In order to determine the stability of the tetraploid state in culture, we established single cell clones of tetraploid cells. Flow cytometry of serially subcultivated tetraploid clones demonstrated that the cells remained tetraploid without reversion to the diploid state [Fig. 1, E and F].

A diploid set of chromosomes is the usual composition of the eukaryotic genome. The diploid state is maintained by the reproduction of DNA and separation of chromosomes during the mitotic cycle. The emergence of an increasing percentage of nuclei with 4C DNA content in association with normal aging and with hypertension may be due either to arrest at the G_2 stage of the mitotic cycle or to the development of true tetraploidy. The presence of reproductively viable tetraploid cells in the normal rat aorta could represent a stem cell population that proliferates preferentially during normal aging and that can be significantly expanded by hypertension. Alternatively, the increased frequency of these cells may be due to continuous conversion of diploid cells with an abnormal mitotic mechanism to the state of tetraploidy (*5*). The role of tetraploid smooth muscle cells in normal growth, aging, and disease is still unknown. Further characterization of the tetraploid cell population including its growth kinetics and interaction with diploid cells may increase our understanding of cellular polyploidy and of vascular physiology.

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6. Sorting was performed with a 5-W coherent argon ion laser (Innova Series) with ultraviolet optics set to a 350- to 354-nm broadband output reflector line, confocally focused to a 15-mm wide spot. The photomultiplier tube was set to 330 at a gain of 10, with effective volts 660 for deflection. A 418-nm long-pass fluorescent filter was used.
7. The cells were subcultured every 6 to 8 days in standard fashion. The medium was removed, and the dish was trypsinized [1:250 trypsin EDTA in buffered saline (Gibco)]. The trypsinization was stopped by addition of fresh medium, and the cell suspension was counted. Cells were plated in fresh medium at an inoculation density of 1×10^4 cells per square centimeter. Cultures were incubated at 37°C with 5 percent CO_2 and 95 percent humidified air.
8. H. M. Shapiro, D. M. Feinstein, A. S. Kirsch, L. Christenson, *Cytometry* **4**, 11 (1983). Cells were centrifuged at 200*g* for 6 minutes and then suspended in nuclear isolation medium (0.6 percent NP-40, 1 m*M* $CaCl_2$, 21 m*M* $MgCl_2$, 0.2 percent bovine serum albumin in tris-buffered isotonic saline, and 8.0 μ*M* Hoechst dye 33342). The nuclear suspension was vortexed gently and cooled on ice for 10 minutes. Samples were filtered through a 50-μm pore size Nitex cloth, and the nuclei were syringed through a 25-gauge needle before flow cytometry. Flow cytometry was performed with a multiple-illumination wavelength, multiparameter flow cytometer system.
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10. Subconfluent cultures were incubated for 30 minutes in the presence of Colcemid (Gibco) at a final concentration of 0.1 μg/ml. Cells were dislodged from the flasks by treating with 0.25 percent trypsin-EDTA and then centrifuged at 150*g* for 7 minutes. The supernatant was discarded, and the cell pellet was suspended in 75 m*M* KCl solution and allowed to stand at room temperature for 10 minutes. The cells were centrifuged and then suspended in a 3:1 (by volume) methanol–acetic acid fixative. After 1 hour, two changes of fixative were made. Air-dried slides were prepared and were stained for 7 minutes in a solution (50 μg/ml) of quinacrine mustard (Sigma). Slides were mounted in tris-maleate buffer (*p*H 5.6) and were observed under a Leitz orthoplan fluorescence microscope equipped with an orthomat camera. Well banded (Q bands) metaphases were photographed.
11. We thank R. Tantravahi of the Cytogenetics Laboratory, Dana-Farber Cancer Institute, for the karylogic analysis, and S. DeMarco for manuscript preparation. This work was supported by NCI grant 5-P01-CA-12662, and NIH grant AG00599.

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Serotonin Selectively Inhibits Growth Cone Motility and Synaptogenesis of Specific Identified Neurons

Abstract. *The motile activity of growth cones of specific identified neurons is inhibited by the neurotransmitter serotonin, although other identified neurons are unaffected. As a consequence, affected neurons are unable to form electrical synapses, whereas other neurons whose growth is unaffected can still interconnect. This result demonstrates that neurotransmitters can play a prominent role in regulating neuronal architecture and connectivity in addition to their classical role in neurotransmission.*

The characteristic morphology and resultant connectivity of adult neurons is due to the combined action of precisely timed intrinsic and extrinsic signals on individual neurons (*1*). Extrinsic signals arising from neighboring neurons can regulate neuronal architecture (*2*), although proximate regulatory agents are not yet defined. One suggestion is that "trophic" substances released from some nerve terminals can control the growth of adjacent neurons (*3*). In light of the demonstration that neurotransmitter can be released from growth cones of growing neurons in culture (*4*), a candidate for such a regulatory agent is the classical chemical transmitter itself (*5*). We now report that the neurotransmitter serotonin can inhibit neurite outgrowth. We demonstrate a growth inhibition specific to individual growth cones by a time-lapse study of the large identified neurons of the snail *Helisoma*. We also demonstrate that this inhibitory action of serotonin prevents the formation of electrical synapses between specific identified neurons with overlapping outgrowth, while connections between neurons whose growth cones are unresponsive to serotonin continue to form (*6*).

These experiments were performed on buccal ganglion neurons 5 and 19 and on pedal ganglion neuron P5, all of which have been studied in terms of growth and connectivity (*6, 7*). Individual neurons were removed from ganglia of adult snails and plated in cell culture (*8, 9*), where neurons undergo a characteristic sequence of outgrowth. Growth cones arise from the cell body and elaborate an extensive network of neurites for 3 to 4 days until a morphological steady state is attained (*6*).

The behavior of growth cones of individual identified neurons is readily analyzed by time-lapse low-light video microscopy (*10*). The activity of growth cones from *Helisoma* neurons characteristically consists of a probing of the environment by filopodia and a ruffling action of lamellipodia. Concurrently, the neurite extends continuously at a nearly

constant rate (Fig. 1A). At the end of the growth period a quiescent phase nerve terminal results that is characteristically bright and shows essentially no motile activity.

Serotonin (Sigma) has a neuron-specific inhibitory effect on neurite outgrowth. Initially we examined this phenomenon by adding a 40-μl dose of serotonin (final concentration of 10^{-8} to $5 \times 10^{-5}M$) to the culture medium. In the neurons 19 studied by this method, nine of ten cells treated with a range of concentrations from 10^{-6} to $5 \times 10^{-5}M$ serotonin showed an abrupt cessation of filopodial probing, a decrease in ruffling action, a decrease in the surface area of the growth cone, and, most strikingly, an inhibition of neurite elongation (Fig. 1A). Serotonin significantly reduced (t = 5.55, $P < 0.0005$) neuron 19's rate of outgrowth from 11.32 ± 4.67 μm/hour (mean ± standard deviation, n = 11 growth cones) to −0.12 ± 4.99 μm/hour (n = 11 growth cones) (*11*). Exposure to carrier medium (50 percent L-15) (*8*) or to medium adjusted to the pH of the serotonin solution did not cause any of these growth inhibitory effects. Neuron 19 in cell culture may have several dozen growth cones on its different neurites. Serotonin causes a systemic inhibition of all growth cones when applied to culture medium at concentrations at or above $10^{-7}M$. In contrast, serotonin has no effect on neuron 5 (n = 10 growth cones from seven neurons) even at concentrations of $5 \times 10^{-5}M$. The growth cones of neuron 5 retained their normal structural features and continued to advance over the substrate; the rate of elongation (15.75 ± 2.99 μm/hour, n = 10 growth cones) being unaffected by serotonin (15.55 ± 3.64 μm/hour, n = 10 growth cones) (Fig. 1A). Thus, serotonin specifically inhibits the motile activity of neuron 19's growth cones without affecting the growth cones of neuron 5.

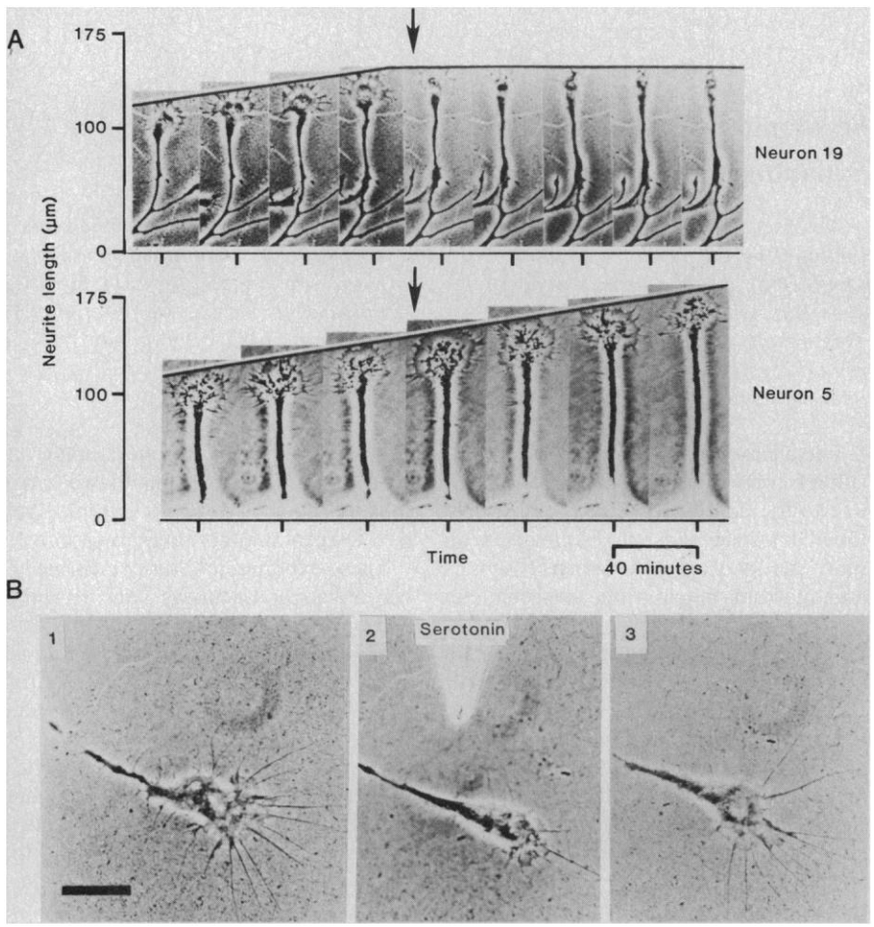

Fig. 1. Photomicrographs of the behavior of intact (A) and isolated (B) growth cones from identified neurons in cell culture. (A) Intact growth cones that are connected to the neuron (not shown) displayed at frame intervals of 40 minutes. Intact growth cones produce neurite outgrowth at a constant rate before serotonin treatment. Application of serotonin (arrows) inhibits the neurite outgrowth of neuron 19 (top) but has no effect on neuron 5 (bottom). (B) A growth cone of neuron 19 isolated by severing the interconnecting neurite with a micropipette (scratch, B1). Isolated growth cones are inhibited by pipette application of serotonin (B2), but after withdrawal of the pipette they resume their characteristic activity (B3). Serotonin's effects on isolated and intact growth cones are virtually indistinguishable, indicating that the growth cones of neuron 19 are directly responsive to serotonin. Calibration bar, 10 μm.

To determine the site responsible for mediating the growth inhibition serotonin was focally applied to specific areas of membrane of neuron 19 while the motile activity of its growth cones was monitored. In these experiments it was important to apply serotonin for short periods of time (less than 50 minutes) to minimize its dispersal throughout the culture medium and thus effectively retain the focal nature of application. Because of this time constraint it was not possible to analyze the rate of neurite elongation quantitatively; instead the inhibitory actions of serotonin were assessed by observing the accompanying structural changes of neuron 19's growth cones. A growth-inhibitory response to serotonin (applied from a micropipette containing 10^{-6} to $10^{-5}M$ serotonin; water pressure head, 7 cm; tip diameter <10 μm was detected only when the serotonin-containing pipette was placed adjacent to the growth cone. This growth-inhibitory response characteristically consisted of filopodial and lamellipodial retraction and a decreased surface area of the growth cone. Application of serotonin directly to growth cones always (n = 10) inhibited motile activity (*12*), an effect which was reversed on withdrawal of the pipette. Focal application of serotonin to a neurite or to the soma (n = 7), on the other hand, never caused such inhibitory effects.

It is possible to show the autonomous reaction of the growth cone to serotonin more dramatically by isolating these organelles from the cell proper. By severing the interconnecting neurite with the tip of a glass micropipette, one produces a viable isolated growth cone (*13*). Pipette application of serotonin to such isolated growth cones of neuron 19 always resulted in the reversible retraction of filopodia and lamellipodia and decreased extension activity (n = 8) (Fig. 1B). Thus, the growth cone itself can detect serotonin and transduce this response into a growth-inhibiting effect. Although this does not exclude additional contributions from the rest of the cell, it seems reasonable to regard the growth cone, in this respect, as an autonomous organelle.

The processes of growth and synaptogenesis are intimately intertwined. Since *Helisoma* neurons must be in an active growth state to form electrical synapses (*6*), we reasoned that serotonin may prevent the formation of these connections

by virtue of its growth-inhibitory characteristics. As a simple test, neurons 5 and 19 were plated in cell culture under conditions known to result in the formation of electrical connections (*6*); additionally serotonin ($10^{-6}M$) was added to the medium (day 1) specifically to inhibit further outgrowth of neuron 19. Later, after the unaffected, growing neurites of neuron 5 had overlapped the steady-state neurites of neuron 19 (Fig. 2A), the resultant connectivity was determined (days 3 and 4). In the presence of serotonin, neuron 19 never formed electrical connections with neuron 5 (mean coupling coefficient 0.00 ± 0.00, $n = 7$) (Fig. 2B) (*11, 14*), whereas in control cultures electrical connections always formed (0.18 ± 0.04, $n = 9$) (Fig. 2B) (*15*). In contrast, serotonin did not prevent the formation of electrical connections between pairs of neuron 5, as would be predicted from this neuron's resistance to serotonin's growth effects. In the presence of serotonin, pairs of neuron 5 always formed electrical connections (0.31 ± 0.09, $n = 5$) (Fig. 2B). Given the previous demonstration that *Helisoma* neurons must be in an active growth state to form electrical connections (*6*) these data indicate that by inhibiting neurite outgrowth, serotonin is able to prevent neuron 19 from forming electrical connections with other neurons that are themselves competent to interconnect.

Serotonin's effects are not restricted to neuron 19. We have examined the response of another neuron, P5. Rather than a total immobilization, as with neuron 19, or no effect, as with neuron 5, serotonin can transiently inhibit P5's motile activity (*16*). This range of effects makes it plausible that, as is the case in chemical synaptic transmission, the nature of the transmitter's effect on outgrowth resides largely in the target neuron. Perhaps as more neurons are examined some will be found whose growth is even enhanced by serotonin.

Although serotonin's locus of action seems restricted to the growth cone, the precise linkage with motility could take several forms; it may act by second messengers such as adenosine 3′,5′-monophosphate (cyclic AMP) or by altering transmembrane ion fluxes. Given the uncertainties of pharmacologically manipulating molluscan neurons, the best resolution to this question may come from direct measurements of both of these candidates. At the other extreme from questions of cellular mechanisms is the role of neurotransmitters in regulating growth in adult nervous systems. Serotonin has been proposed to have a regulatory role in neurogenesis (*17*). Could appropriately located release sites of serotonin in situ also regulate the quantity of neurite outgrowth and the actual form of a dendritic tree? Perhaps this is the basis for the kinds of effects seen on the plasticity of neuronal connections in the vertebrate visual system by another monoaminergic neurotransmitter, noradrenalin (*18*).

Numerous investigations have demonstrated that macromolecules can play important roles in the elaboration of neuronal architecture and connectivity. Our demonstration that a neurotransmitter is

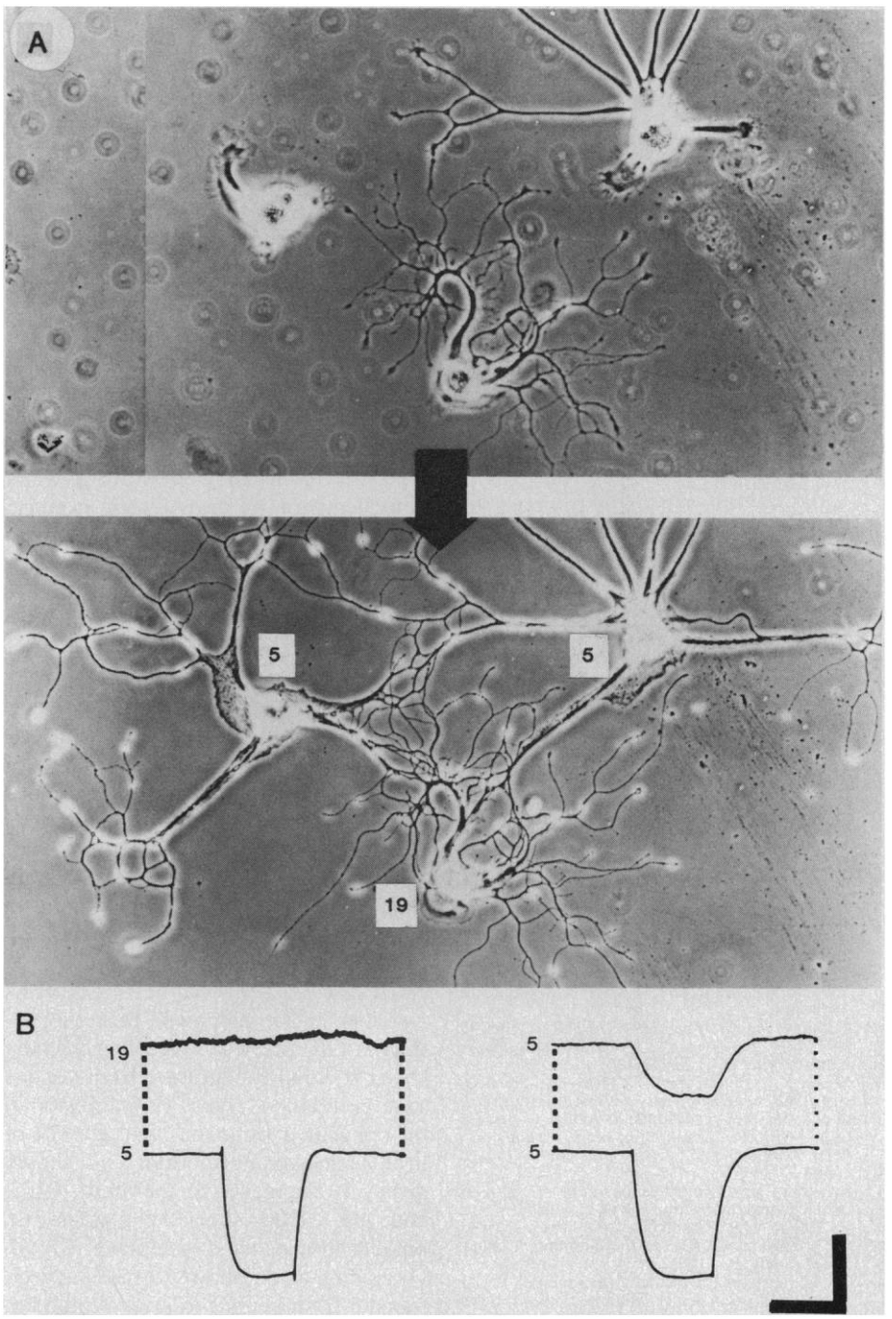

Fig. 2. Serotonin prevents the formation of specific electrical connections. (A) Serotonin ($10^{-6}M$) was added to the medium (day 1) to inhibit the outgrowth from the previously growing neuron 19. Serotonin was added before neuron 19 began to overlap with the growing neurites of neuron 5 (top). By day 3 there was little additional outgrowth of neuron 19 compared with a major elaboration of the arbor of the neurons 5 (bottom). This continued outgrowth of neuron 5 caused an extensive overlap of neurites between both neurons 5 and between neurons 5 and 19. (B) Direct current applied intracellularly to neuron 5 does not pass into neuron 19 but does pass into the paired neuron 5. Thus, serotonin's inhibition of outgrowth of neuron 19 thereby prevents the formation of specific electrical connections. Calibration: Horizontal, 2 seconds. Vertical left: neuron 19, 5 mV; neuron 5, 20 mV; vertical right: top neuron 5, 10 mV; bottom neuron 5, 20 mV.

able to regulate growth and, consequently, connectivity indicates that rather common simple molecules may also play prominent roles in regulating the pattern of neuronal connectivity.

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15. Serotonin significantly reduced the strength of the electrical connection between neurons 5 and 19 [$t(8) = 4.15$, $P < 0.002$; t-test] and increased the frequency of zero coupling values [$\chi^2(1) = 9.0$, $P < 0.01$].
16. Of 12 neurons P5 studied, serotonin (10^{-6} to $5 \times 10^{-5}M$) transiently inhibited the growth cone motile activity of eight neurons for periods of up to 100 minutes and totally inhibited the activity of the other four.
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Extrapulmonary Gas Exchange Enhances Brain Oxygen in Pigeons

Abstract. *Blood in mouth, nose, and eye tissues of birds cools by evaporation, then flows to a cephalic vascular heat exchanger, the ophthalmic rete. There, acting as a heat sink, blood from the evaporative surfaces cools arterial blood flowing countercurrent to it toward the brain. The brain thus remains cooler than the body core. Data for unanesthetized domestic pigeons* (Columba livia) *suggest that in addition to losing heat, blood perfusing the evaporative surfaces also exchanges oxygen and carbon dioxide with air. In the heat exchanger, this blood apparently gives up oxygen to, and gains carbon dioxide from, arterial blood. The consequent increase in oxygen and decrease in carbon dioxide in the brain's arterial blood enhance diffusion of these gases in, and oxygen supply to, the brain. Such events may help birds maintain the brain's oxygen supply during the high systemic demand of exercise and at the reduced oxygen availability of high altitude.*

Evaporation from mouth, nose, and eye surfaces in birds cools the blood flowing just beneath these surfaces. The cooled blood then courses to the ophthalmic rete (OR), a network of small arteries and veins caudal to each eye (Fig. 1). Arterial blood in the OR conducts heat to venous blood flowing countercurrent to it, then proceeds to the brain at reduced temperature. This protects the brain from overheating when body temperature rises during exercise or high temperature exposure (*1*).

Oxygen and carbon dioxide tensions (P_{O_2} and P_{CO_2}) in avian arterial blood at sea level are about 85 and 30 torr, compared to 159 and 0 torr in air (*2*). Blood circulating in the dense vascular beds beneath the moist cephalic surfaces must therefore encounter large gradients for these gases, and it seemed reasonable to infer that air and blood at these surfaces might exchange O_2 and CO_2, as well as water vapor and heat. Venous blood flowing to the OR would then not only have a lower temperature but would also have an increased P_{O_2} and a reduced P_{CO_2}. Our preliminary calculations indicated that P_{O_2} and P_{CO_2} in the OR veins would be higher and lower, respectively, than in OR arteries, suggesting that the OR is a site of O_2 diffusion from venous to arterial blood, and of CO_2 diffusion in the opposite direction, as well as a site of heat exchange. Furthermore, as shown in Fig. 1, changes in temperature, P_{CO_2}, and pH would affect hemoglobin O_2 binding in a manner conducive to both O_2 loading at the moist surfaces and O_2 transfer from venous to arterial blood in the OR (*3*).

The data presented here support this idea. They show that P_{O_2} in cerebral arterial blood of domestic pigeons (*Columba livia*: mean mass, 0.34 kg) is greater than in carotid arterial blood and suggest that the P_{O_2} difference is correlated with the extent to which cephalic mucosal surfaces contact air. An elevated P_{O_2} in cerebral arteries would increase the P_{O_2} in brain capillaries, improving O_2 diffusion to tissue. If arterial O_2 saturation also increases, total O_2 flow to brain would also increase.

To test the idea, we compared gas tensions in arterial blood before it entered the OR (pre-OR) and after it left the OR (post-OR) to determine whether enhancement of gas exchange occurs in the OR itself. Pre-OR arterial gas tensions were measured in carotid blood. However, post-OR arteries were inaccessible for sampling. We therefore reasoned that gas tensions in cerebrospinal fluid (CSF) sampled near the lateral ventricle's choroid plexus, a site of CSF formation (*4*), would represent corresponding values in choroid plexus blood, at least some of which comes from the OR. Accordingly anaerobic samples of CSF and arterial blood were simultaneously sampled from unanesthetized birds, and P_{O_2}, P_{CO_2}, and pH were measured (*5*).

Figure 2 shows results for 11 control birds at room temperature (experiment 1). Variances for mean differences were not significantly different from each other; we therefore used t-tests for comparing means. The mean P_{O_2} of the CSF was 114 torr (±3 standard deviation). This is 39 percent greater [$t(10) = 24.8$; $P < 0.0001$] than the mean P_{O_2} in carotid blood [82 torr (±3)]. We also found that P_{CO_2} was significantly lower in CSF [23.3 torr (±2.2)] than in carotid blood [31.4 torr (±2.3)], a 26 percent decrease [$t(10) = -8.5$, $P < 0.0001$]. The changes in cerebral arterial P_{O_2} and P_{CO_2} make possible increases in O_2 diffusion to and CO_2 diffusion from brain tissue.

Our results may also be explained by possible carbonic anhydrase activity in the ependymal cells of the choroid plexus. It has been suggested that the ependymal carbonic anhydrase reaction produces HCO_3^-, which moves into CSF, and H^+, which enters choroid capillary plasma (*6*). Erythrocytic bicarbonate