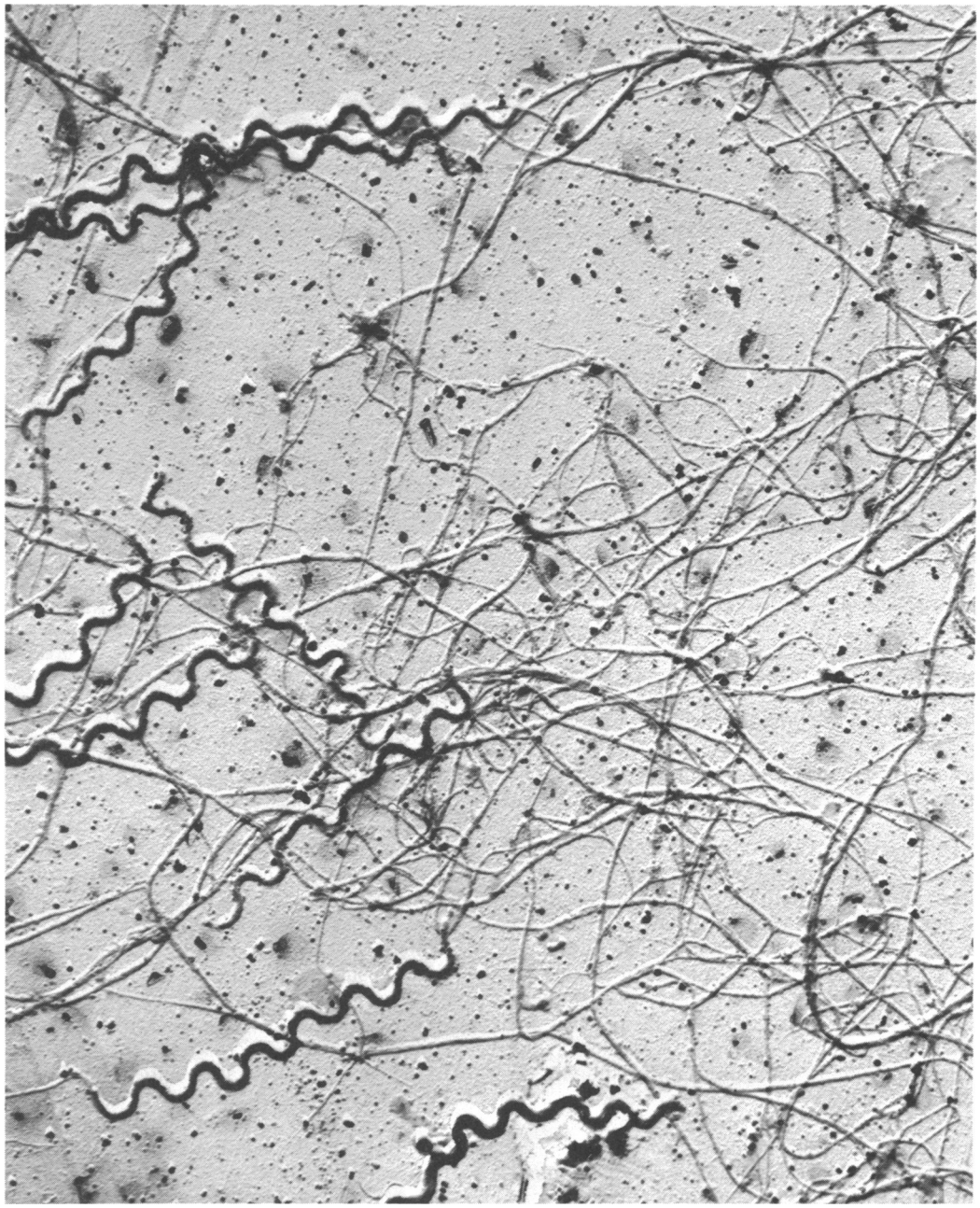

FIG. 3. *Electron micrograph of starting sample of T. pallidum extract for zonal centrifuge run.* × *13,750.*

DISCUSSION

The evaluation of an isolation method depends on the method used to determine the purity of the final preparation. This requires that we have an assay or analysis that can be related to the number of organisms present. Total protein, total nitrogen, or dry weight per organism would answer the purpose. By dividing the total protein value by the numerical count of the suspension, we obtain the protein value per organism. From Table 1, it can be seen that the purest preparation (fraction 12) contained 0.592×10^{-6} μg of protein per organism counted, a 45-fold reduction from the 26.76×10^{-6} μg of protein in the starting ma-

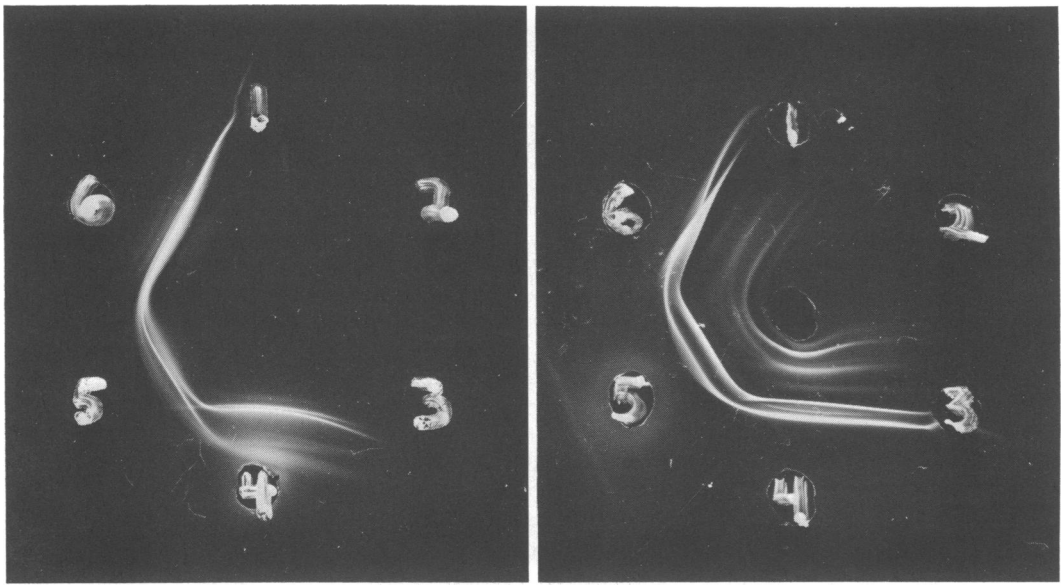

FIG. 4. *Immunodiffusion patterns of rabbit testicular syphiloma extract after zonal centrifugation. Left: center well, goat anti-rabbit testicular homogenate; wells 1 through 3, concentrated T. pallidum sonically treated solution from zonal centrifuge; well 4, normal rabbit testicular homogenate, sonically treated solution; well 5, effluent from flow-through stream; well 6, concentrated T. pallidum sonically treated solution before zonal centrifugation. Right: center well, goat anti-rabbit whole serum; wells 1 through 3, concentrated T. pallidum sonically treated solution from zonal centrifuge; well 4, concentrated T. pallidum sonically treated solution before zonal centrifugation; well 5, normal rabbit testicular homogenate, sonically treated solution; well 6, effluent from flow-through stream.*

terial. Apparently a substantial part of the impurities from the testicular tissue were eliminated.

Our extraction procedure immobilized most of the *T. pallidum* in a matter of hours. Any survivors were killed by the cesium gradient, as evidenced by animal inoculation. The effect of the procedure on the morphology and composition of *T. pallidum* requires further study. The high osmotic pressure of the gradient material may damage the organism when the CsCl is removed by dialysis. In studies on the effect of various ions used in prolonging *T. pallidum* motility in vitro, cesium ions could partially replace potassium ions; however, neither rubidium nor cesium could replace sodium ions (9). These findings are in agreement with the lack of gross morphological damage observed microscopically and in electron micrographs.

The wide range of buoyant density of *T. pallidum* in CsCl may be due in part to the interaction of cesium with organisms in different physiological states. Portions of the outer membrane may be lost from some treponemes during this procedure, thus changing the sedimentation rate. Also, dehydration of the organism in high concentrations of cesium salt would affect the density. In other studies, Foster and Thomas (*unpublished data*) noted that osmotic effects in sucrose gradients possibly alter the density. In addition, we suggest that this range in density may reflect varying concentrations of lipid in the infectious moiety, and this may be influenced by the host in which it is grown or physical factors to which it is subjected.

Rathlev and Pfau (13) previously reported the purification of *T. pallidum* by isopycnic centrifugation in a potassium tartrate gradient. However, the relationship of the study to the present work is difficult to assess since no analytical data, electron micrographs, or information on the banding density of the organism were presented.

The zonal centrifuged treponemes could be stained in the fluorescent-antibody procedure with syphilitic sera, suggesting that there remained intact at least a major portion of *T. pallidum* antigenic determinants reactive in the fluorescent-antibody procedure. Removal of rabbit globulin from the treponemes was also assayed by the fluorescent-antibody procedure. The 1+ level of reactivity with FITC-labeled antiserum to rabbit globulin that was present before centrifugation could not be

TABLE 1. *Analysis of fractions separated by zonal centrifugation of syphiloma extract*[a]

Fraction no.	Treponemes/ml	Total protein (μg/ml)	Total protein/treponeme (μg)
1	0.0	295.00	0.0
2	0.11×10^6	280.00	$2{,}545.45 \times 10^{-6}$
3	0.06×10^6	265.00	$4{,}416.66 \times 10^{-6}$
4	0.06×10^6	270.00	$4{,}500.00 \times 10^{-6}$
5	0.22×10^6	218.75	994.32×10^{-6}
6	0.28×10^6	232.50	830.35×10^{-6}
7	0.33×10^6	255.00	742.42×10^{-6}
8	0.88×10^6	231.25	262.78×10^{-6}
9	3.74×10^6	232.50	62.16×10^{-6}
10	10.70×10^6	145.00	13.55×10^{-6}
11	46.20×10^6	126.25	2.73×10^{-6}
12	225.50×10^6	133.50	0.592×10^{-6}
13	45.10×10^6	92.50	2.05×10^{-6}
14	4.40×10^6	73.75	16.76×10^{-6}
15	4.18×10^6	37.50	8.97×10^{-6}
16	6.82×10^6	31.25	6.05×10^{-6}
17	7.76×10^6	35.62	4.59×10^{-6}
18	0.72×10^6	21.25	29.51×10^{-6}
19	0.50×10^6	13.75	27.50×10^{-6}
20	0.33×10^6	12.50	37.88×10^{-6}
21	0.83×10^6	11.25	13.55×10^{-6}
22	0.06×10^6	5.00	83.33×10^{-6}
23	0.0	6.25	0.00
24	0.06×10^6	3.12	52.00×10^{-6}
25	0.17×10^6	1.88	11.06×10^{-6}
26	0.06×10^6	1.88	11.06×10^{-6}
27	0.0	0.0	0.0
28	0.0	0.0	0.0
29	0.0	0.0	0.0
30	0.0	0.0	0.0
31	0.0	0.0	0.0
32	0.0	0.0	0.0

[a] Crude extract contained 18.5×10^6 treponemes/ml, 495 μg of protein/ml, and 26.75×10^{-6} μg of protein/treponeme.

demonstrated after gradient centrifugation. This suggests that the zonal procedure removed rabbit globulin that was closely associated with *T. pallidum*. Furthermore, based on column chromatography in Sephadex G-200, the bulk of the serum components (19*S*, 7*S*, and 3 to 4*S*) did not enter the gradient but passed in the effluent stream. The immunodiffusion technique used can detect as little as 8 μg of rabbit protein per ml, as evidenced by control experiments with serial dilutions of normal rabbit serum. Our purpose was to detect soluble components of rabbit tissue and serum proteins that were not eliminated by the zonal procedure. Results of immunodiffusion suggested that these components were removed. However, we offer this with caution since, by more sensitive radioassay methods, trace amounts of protein may have been detected.

From these preliminary studies, it appears that continuous-flow zonal rotors offer a rapid and useful technique for isolating and concentrating the causal agent of syphilis from multiliter quantities of solution. The limitation in the procedure appears to be the overlapping densities of testicular particles and treponemes, resulting in a preparation contaminated with, presumably, microsomes and mitrochondria since these particles sediment in the same density range (7).

To date, 48 zonal runs have been made, and extract volumes have varied from 1 to 13.5 liters. The per cent yields for these runs have varied from 10 to 54% recovery from the initial suspensions. The low yields encountered could be attributed in part to delays in centrifugation. Immediate processing of the fresh treponemal suspension resulted in higher yields, possibly because temporary storage promotes aggregation and spontaneous changes of the organisms, rendering them less applicable to the zonal procedure.

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