jority of the cells in a confluent culture are in G_1, whereas in a nonconfluent culture 30 to 60 percent of the cells are in the S phase (Fig. 1B).

Effective vaccines against *T. cruzi* and *T. gondii* have not been developed. The demonstration of vertebrate cell cycle modulation of infection and the probable existence of specific receptors for these parasites provides a new approach to the problem of disease prevention. Once isolated and characterized, the S-phase surface components responsible for the enhancement of infection could be used to block the complementary receptor on the parasites. This would interrupt the obligate intracellular vertebrate cell cycle and possibly produce sterile immunity in the host.

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3. T. Terisma and L. J. Tolmach, *Exp. Cell Res.* **30**, 344 (1963). To obtain a sufficient quantity of mitotic cells, eight flasks (150 cm^2) were seeded with HeLa cells in Dulbecco's minimum essential medium supplemented with 10 percent heat-inactivated fetal bovine serum. When the cultures reached 50 to 70 percent of confluence, mitotic cells were harvested by the shake-off technique. The initial shake-off, containing loosely attached and early G_1 cells, was discarded. At 15-minute intervals thereafter the cultures were again shaken and the mitotic cells were pooled and stored at 4°C. Generally, two to four shake-offs were required to obtain a sufficient quantity of mitotic cells. After centrifugation at 67*g* for 10 minutes, the cells were diluted in fresh medium to a concentration of 10^4 cells per milliliter. The mitotic index of the initial (time 0) synchronized HeLa cell population was quantified from slides prepared by fixing a portion of the cell suspension in acetic acid and absolute ethanol (1:3). After washing the cells by centrifugation (67*g*, 10 minutes) in absolute ethanol, they were stained with 1 percent toluidine blue in 1 percent butanol, cleared in xylene, and mounted in Permount. The percentage of cells undergoing mitosis (> 85 percent) was calculated by classifying 100 to 200 cells. At subsequent intervals (3 to 24 hours) the mitotic index was calculated for the cover-slip cultures that were exposed to parasites.
4. HeLa cell cultures grown as described in (*3*) were rinsed with Dulbecco's minimum essential medium without serum and treated for about 30 seconds with Dulbecco's phosphate-buffered saline (without calcium or magnesium) containing 0.25 percent trypsin and 0.10 percent EDTA. A sharp tap on the flask detached the cells, which were immediately diluted tenfold in medium supplemented with 10 percent fetal bovine serum to inactivate the trypsin. After centrifugation (67*g*, 10 minutes), the cells were diluted for use as described in (*3*).
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Quantitative Autoradiography of [^{3}H]Muscimol Binding in Rat Brain

Abstract. *A simple quantitative autoradiographic technique for the study of neurotransmitter receptors that includes the use of a tritium-sensitive film permits saturation, kinetic, and competition studies of brain samples as small as 0.01 cubic millimeter. This technique was used to study [^{3}H]muscimol binding in rat brain. Unilateral γ-aminobutyric acid receptor supersensitivity was observed in the substantia nigra pars reticulata after production of localized lesions of the ipsilateral corpus striatum.*

The use of tissue homogenates in binding studies of the brain has provided a wealth of information about the location, pharmacology, and regulation of synaptic neurotransmitter and drug receptors (*1*). However, the use of homogenate techniques to study the details of regional variation in receptor numbers has been limited by the requirement that milligrams of tissue are needed for each assay. Studies of such structures as the rat's red nucleus (weight, 0.6 mg) or medial habenula (weight, 0.2 mg)(*2*) are thus impossible unless tissues from several animals are pooled. Therefore, light microscopy has been used with autoradiographic techniques to determine the location of receptors for diffusible ligands in the brain (*3*). Although these techniques provide information about variations in receptor density within an area of the brain, quantitation of the autoradiograms must be done by grain counting (*4*), which is laborious and difficult to standardize from one section or experiment to another. Quantitative techniques for autoradiography with x-ray film have been developed for other diffusible substances—for example, investigation of local cerebral glucose metabolic rates with 2-[^{14}C]deoxyglucose and ^{14}C-sensitive x-ray film—has gained widespread use (*5*).

We now report the use of tritium-sensitive film for quantitative autoradiography of [^{3}H]muscimol binding in rat brain. Muscimol, a potent γ-aminobutyric acid (GABA) receptor agonist, binds to synaptic GABA receptors (*6*). Our technique enables us to obtain directly from the film complete saturation, kinetic, and competition data comparable to those obtained from homogenate studies for regions of brain as small as 10 μg.

Frozen sections of rat brain (20 μm) were mounted on subbed slides and kept frozen at −20°C until use. Mounted sections were first subjected to three consecutive 5-minute washings in ice-cold 50m*M* tris citrate buffer (*p*H 7.0 at 4°C) to remove as much endogenous GABA as possible. The washed sections were then dipped in varying concentrations (5 to 80 n*M*) of [^{3}H]muscimol (15 Ci/mmole)(Amersham) in 50 m*M* tris citrate buffer (*p*H 7.0 at 4°C), with or without various concentrations of drugs. Nonspecific binding in the presence of 0.1 m*M* GABA was determined in adjacent sections. After incubation for 30 minutes at 4°C, sections were subjected to three consecutive 5-second washings with ice-cold buffer and then were blown dry. Slides were mounted along with appropriate standards in an x-ray cassette, and a sheet of Ultrofilm ^{3}H (LKB) was apposed to the sections. After a 12-day exposure at 4°C, the film was developed in D-19 (Kodak) for 5 minutes at 20°C, fixed, and dried. For quantification, the autoradiograms were projected in a photographic enlarger. Density readings were made with a photosensitive diode located at the center of the enlarger's image plane (*7*); readings of different regions were made by moving the autoradiographic film across the enlarger's film plane. Eight readings of the equivalent of 10-μg samples from each region were averaged, and the concentration of radioactivity in the region of the section that had underlain the film was determined by a computer-generated polynomial regression analysis, which compared the film densities produced by the sections with those produced by the standards.

In preliminary experiments, the correlation between densities produced by commercially available ^{14}C plastic standards and sections of brain paste containing known amounts of [^{3}H]formalde-

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hyde or [^{3}H]quinuclidinyl benzilate was determined. A similar relationship had already been determined for tissues containing known amounts of ^{14}C and plastic standards (*8*). The calibration values produced by the plastic standards were reproducible to within ± 5 percent. Use of ^{14}C standards obviates the need for recalibration of standards made from tissue containing ^{3}H to correct for isotopic decay.

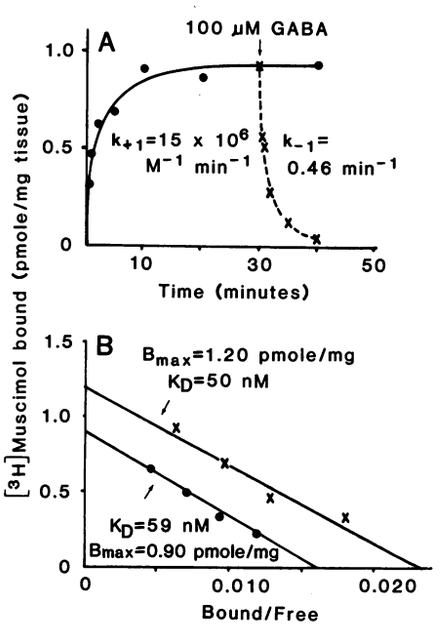

Fig. 1. Kinetic and saturation studies of [^{3}H]muscimol binding by quantitative autoradiography. (A) Time course of [^{3}H]muscimol binding. Sections of frozen rat brain (20 μm) were dipped in 50 n*M* [^{3}H]muscimol (15 Ci/mmole; Amersham), with or without 100 μ*M* GABA, for various lengths of time; the slides were then rinsed, dried, and apposed to Ultrofilm ^{3}H (LKB) for 12 days at 4°C. After the film was developed, densities were measured in layer IV of the cerebral cortex and related to standards to determine the amount of tritium bound per milligram of tissue at any given time (●). Nonspecific binding in the presence of 100 μ*M* GABA was subtracted from each point. At 30 minutes, GABA was added to the incubation medium to yield a final concentration of 100 μ*M*. The decline in bound [^{3}H]muscimol was then determined at various times thereafter (X). Each point represents the mean of eight determinations in the region. The experiment has been replicated three times. (B) Scatchard analyses of saturation experiments performed on rats with unilateral kainic acid lesions of the striatum. Twelve days after production of striatal lesions, serial sections of frozen rat brain were dipped in 5 to 80 n*M* [^{3}H]muscimol, with or without 100 μ*M* GABA, and processed as described above. Densitometric readings were taken in the substantia nigra pars reticulata (SN). The SN on the lesioned side (X) showed a 30 percent increase in the number of binding sites as compared to the control side (●), with little change in K_D. Each point represents the mean of eight determinations in the region. The experiment has been replicated six times.

This technique was used for quantitative studies, with data taken directly from the film (Fig. 1). Binding was saturable in all areas studied. The range of affinities (K_D's) was small (mean ± standard error = 45 ± 2 n*M*; *N* = 20). Kinetic data indicate that binding reached equilibrium by 30 minutes and had a rapid rate of dissociation (Fig. 1), the data indicating an association rate (k_{+1}) of 15×10^6 M^{-1} min^{-1} and a dissociation rate (k_{-1}) of 0.46 min^{-1} ($k_{-1}/k_{+1} = K_D$ = 30 n*M*). The value of K_D (30 n*M*) calculated from the kinetic data corresponds well with that obtained from equilibrium saturation data and is similar to that obtained in homogenate studies of lightly washed tissues and to the lower affinity site value obtained from the homogenate studies of extensively washed or tritonized tissues (*6*). It is unknown whether this slightly lower affinity is secondary to some residual endogenous modulator or to the difference in tissue preparation. [^{3}H]Muscimol binding was highest in layer IV of the cerebral cortex, the granule cell layer of cerebellum, the stratum moleculare of the dentate gyrus, the substantia nigra pars reticulata, and parts of thalamus (Fig. 2).

Competition studies of [^{3}H]muscimol binding showed a similar pharmacology to that observed in homogenate studies (*6*). Both GABA and the convulsant alkaloid and GABA antagonist (+)-bicuculline caused nearly complete displacement of bound [^{3}H]muscimol at 100 μ*M*, whereas picrotoxin at the same concentration was ineffective. Taurine and β-alanine caused partial displacement only at high concentrations (20 and 10 percent, respectively, at 100 μ*M*). The uptake blocker, 2, 4-diaminobutyric acid (100 μ*M*), was ineffective in displacing [^{3}H]muscimol, as were similar concentrations of glutamate, aspartate, and glycine.

Having demonstrated that our autoradiographic technique could provide quantitative data about central nervous system GABA receptors, we wanted to determine whether the technique could be used to demonstrate changes in brain receptors after the production of lesions. We investigated [^{3}H]muscimol binding in the substantia nigra after unilateral lesions were produced in the corpus striatum with kainic acid (Fig. 2). Neurons in this part of the striatum project to the medial and intermediate portions of the rostral two-thirds of the pars reticulata of the substantia nigra (*9*). Homogenate studies showed that GABA receptors in the substantia nigra as a whole become supersensitive after the production of striatal lesions (*10*); these homogenate studies indicated that the supersensitivity was secondary to an increase in the number of receptors in the substantia nigra 1 week after the lesion was produced. Lesions of the corpus striatum just anterior to the level of the anterior commissure in rats were produced by electrophoretic application of 3 nmole of

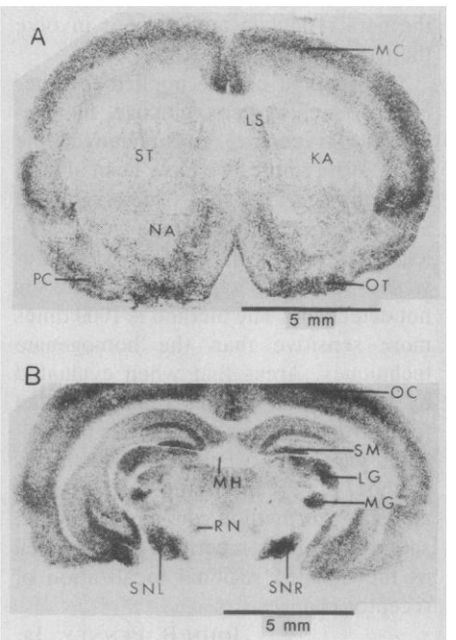

Fig. 2. Autoradiograms of [^{3}H]muscimol binding in rat brain. Male Sprague-Dawley rats were injected in the right striatum with 3.0 nmole of kainic acid at various times before being killed. The brain was removed, frozen, and sectioned onto subbed slides. Slices were washed and incubated as described in the text in 5 to 80 n*M* of [^{3}H]muscimol to obtain regional saturation data. (A) Coronal section through the kainic acid lesion site demonstrating the lack of local receptors 30 days after the lesion was produced. (B) Coronal section through the midbrain demonstrating increased receptor binding in the ipsilateral substantia nigra pars reticulata 12 days after production of the lesion. Areas for which receptor numbers (B_{max} given as picomoles per milligram of tissue) and affinities (K_D given as nanomoles per liter) have been measured are *KA*, kainic acid lesion site (no detectable specific binding); *LG*, lateral geniculate nucleus (B_{max} = 1.1, K_D = 41); *LS*, lateral septum (B_{max} = 0.4, K_D = 35); *MC*, motor cortex (B_{max} = 1.1, K_D = 46); *MG*, central body of medial geniculate nucleus (B_{max} = 1.2, K_D = 37); *MH*, medial habenula (B_{max} = 0.5, K_D = 42); *NA*, nucleus accumbens (B_{max} = 0.5, K_D = 38); *OC*, occipital cortex layer IV (B_{max} = 1.6, K_D = 44); *OT*, olfactory tubercle (B_{max} = 0.7, K_D = 49); *PC*, pyriform cortex (B_{max} = 0.7, K_D = 48); *RN*, red nucleus (B_{max} = 0.7, K_D = 58); *SM*, stratum moleculare of dentate gyrus (B_{max} = 1.4, K_D = 43); *SNL*, left substantia nigra pars reticulata (B_{max} = 0.9, K_D = 59); *SNR*, right substantia nigra pars reticulata (B_{max} = 1.2, K_D = 50); and *ST*, corpus striatum (B_{max} = 0.4, K_D = 46).

kainic acid (Fig. 2) (*11*); we looked at the GABA receptors in the pars reticulata of the substantia nigra 12 days later. [^{3}H]Muscimol binding was 20 to 60 percent above that on the control side (Fig. 1). The change was secondary to an increase in receptor number, with no change in K_D. The magnitude of the change was correlated with the size of the striatal lesion and its location. The supersensitivity was confined to the rostral, medial, and intermediate parts of the pars reticulata and did not involve the pars compacta.

This method can be applied to other ligands, such as deoxyglucose, flunitrazepam, strychnine, and phencyclidine (*12*). Furthermore, we have been able to study tissues after 2-[^{14}C]deoxyglucose administration. The labeled glucose analog is eluted in the successive buffer washes, and radioactivity in the blanks is not detectable. The method is 1000 times more sensitive than the homogenate techniques. Areas that when evaluated by homogenate studies require pooled tissue from microdissections can be evaluated easily in a single animal with this method. For receptor studies in lesioned animals, this method can supply kinetic, saturation, and competition data, as well as the detailed regional localization of receptor changes.

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Stimulation of Intestinal Calcium Transport and Bone Calcium Mobilization by Prolactin in Vitamin D–Deficient Rats

Abstract. *In vitamin D–deficient rats intestinal calcium transport increased significantly 4 hours after an injection of prolactin, reached a maximum after 8 hours, and declined to preinjection levels after 24 hours. Similarly, in vitamin D–deficient rats fed a diet low in calcium or phosphorus prolactin stimulated an increase in serum calcium in both groups and an increase in serum phosphorus in the rats fed the diet low in phosphorus. Thus it appears that prolactin affects organs involved in calcium regulation in a manner that is independent of the vitamin D endocrine system.*

For some time it has been accepted that intestinal calcium transport responds solely to the active form of vitamin D—1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]—or to one of its analogs (*1–3*). Liberation of bone calcium is believed to result from a combined action of the vitamin D endocrine system and parathyroid hormone (*1–4*). However, it is also known that prolactin can stimulate absorption of calcium by the intestine (*5, 6*). This has been believed to result from prolactin stimulating the conversion of 25-hydroxyvitamin D_3 (25-OH-D_3) to 1,25-$(OH)_2D_3$ (*7*). Recently it was shown that female rats severely deficient in vitamin D can reproduce and lactate, demonstrating that calcium can be obtained by the pregnant female from intestinal and bone sources (*8–10*). The loss of mineral from bone during reproduction and lactation in the vitamin D–deficient mothers was similar to that in vitamin D–replete control mothers (*9*). Also, intestinal calcium transport increased in the vitamin D–deficient mothers (*10*). It was suggested that some humoral factor might be involved in the liberation of calcium from intestine and bone (*9, 10*). The most likely hormone that could satisfy this role is prolactin, which is secreted at precisely the appropriate time and is maintained at elevated concentrations in the circulation during pregnancy and lactation (*11–13*).

To test the possibility that prolactin affects intestinal calcium transport and the liberation of calcium from bone in a manner that is independent of vitamin D, weanling male albino rats (Holtzman) were placed for 10 to 16 weeks on a diet low in vitamin D (*14*). That this regimen led to vitamin D deficiency was revealed not only by hypocalcemia and lack of growth but also by the lack of detectable levels of 25-OH-D_3 and 1,25-$(OH)_2D_3$ in plasma (*15, 16*). These rats were then divided into groups of six to nine ani-

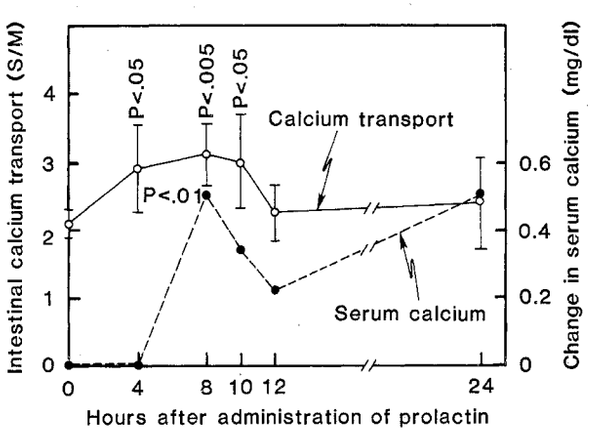

Fig. 1. Changes in intestinal calcium transport and serum calcium concentration in vitamin D–deficient male rats following an injection of prolactin (250 μg per 250 g of body weight). The data (means ± standard deviations) are expressed as either the rise in serum calcium or as the ratio of ^{45}Ca in the serosal medium (*S*) to ^{45}Ca in the mucosal medium (*M*). Before incubation the ^{45}Ca concentration was equal in both the serosal and mucosal media.

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