plain the trypanosome not having been found previously.

The trypanosome found in *B. boreas* appears specific for toads. Intraperitoneal inoculations of 0.5 ml of culture overlay produced infections in all of five uninfected toads from outside the study area. Trypanosomes were not seen in wet smear mounts until 3 weeks after inoculation and parasitemias remained low. Similar inoculations failed to infect one *Sceloporus occidentalis*, two *Rana aurora*, two *R. catesbiena*, three *R. pipiens*, and two *Hyla regilla*.

Preliminary studies suggest that the incidence of infection of toads increases with age (*9*). The overall low incidence of infection in toads parallels the previously determined low infection rates in wild-caught sandflies (*1*).

Discovery of this host-parasite-vector system in California raises the possibility that similar systems involving *Leishmania* species may also be present here, and it also should assist in clarifying interpretative problems associated with infections of wild-caught sandflies in the Americas and elsewhere. For example, the high rates of infection with flagellates in wild-caught sandflies in Panama, especially the large proportion with hindgut infections (*10*), may be attributable to infection with nonmammalian parasites (species of either *Leishmania* or *Trypanosoma*). Previous explanations implied that these sandflies were infected with mammalian *Leishmania* (*11*). However, all evidence so far indicates that posterior station infections in sandflies are not associated with *Leishmania* of man (*12*). As the hindgut forms of the *B. boreas* trypanosome are morphologically similar to those of various *Leishmania* species in their vectors, the relationships of anuran, reptilian, and mammalian flagellates should be considered in epidemiological studies of leishmaniasis.

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13. We thank D. Heyneman, University of California Medical Center, San Francisco, and R. H. Dadd and C. J. Weinmann of our department for their helpful comments on the original transcript. S.C.A. is a NIH trainee (grant TO1 AI00218-06).

28 June 1968

Neuronal Perikarya of Rat Brain Isolated by Zonal Centrifugation

Abstract. *Cerebral cortex and hippocampus of rat were disrupted in a specially designed tissue-press and prepared as a 2 percent suspension in 10 percent buffered Ficoll medium and fractionated by density-gradient centrifugation in the B XIV zonal rotor. The suspension was injected into a buffered 30 percent Ficoll, 58 percent sucrose discontinuous gradient previously loaded in the B XIV zonal rotor spinning at 3500 revolutions per minute. Intact neuronal perikarya were recovered as a discrete band in the dense sucrose zone of the gradient after centrifugation at 35,000 revolutions per minute for 45 minutes.*

Among the many techniques used to isolate nerve cells from glial cells (*1*) the microdissection methods of Hyden (*2*) and Lowry (*3*) and the sieving-fishing method by Roots and Johnston (*4*) can provide pure neuronal perikarya, but not in numbers sufficient for macroscale chemical characterization. More recently methods have been described in which density-gradient and swinging-tube ultracentrifugation techniques are used (*5*). We now report a technique by which brain tissue is disrupted with a tissue-press. Intact neurons and glial cells can be separated in the B XIV zonal ultracentrifuge head

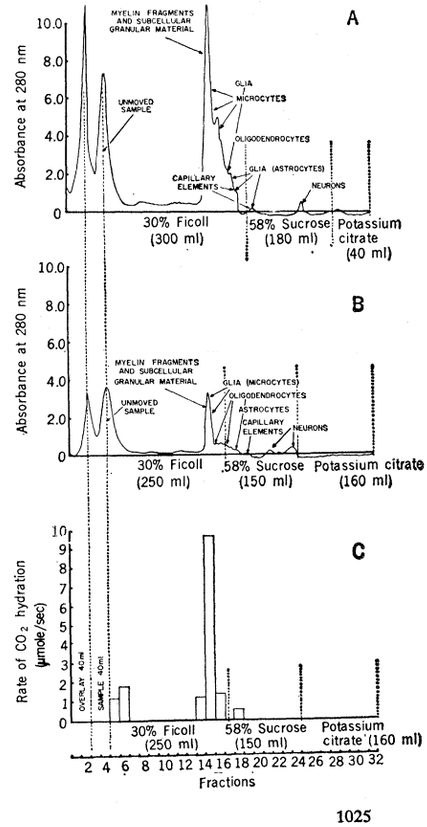

Fig. 1. Rate-zonal separation of cerebral cortex and hippocampus tissue of rat. (A) Separation of cerebral cortex after centrifugation at 53,840 × 10^6 $\omega^2 t$, including acceleration and deceleration. (B) Separation of hippocampus tissue after centrifugation at 50,002 × 10^6 $\omega^2 t$, including acceleration and deceleration. (C) Occurrence of net carbonic anhydrase activity (nonenzymatic hydration of CO_2, 1.85 μmole sec^{-1}, is subtracted) in fractions of separated rat hippocampus tissue of Fig. 1B. The serially displaced gradient 20-ml fractions monitored at 280 nm were recorded on linear scale chart paper with a recorder sensitivity setting of 0 to 12.5 units (that is, 0 to 100 percent absorbance at 280 nm was equivalent to 0 to 12.5 optical density units on the chart). Peak 1 contains fragmented myelin and amorphous subcellular constituents of remaining unmoved sample. Peak 2 contains glial constituents (microcytes, oligodendrocytes, and astrocytes) separating centrifugally in the 30 percent Ficoll zone with the larger astrocytes and capillary elements of brain penetrating into the 58 percent sucrose zone. Peak 3 contains intact neuronal perikarya banding discretely from peak 2, in the 58 percent sucrose zone.

by means of a modification of the discontinuous gradient technique of Rose (*5*). The method is more flexible, rapid, and precise than gradient swinging-tube methods.

Male Sprague-Dawley rats (200 to 250 g) were decapitated. The whole brain was quickly removed and dissected over ice to remove the cerebellum and olfactory lobes. The remaining cerebral hemispheres and midbrain tissue was then quickly disrupted without use of buffer solutions in a specially designed tissue-press. The press, made of brass with a detachable bottom section (assembled dimensions 1⅜ inches in diameter by 6 inches in length), is equipped with removable perforated stainless steel supports in the bottom section for insertion of stainless steel grids. The press was adapted for separation of whole cells by insertion of first, a 200-mesh (75 μm) stainless steel grid and then a 400-mesh (37 μm) grid. Inasmuch as only larger neurons are approximately 40 μm in diameter, this combination of grids permitted cellular passage and, therefore, successful disruption of cerebral cortex in a single step. The recovered tissue was quickly weighed and made up as a 10 percent tissue suspension in a medium containing, in final concentration, 10 percent Ficoll (*5, 6*), 0.005*M* $CaCl_2$, and 0.05*M* tris (hydroxymethyl) aminomethane buffer (*p*H 7.8). (All percentages used here were based on weight per volume.) This suspension was then finely dispersed by five to ten passes through the orifice of a 35-ml syringe. The suspension was diluted to 2 percent with additional 10 percent Ficoll medium and was filtered once through a microsyringe filter holder fitted with two 400-mesh (37-μm pore) grids. The filtrate (usually 40 ml) was used as the standard preparation for fractionation by zonal density-gradient centrifugation (*7*).

Our experiments are the first to use the B XIV rotor (*8*) for brain-cell fractionation. The rate-zonal separations of cerebral cortex and hippocampal tissue shown in Fig. 1, A and B, were made in 560 ml of a discontinuous gradient composed of 30 percent Ficoll medium (density 1.102) containing, in final concentration, 0.005*M* $CaCl_2$, 0.05*M* tris buffer (*p*H 7.8); 58 percent sucrose (density 1.282) containing 0.005*M* $CaCl_2$, and 0.05*M* tris buffer (*p*H 7.8); and potassium citrate (density 1.49) underlay or wall cushion. While spinning at 3500 rev/min the rotor was loaded from the edge with various portions of the discontinuous gradient (Fig. 1). The tissue sample (40 ml) was then added through the central core of the rotor and was overlaid with 40 ml of 10 percent Ficoll medium. The seal was then removed from the spinning rotor, and the inlet was capped. Centrifugation at 35,000 rev/min lasted 45 minutes [approximately $40{,}000 \times 10^6\ \omega^2 t$, not including acceleration and deceleration time (see *9*)].

After deceleration to about 3500 rev/min, the lid was opened and the cap was replaced by the seal. While the rotor was still spinning at approximately 3500 rev/min its contents were displaced by potassium citrate pumped to the rotor edge. The absorbance of the solution coming out of the rotor was monitored continuously at 280 nm by a Gilford attachment on a Beckman DU spectrophotometer with a 0.2-cm flow cell (Fig. 1). The volume of each fraction collected was 20 ml. Usually 32 fractions were collected in a single experiment.

The fractions were then separated into two 10-ml aliquots, and the contents were diluted with 0.25*M* sucrose sedimented at 30,000 rev/min for 30 minutes in the angle 30 rotor of the Spinco Model L ultracentrifuge. Samples of the sedimented tissue were stained with Paragon polychromatic stain (*10*) for light-microscopic examination, fixed and embedded with epoxy resin for electron-microscopic examination, or assayed for carbonic anhydrase activity (*11*).

Examples of light microscopy of pellets correlated with various peaks or bands are given in Fig. 2. The first peak to be collected, fractions 0 to 6, contained the remains of the standard preparation which failed to move from the origin. This band consisted of highly fragmented sheath material and subcellular granules, but no intact cells. The second peak, contained mainly in fractions 14 to 21, occurred at the outer edge of the 30 percent Ficoll zone of the gradient and was heterogeneous. Fractions 14 and 15 contained flocculated subcellular granules and myelinated fragments which disappeared within two tube fractions. Fractions 16 to 21 (Fig. 1A) and 16 to 19 (Fig. 1B), which extended into the beginning of the 58 percent sucrose zone of the gra-

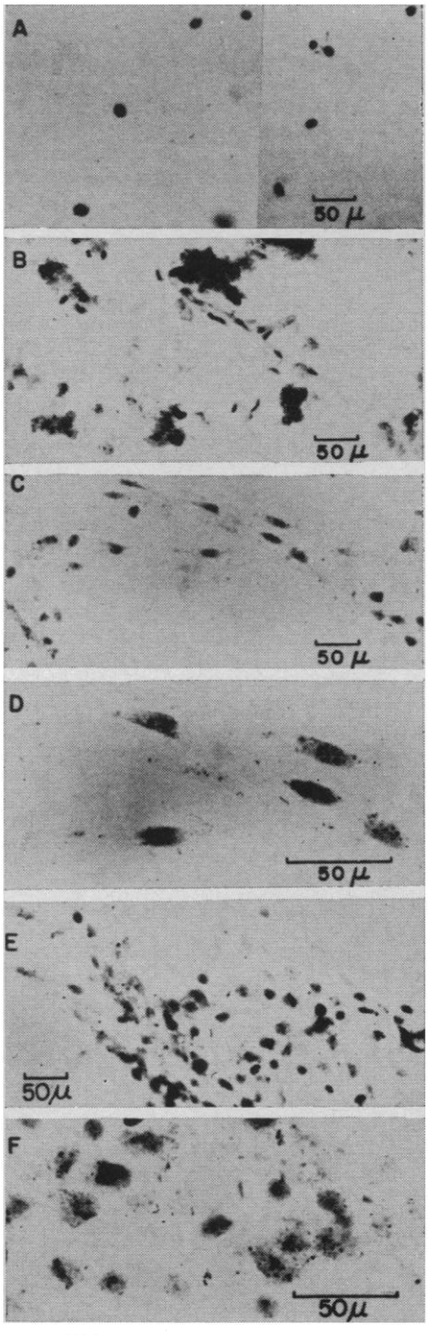

Fig. 2. Composition of recovered sediment stained with Paragon. (A) From upper basipetal portion of peak 2 [fractions 17 and 18 (Fig. 1A) and 16 to 18 (Fig. 1B)], showing recovered small glial cells (microcytes and oligodendrocytes) of brain. (B) From the lower basipetal position of peak 2 (fractions 18 to 20 in Fig. 1A, and 18 and 19 in Fig. 1B), showing the larger glial cells (astrocytes) separating together with the capillary tubes and endothelial nuclei of brain tissue. (C and D) From peak 3 (fraction 25, Fig. 1A), showing low- and high-power magnification of recovered intact neuronal perikarya of rat cerebral cortex after centrifugation at $53{,}840 \times 10^6\ \omega^2 t$, including acceleration and deceleration (*9*). (E and F) From peak 3, (fraction 24, Fig. 1B) showing low- and high-power magnification of recovered intact neuronal perikarya of rat hippocampus tissue after centrifugation at $50{,}002 \times 10^6\ \omega^2 t$ including acceleration and deceleration (*9*).

dient, was enriched with glia. Small glial cells (microcytes and oligodendrocytes) appeared in fractions 17 and 18 (Fig. 1A) and 16 to 18 (Figs. 1B and 2A), whereas larger glial cells (astrocytes) appeared in fractions 18 to 20 (Fig. 1A) and 18 to 19 (Fig. 1B). The latter fractions also contained capillary tubes and endothelial nuclei which were seen particularly in highest incidence with astrocyte cells (Fig. 2B). The third peak [fractions 25 (Fig. 1A) and 24 (Fig. 1B)], containing purified neuronal perikarya (Fig. 2, C–F) was banded at or near the interface between 58 percent sucrose and potassium citrate, and was cleanly separated from the tail of the second peak. Fraction 22 (Fig. 1B), which precedes fraction 24 (Figs. 1B and 2, E and F) is the result of a moving peak whose purified neuronal contents (Fig. 2, E and F) disappear at $50{,}002 \times 10^6\ \omega^2 t$, including acceleration and deceleration, into the interface of 58 percent sucrose and potassium citrate forming fraction 24. The purity of the recovered neuronal fraction was also assessed by measuring carbonic anhydrase activity (*12*), which is a marker of glial cell concentrations in brain tissue (*11*). The distribution and highest occurrence of enzyme activity (Fig. 1C) are associated with the glial-enriched band (tail of second peak); activity was not detected in the neuronal fraction (third peak).

Our experiments illustrate the potential resolving power and flexibility of zonal ultracentrifugation for fractionation of brain into whole cells. It may become practical to obtain increasingly purified cellular populations of brain in quantities necessary for classical biochemical measurements of metabolism.

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1. Neurons and glia vary in their shape, in mass, and in relation to each other in an intact brain. Thus, it is difficult to relate neurochemical data of a gross sample to its anatomical function. It is therefore relevant to attempt to separate the neurons from the glia and to characterize the chemical properties of each cell type.
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12. Carbonic anhydrase activity was determined by the electrometric method of R. E. Davis [*J. Amer. Chem. Soc.* **80**, 5209 (1958)].
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27 May 1968

Mice: Fighting by Neonatally Androgenized Females

Abstract. *Administration of testosterone propionate to female mice on the day of birth resulted in increased fighting after administration of testosterone during adulthood. This fighting, comparable to fighting among normal male mice, suggests that early androgenic stimulation organizes neural structures mediating aggression in the mouse.*

When two male mice that have been socially isolated for some time are placed together, aggression is invariable. Relatively little is known about the development of the neuroendocrine determinants of this behavior. One report (*1*) indicates that, if the male mouse is castrated before puberty, fighting is rare. Treatment of the castrate with exogenous testosterone results in arousal of the propensity to fight. Unlike males, adult female mice do not fight when testosterone is administered (*2*). This difference in response by male and female mice to testosterone may reflect differences in the nature or sensitivity of brain systems underlying aggression. Much research on the neural mechanisms underlying sexual behavior (*3*) indicates that males do differ from females with respect to some brain characteristics, and that these differences result from differential androgenic stimulation during a limited critical period of development. I sought to determine whether or not differences between adult males and females in fighting also are determined by the presence or absence of early stimulation by androgens.

Some Swiss-Webster litters of mice were injected subcutaneously with 0.5 mg of testosterone propionate within 24 hours of birth. The result was genital virilization characterized by small vaginal opening and hypertrophied clitoris in all females. Control litters received injections of peanut oil within 24 hours of birth, without genital effect. The young mice were kept with the mother until 30 days of age, when all were weaned and gonadectomized; they remained in the litter of birth until 60 days of age, when the males that had received testosterone at birth were eliminated from the study, the remainder being individually caged.

The remainder constituted three groups: (i) males treated with oil at

Table 1. Percentages of pairs of mice fighting at least once, and mean latencies to fighting when fighting occurred.

Group	Pairs		Latency (min)
	No.	Fighting (%)	
OM	16	100.0	5.1
TPF	16	93.8	5.0
OF	12	8.3	