# Drugs in the Brain

## Autoradiography and radioassay techniques permit analysis of penetration by labeled drugs.

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The brain has been a source of continuing interest and investigation throughout recorded history. It has been studied by anatomists, physiologists, biochemists, and psychologists and, in its response to drugs, by pharmacologists. The multiple-disciplinary approach has not only revealed the magnificent organization and infinite complexity of the brain but has constantly stimulated investigators to extend their knowledge of its structure and function. The enormous degree of structural differentiation in the brain is obvious on gross inspection, and examination by light and electron microscopy reveals wide variety in structure and arrangement at the cellular and subcellular level. The amount of blood flowing through the various anatomical subdivisions of the brain is neither uniform nor constant. More blood flows through gray matter than through white, the effects of physiologically controlled changes in blood flow being disproportionate for the two types of tissue.

In addition, evidence has accumulated to demonstrate a regional biochemical topography in brain for both enzyme and substrate. Enzyme activity clearly varies in different regions. For example, levels of acetylcholine esterase may differ from one region to another by a factor of 50, whereas levels in the same regions of similar species may be remarkably constant (*1*). The histological complexity of the brain led Lowry *et al.* to develop elegant microbiochemical techniques for analysis of brain samples as small as 1 microgram (*2*). Robins *et al.* utilized these methods in mapping the concentrations of as many as nine enzymes (*3*). They were able to demonstrate finite differences in concentration in such subdivisions as the outer layers of the cerebral and cerebellar cortex. The concentrations of endogenous, active substances have also been found to vary with the brain region examined. Concentrations of noradrenaline have been found to be highest in the hypothalamus and the area postrema (*4*). Serotonin is highly localized; structures of the limbic system contain distinctly more serotonin than the neocortex (*5*).

Studies with exogenous substances, such as drugs, frequently ignore the specific and potentially unique response of the functionally specialized tissues in discrete anatomic loci of the brain. Specificity has been shown in the monkey at the gross level with certain 8-aminoquinolines (*6*, *7*) which are striking in their ability to produce structural changes within selected anatomical areas of the brain (Fig. 1). Certain arsenical compounds have also been found to produce specific necrotizing lesions in the lateral geniculate bodies of monkeys (*8*). Marked changes in the monkey brain have been noted after poisoning with methyl alcohol, necrosis and hemorrhage being produced in the putamen and caudate nucleus (*9*). Carbon disulfide produces a specific bilateral degeneration of the globus pallidus and substantia nigra which is not related to vascular damage (*10*). What, then, are the determinants which govern such localized destructive lesions? No answers are available, yet these examples suggest that certain specific relationships exist between tissue and toxin. Richter has commented (*7*) that it is "difficult to escape the conclusion that there is some specific selective affinity, chemically direct or indirect, between the toxin and the chemical organization of the part of the brain destroyed."

These extreme examples of localized necrotizing action on specialized nervous tissue raise certain questions about any drug which affects the central nervous system. Is the action on nervous tissue per se, or does the drug produce a response in the brain by some secondary effect? If a drug enters the brain, what factors determine the entry, into what structures does it move most readily, and where does it concentrate? The question should be asked, Do localized concentrations of a drug have significance only in relation to regional brain characteristics or do they, in addition, have pharmacological significance?

The problem of entry and concentration of drugs in brain loci is often sidestepped, although the blood-brain barrier is frequently cited to explain a series of pharmacological reactions which are not otherwise understood. Nor is the barrier itself uniform throughout the brain. It is poorly established in the area postrema, the periventricular nucleus, the supraoptic nucleus, the pituitary, and the pineal body. The blood-brain barrier appears to be a combination of factors, including capillary endothelium, surrounding glial cells, and perhaps other anatomical components, all of which may bar or admit drugs to the brain parenchyma.

Analyses of drugs in whole brain or whole-brain homogenates, while important first steps, may actually obscure subtle variations of correlative value. It is important, therefore, to measure drug concentration in discrete areas of the brain with respect to time after administration, and to explore the possible influences of such factors as disease and maturational, nutritional, physiological, and biochemical states.

Techniques are presently available which can assist in elucidating the problem of entry of drugs into the brain and its structural subdivisions, under conditions in which pharmacological levels of a drug are employed. The techniques of autoradiography and radiochemical assay offer means for studying this problem, through utilization of animals given low or trace levels of active substances. Autoradiography provides an especially valuable integrated visual description of penetration and localization phenomena in discrete anatomic loci and also provides a qualitative comparison of content from structure to structure. Thus, relative changes between areas may be related to time after administration as

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well as to developmental age and physiologic or pathologic state (*11*).

We have found that drugs that are classified together because of similar basic chemical structures, such as the barbiturates, may have profoundly different penetration patterns. No definitive generalizations can be made until more specific and detailed information is obtained on many more compounds. We give here a brief summary of our work on the penetration of cat brain by a number of synthetically labeled substances, and a discussion of some of the factors which influence penetration and localization. We refer to the methodology of other workers by citation. A brief description of the technique we have utilized is given below (*12*).

### Factors Influencing Entry into Brain

*The circulation.* The brain possesses few metabolic reserves, and a substantial flow of nutrient and oxygen (20 percent of the total oxygen consumption of the body) must be supplied to it. Because of the large amount of blood that circulates through the brain [54 $cm^3$ per 100 g per minute in adult man (*13*)], it is tempting to assume that the penetration of drugs into its parenchyma may be related directly to the quantity of blood supplied. Such assumptions ignore the minute amounts of exogenous substances usually accepted by the brain in spite of the fact that approximately 15 percent of the total blood flow passes through the brain. Because of the importance of the cerebral circulation in the metabolic functions of the brain, many attempts have been made, with both anatomical and physiological techniques, to estimate how much blood is supplied to the various subdivisions of cat brain. In general, dynamic studies have been limited to structures, such as the pial vessels, which can be easily exposed. Capillary counting and extrapolation of data have served for describing the circulation of the deeper structures. The entire problem is complicated by the geometry of the brain and its enclosure within the bony skull.

The techniques of autoradiography and densitometric assay have been successfully applied by Kety, by Sokoloff, and by their co-workers (*14, 15*) in the development of a method capable of providing data on blood flow in selected regions of the cat brain. These workers infused the radioactive gas trifluoroiodomethane and froze the head in liquid nitrogen after decapitation. They were able to measure blood flow in 28 regions of the brain and cord and demonstrated a striking nonuniformity of flow among structures of the adult cat brain. They found that gray matter was more vascular than white matter, and that the colliculi and the lateral and medial geniculate bodies, as well as the cerebral cortex, were among the regions of most active blood flow. These variations are shown in the autoradiogram reproduced in Fig. 2.

We have supplemented the data of these workers, utilizing iodine-131 serum albumin (RISA, Abbott) to demonstrate the relative vascularity of various areas of the cat brain. Autoradiography and densitometric measurement of the exposed film gave values for static vascularity that were in good agreement with those obtained

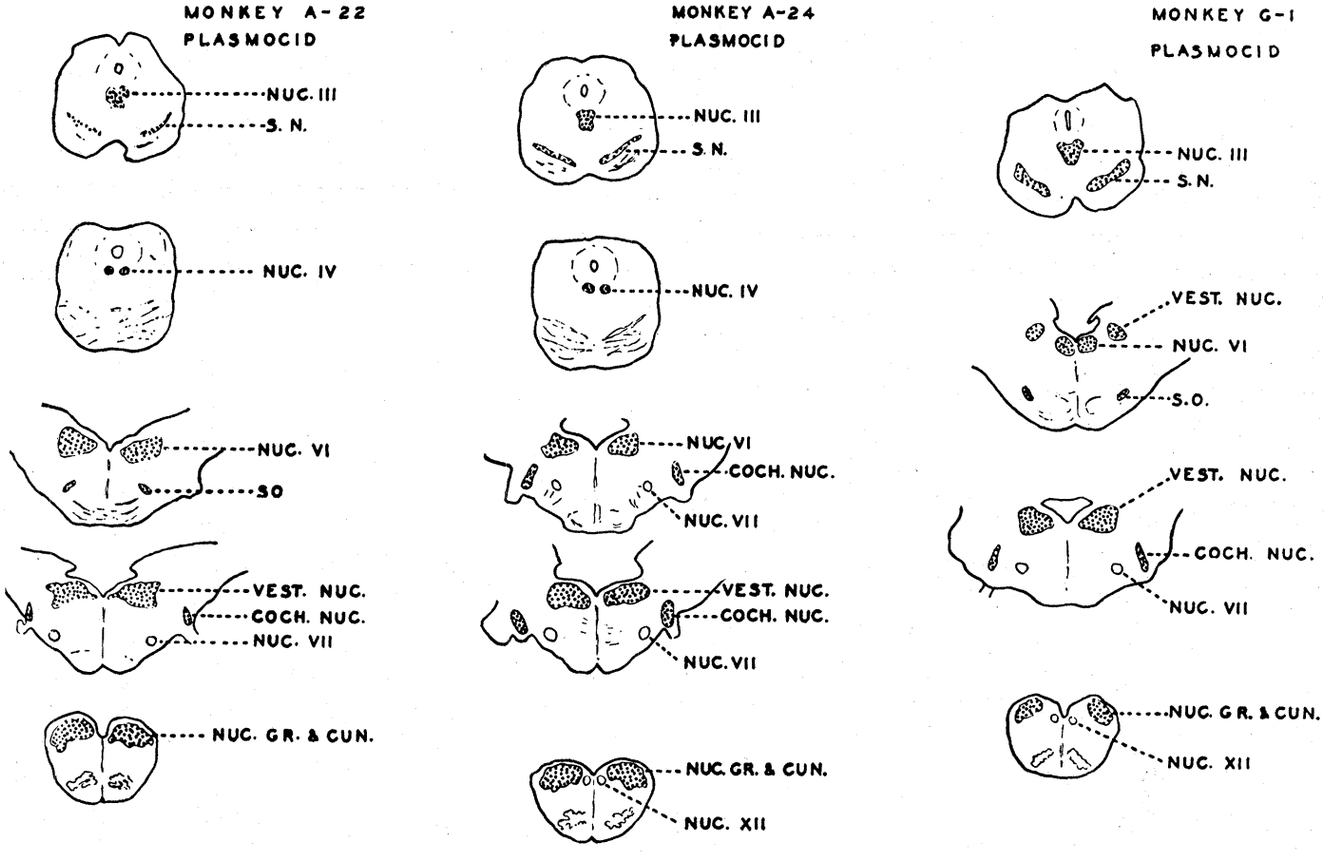
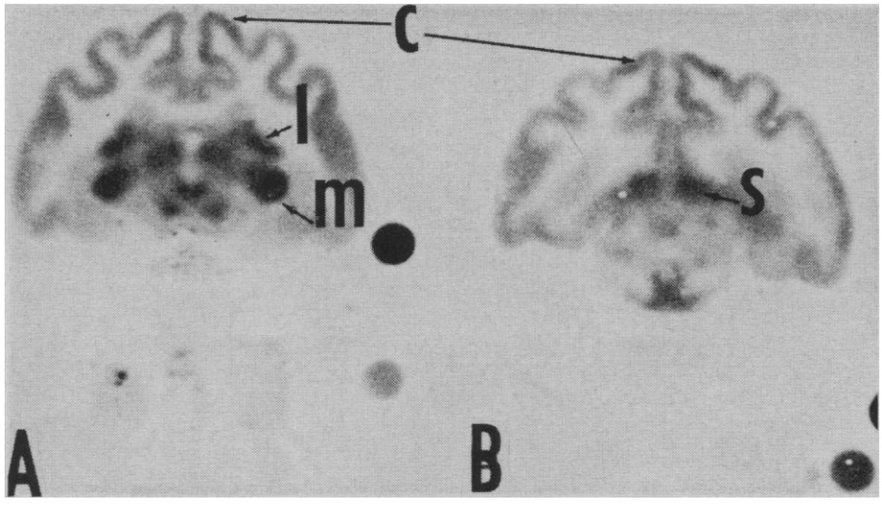
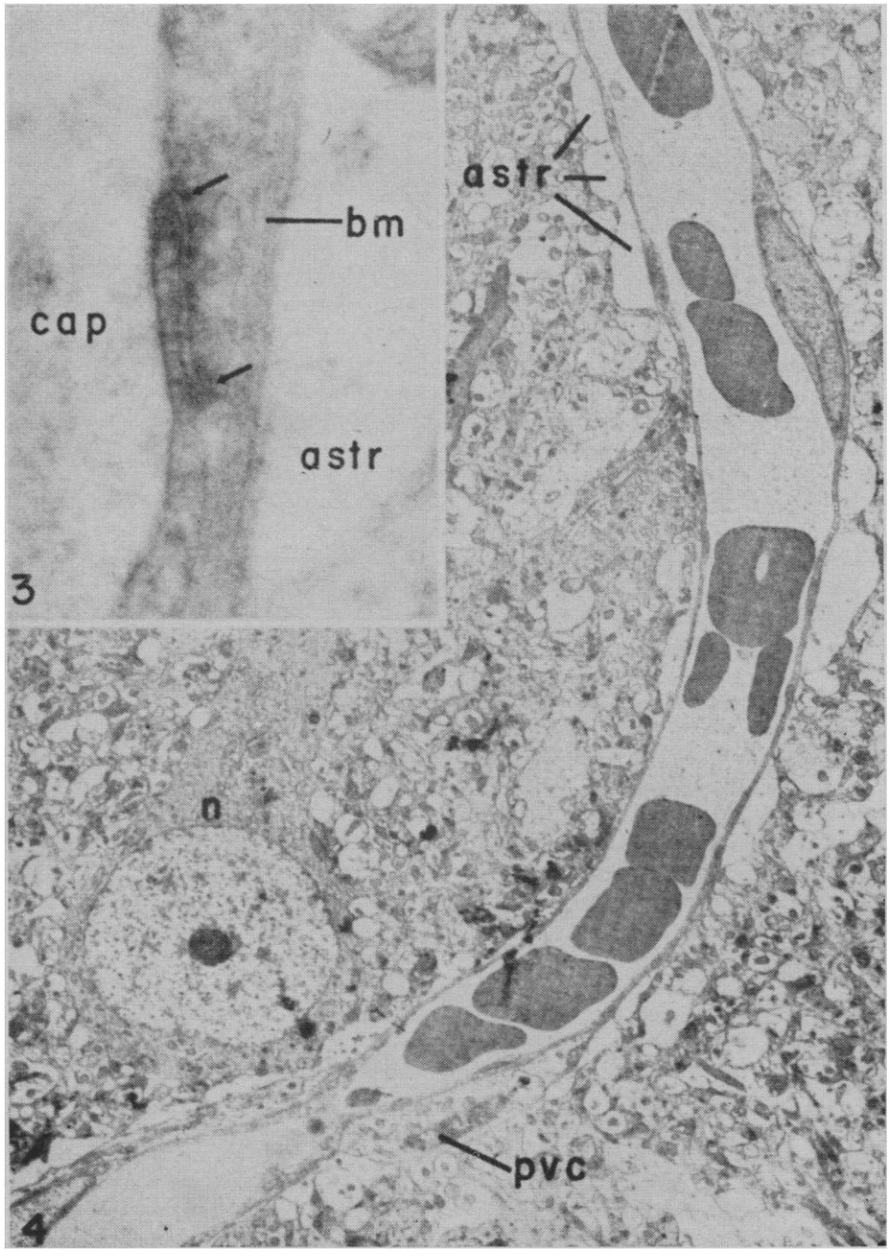



Fig. 1. Projection drawings from serial sections, showing the distribution of necrotizing lesions in the brain-stem nuclei of plasmocid-treated monkeys. [From R. B. Richter, *J. Neuropathol. Exptl. Neurol.* **8**, 155 (1949)]




previously by capillary-counting techniques and re-emphasized the abundant vascularity of geniculates and colliculi (*16*).

Detailed determination of blood flow and vascularity for the brains of immature cats has not been reported, although each of the afore-mentioned techniques should be applicable. Measurements of blood flow and vascularity are germane to the question, Does local circulation constitute a major determinant in the penetration and concentration of drugs in the central nervous system? Before reporting our findings on these relationships, we discuss briefly certain other characteristics of brain which may also play a role in influencing drug penetration.

*Solute spaces.* Electron-microscopic studies reveal that there is very little extracellular space in the brain, and that the membranes of cellular elements are in intimate contact with each other and with the capillaries (*17*). Maynard *et al.* (*18*) report that brain capillaries have a unique, thick, and solid arrangement of endothelial cells, the pericapillary structure being composed largely of astrocyte feet applied directly to the capillary wall. Maynard and her co-workers have also shown that the extracellular spaces found by earlier workers are, in fact, watery glia, and that the capillary endothelium is in close apposition to the basement membrane. Figure 3 demonstrates this concept and indicates what, with less refined techniques, could have been mistaken for extracellular spaces. Calculations based on the assumption that the chloride ion is entirely extracellular show this chloride space to account

Fig. 2 (top). Autoradiograms of coronal slices of cat brain, showing regional variations in blood flow as indicated by distribution of $I^{131}$-labeled trifluoroiodomethane, an inert gas. Areas of greater density are areas of greater blood flow. *c*, striate cortex; *l*, lateral geniculate ganglia; *m*, medial geniculate ganglia; *s*, superior colliculi. [From L. Sokoloff, "Local blood flow in neural tissue," chap. 6 in *New Research Techniques of Neuroanatomy*, W. Windle, Ed. (Thomas, Springfield, Ill., 1957) (anatomical labels ours)]. Fig. 3 (bottom). Electron photomicrograph of the cerebral cortex of an adult rat. Note the virtual absence of visible extracellular space. *cap*, lumen of the vessel; *astr*, astrocytic cytoplasm; *bm*, basement membrane; *pvc*, flattened perivascular cell; *n*, large neuron. [From E. A. Maynard, R. L. Schultz, D. C. Pease, *Am. J. Anat.* **100**, 409 (1957), plate 2]

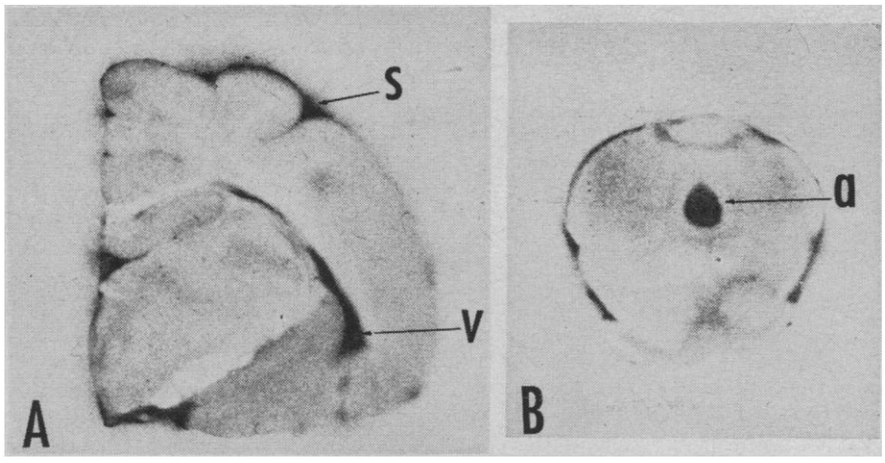



Fig. 4. Autoradiograms of the cerebral hemisphere (*A*) and midbrain (*B*) of a cat 4 hours after injection of $S^{35}$-labeled sodium sulfate. Cerebrospinal fluid is shown by the heavy radiodensity in subarachnoid spaces (*S*), ventricles (*V*), and aqueduct of Sylvius (*a*). The fluid is sharply confined by the anatomical boundaries of these structures. Anatomical structures within the brain show almost equal radiodensities. [From L. J. Roth, J. C. Schoolar, C. F. Barlow, *J. Pharmacol. Exptl. Therap.* **125**, 128 (1959)]

for 30 percent of the weight of brain. Experiments in which $S^{35}$-labeled sulfate (*19*), inulin (*20*) and sucrose (*21*) were used as indicators of extracellular spaces yielded values more in line with the electron-microscopic findings.

Using the autoradiographic and radioassay techniques and $S^{35}$-labeled sulfate as an extracellular indicator, we have re-examined this problem in cats, utilizing ureter ligation to maintain a near-plateau concentration of sulfate in plasma (*22*). This resulted in a constant level in brain between 4 and 8 hours after administration. At this time the concentration of sulfur-35 is of the same magnitude (2.5 to 3.8 percent) in all regions; correction for metabolic incorporation alters these percentages very little.

The autoradiograms (Fig. 4) support the tissue assay data and show little distinction in level among anatomical structures and no relation to variations in vascularity or blood flow. The area postrema is the only parenchymal region of high autoradiographic density, while the cerebrospinal fluid in subarachnoid spaces and in the ventricular system is also prominent. The sharp line of demarcation between the brain parenchyma, together with the high autoradiographic density of cerebrospinal fluid, indicates that the higher concentration of sulfate is sharply confined within the boundaries of the subarachnoid space. It also illustrates the superiority of the autoradiographic technique in elucidating such phenomena.

Thus, the extracellular space previously thought of as bathing the cellular elements of the brain is found, by these physiological indicators, as well as by the electron microscope, to be very small. It is possible that the small extracellular space in the brain of adult cats is a significant factor affecting the entry and accumulation of certain substances in the brain. We have extended our observation on the sulfate space to the immature kitten, and some of our findings have differed strikingly from findings in the adult animal. The sulfate space is appreciably greater in the first month of life and, in addition, marked regional differences are observed (Fig. 5*B*).

Urea has been used frequently as a measure of the total body water because of its presumed easy access to all fluid compartments of the body

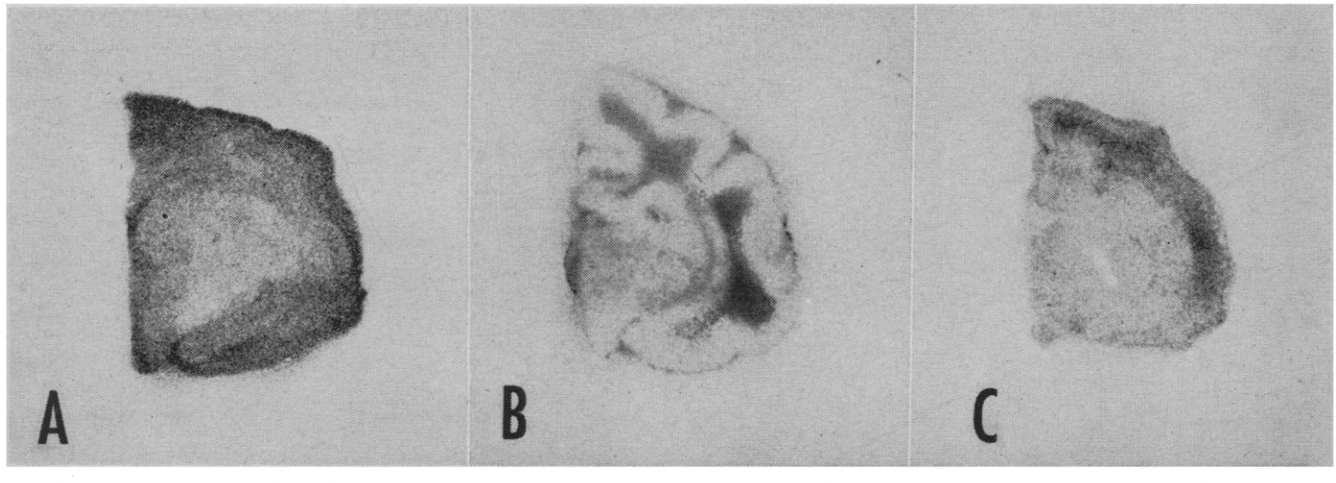



Fig. 5. Autoradiograms of cerebral hemispheres of newborn kittens. (*A*) Hemisphere 1 hour after injection of $C^{14}$-labeled urea, showing equal radiodensities in the cerebral cortex and in white matter, a finding that correlates well with the findings that these structures have equal water content and that significant amounts of myelin in white matter are lacking. [From J. C. Schoolar, C. F. Barlow, L. J. Roth, *J. Neuropathol. Exptl. Neurol.* **19**, 216 (1960)] (*B*) Hemisphere 6 hours after injection of $S^{35}$-labeled sulfate, showing greater radiodensity in primordial white matter than is found in the adult animal (Fig. 3). At 6 hours the sulfate space (after correction has been made for metabolic incorporation of sulfur-35 in plasma and brain) accounts for 16.8 percent of the weight of the cerebral cortex and 33.5 percent of that of the cerebral white matter, as compared to values of slightly less than 4 percent in adult animals. [From C. F. Barlow, N. S. Domek, M. A. Goldberg, L. J. Roth, *A.M.A. Arch. Neurol.*, in press] (*C*) Autoradiogram of hemisphere after injection of $C^{14}$-labeled phenobarbital, showing the greater density in primordial white matter. [From N. S. Domek, C. F. Barlow, L. J. Roth, *J. Pharmacol. Exptl. Therap.* **130**, 285 (1960)]

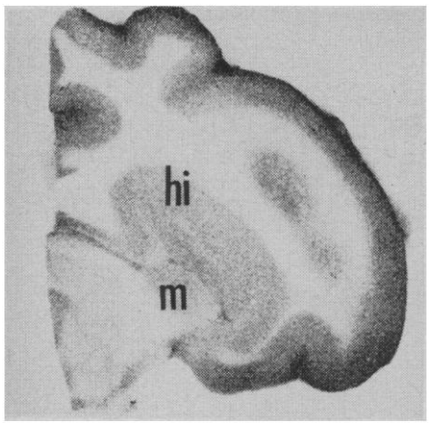



Fig. 6. Autoradiogram showing penetration of brain by $C^{14}$-labeled urea 6 hours after injection and definition of anatomical structure, as indicated on the developed film. The cerebral cortex shows the highest radioactivity; white matter, the lowest. Hippocampus (*hi*) and the very vascular medial geniculate body (*m*) show intermediate activity. [From J. C. Schoolar, C. F. Barlow, L. J. Roth, *J. Neuropathol. Exptl. Neurol.* **19**, 216 (1960)]

(*23*). We have, therefore, utilized $C^{14}$-labeled urea as an indicator of the behavior of a substance which would be expected to enter all the extra- and intracellular water of brain tissue. For this purpose urea has the advantages of being nonionic, water-soluble, and presumably not significantly metabolized by brain, and of having little or no tendency to bind to plasma or tissue protein. Autoradiograms and radioassay of dissected areas of the cat brain, after a single injection of $C^{14}$-labeled urea in trace amounts, clearly indicate a nonuniform penetration pattern, with maximum levels in brain and cerebrospinal fluid not attained before 6 hours. Furthermore, the rate of penetration into gray matter exceeds that for white matter (Figs. 6 and 7). It seems likely that regional difference with respect to penetration is related to the barrier posed by the multiple membranes of the numerous laminated myelin sheaths that are found in white matter (Fig. 8). This concept is supported by our observation of equal rate of entry into cortex and white matter of the newborn kitten (Fig. 5*A*). At this age the total water contents of cortex and of white matter are similar, and myelin has not yet developed. It would appear that vascularity and blood flow play a minor role in urea penetration, since regions of high vascularity, such as the colliculi and geniculate bodies, contain a smaller concentration of labeled urea than the equally or less vascular cerebral cortex at all times after administration (*24*).

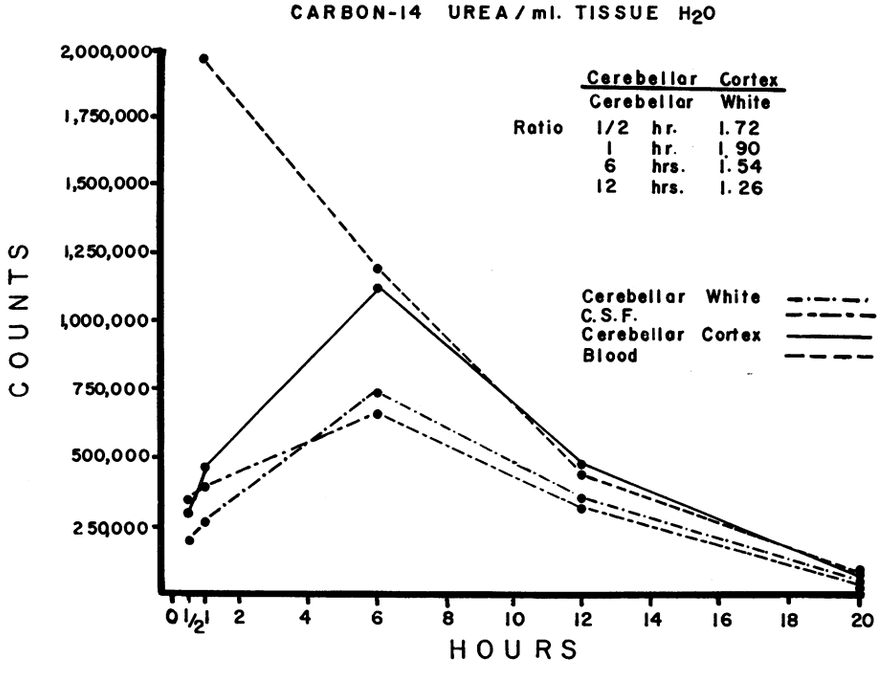



Fig. 7. Graph showing radioactivity in blood, cerebrospinal fluid, cerebellar cortex, and cerebellar white matter at different times after injection of urea. Note that it requires 6 hours for the level of activity in the cerebellar cortex to approach that in blood, and that the urea enters white matter more slowly and perhaps less completely. [From J. C. Schoolar, C. F. Barlow, L. J. Roth, *J. Neuropathol. Exptl. Neurol.* **19**, 216 (1960)]

*Blood-brain barrier.* There can be no doubt that the brain possesses a unique and specialized mechanism for excluding many substances presented to it by the circulation. The brain may exclude certain compounds completely, or admit them in only small amounts, while other organs become easily saturated.

The anatomical and physiological nature of the blood-brain barrier is a matter for active research and discussion. Some view the barrier as lying between the circulating plasma within the capillaries and the extracellular fluid (*25*), while others think the capillary endothelium (*26*) or the perivascular glia (*27*) constitute the barrier. The watery constitution of these glial cells, which fill the interstices in the neuropil, in no way detracts from their potential importance in controlling exchange between capillaries and neurons. The nature of the barrier differs in the various regions of the brain, such as areas of gray matter, areas of white matter, and choroid plexus. Substances normally excluded from the brain may penetrate the parenchyma readily if injected directly into the ventricles (*28*). Feldberg and Fleischhauer have shown that this may be an active process, since bromphenol blue failed to penetrate from the cerebrospinal fluid in the nonliving cat, whereas it penetrated deeply in the living animal (*29*). We have demonstrated repeatedly a marked difference in the penetration of gray matter and white by drugs, as well as by urea and sulfate, and have ascribed this difference in part to the lipoid lamellae of the white matter, suggesting an additional anatomical aspect of the blood-brain barrier. Moreover, boundaries of the small extracellular space probably restrict the entry of certain substances such as inulin, sucrose, and sulfate ion to that space. The physical (*30*) and metabolic (*31*) characteristics of the compound or ions under investigation are also influential factors in controlling penetration. Alteration in penetration may also be influenced by the inhalation of carbon dioxide, with concomitant variations in acid-base balance (*32–34*).

Nor can the age of the animal be ignored, since degree of myelinization, water content of various anatomical

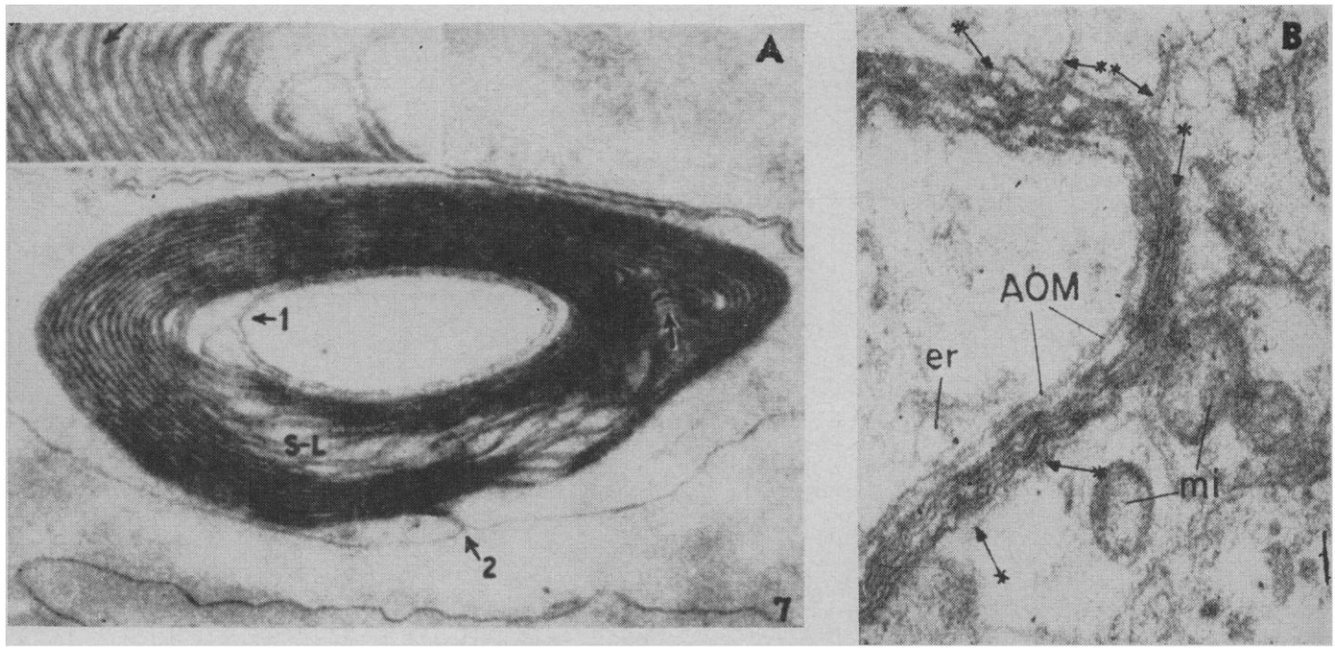



Fig. 8. Electron photomicrographs showing the laminated membranes characteristic of myelin sheaths of the peripheral (*A*) and the central (*B*) nervous systems. [*A*, from J. D. Robertson, *J. Biophys. Biochem. Cytol.* **4**, 349 (1958); *B*, from E. DeRobertis, H. M. Gerschenfeld, F. Wald, *ibid.* **4**, 651 (1958)]

divisions, cellular architecture, and biochemical organization of the brain vary with the degree of maturity (*31*). Each of these factors may profoundly influence the development of barriers and fluid spaces, and thus they may alter the penetrability of different structures in dissimilar ways. Our studies of penetration of kitten brain by $C^{14}$-labeled phenobarbital (*35*), $C^{14}$-labeled urea (*29*), and $S^{35}$-labeled sulfate (*22*) clearly demonstrate these differences (Fig. 5).

In view of the foregoing observations, it would appear that a whole series of anatomical, physiological, biochemical, and maturational factors, all unique to brain and many of them showing regional variation, must be taken into account in interpreting barrier phenomena. Herlin states (*36*): "It is practical to include in the term 'barrier' all the phenomena which prevent, reduce, delay, or even actually facilitate the penetration of a substance into the CNS [central nervous system]. The penetration might occur by dialysis, ultrafiltration, osmosis, the Donnan equilibrium, electrical charges, lipoid solubility, special tissue affinity or metabolic activity. A more limited definition of this term would imply a more exact knowledge of the barrier mechanism than one really possesses. If one had such exact knowledge, it would be unnecessary to use the term 'barrier'. It would be preferable to describe what happens. In reality, the barrier mechanisms may be different for each substance." These statements seem to be especially applicable to the compounds we have investigated.

## Studies with Labeled Drugs

Our investigations of the penetration of labeled barbiturates (phenobarbital and thiopental) into the central nervous system and of their accumulation there clearly demonstrate that the prevalent concept of uniform entry and distribution of barbiturates is no longer tenable if one examines drug entry as a function of time after injection and maturity of the animal, with due regard for the anatomical subdivisions of brain (*35, 37*). Sampling of such areas as cerebral hemispheres, diencephalon, brain stem, and cerebellum, without regard for their anatomical subdivision, obscures the differences which become apparent when their substructures are considered separately. By using autoradiography and radioassay techniques which delineate these subdivisions, we found a distinctly nonuniform distribution of phenobarbital, gray matter having been penetrated more rapidly than white (Fig. 9)—a finding which indicates that penetration by phenobarbital, like penetration by urea, is retarded by white matter in the adult cat. An inverse relationship exists in the kitten brain (Fig. 5*C*), where the primordial white areas are more readily penetrated. Presumably this is due to the incomplete formation of the myelin barrier and, perhaps, to the higher content of extracellular water in white matter in the immature brain. As the animal develops and myelination proceeds, the relative ease of penetration of white matter and gray reverses.

It is important to note that no metabolites of phenobarbital were found in the brain, and that while only one intravenous injection of the labeled drug was administered, a nearly constant level was maintained in the circulating plasma. Ultrafiltrates of plasma containing phenobarbital show that this drug is present in the cerebrospinal fluid in the same concentrations as in an ultrafiltrate of plasma. Phenobarbital is found in cerebral cortex and cerebral white matter at levels which indicate a degree of binding quantitatively similar to that existing in plasma, the total levels in cortex being somewhat above plasma levels after 3 hours (see Fig. 9). Although the level of phenobarbital in gray matter was comparable to that in plasma throughout the period of study, cerebral white matter and other myelinated structures, such as the medullary pyramids, did not achieve plasma levels for from 3 to 6 hours. It is evident that the rate of penetration is unrelated to vascularity, since the

highly vascular colliculi and geniculates contained less of the drug than would be expected if vascularity were a controlling factor. The eventual distribution at later time periods was nearly uniform.

Phenobarbital, a barbiturate with slow onset of action but of long duration, contrasts directly with thiopental (Pentothal), which is widely used in anesthesiology because it rapidly produces sleep of short duration. This remarkably early onset of action has been explained on the basis of thiopental's lipoid solubility and consequent rapid penetration into all areas of the brain. The short duration of action has been ascribed to early removal of the drug from the brain into the fat depots of the body (*38*). That fat constitutes the primary depot for removing thiopental from the circulation has been questioned by Goldstein and Aranow, who have demonstrated that total body water, muscle, and other binding sites play the more dominant role (*39*).

Examination, by the techniques of autoradiography and radioassay, of the penetration of thiopental into the brain of a cat reveals an interesting pattern of entry and accumulation not previously noted (*37*). Thiopental is unique among the drugs we have studied in that its distribution is suggestive of the known pattern of vascularity in cortex, geniculates, colliculi, and white matter at 1 and 5 minutes after injection (Fig. 10). We have found, as did Goldstein and Aranow (*39*), an early and high

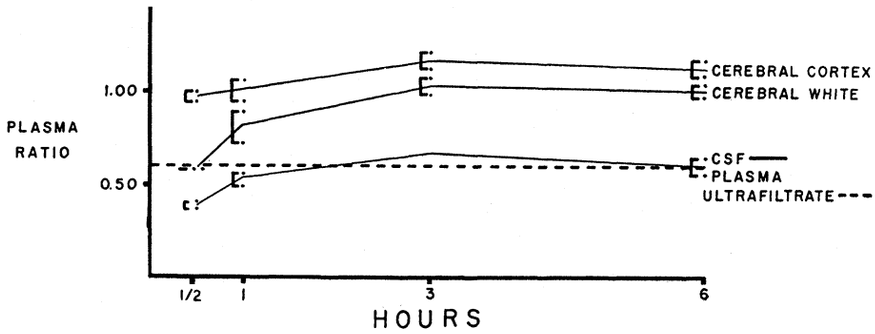



Fig. 9. Levels of $C^{14}$-labeled phenobarbital in cerebral cortex, cerebral white matter, and cerebrospinal fluid as compared with levels in total and unbound plasma. Values are expressed as ratios relative to values for plasma. It may be noted that levels in the cerebrospinal fluid and in the plasma ultrafiltrate are quantitatively comparable, while the total amounts in cortex and in white matter are comparable to the total level in plasma. Equilibration is more slowly attained in white matter than in cortex. [From N. S. Domek, C. F. Barlow, L. J. Roth, *J. Pharmacol. Exptl. Therap.* **130**, 285 (1960)]

Table 1. Influence of carbon dioxide on penetration of test substances. Concentration in brain is expressed as a fraction of levels in plasma. The values are based on radioassay data from M. Goldberg, C. F. Barlow, and L. J. Roth (*34*). Note the disproportionate increase in penetration of white matter by salicylic acid under conditions of hypercapnia. *Gray*, cerebral cortex; *white*, cerebral white matter.

| Area | Concentration in brain | | Relative penetration* |
|---|---|---|---|
| | Hypercapnic acidosis | Hypocapnic alkalosis | |
| | *Urea (1 hr)* | | |
| Gray | 0.40 | 0.16 | 2.50 |
| White | 0.12 | 0.05 | 2.40 |
| | *Phenobarbital (½ hr)* | | |
| Gray | 1.20 | 0.72 | 1.67 |
| White | 1.08 | 0.40 | 2.70 |
| | *Salicylic acid (1 hr)* | | |
| Gray | 0.44 | 0.11 | 4.00 |
| White | 0.38 | 0.04 | 9.50 |

* Relative penetration: brain fraction under conditions of hypercapnia to brain fraction under conditions of hypocapnia.

concentration of thiopental in the brain. However, by considering this organ in more anatomical detail, we have demonstrated the nonuniformity of the brain with regard to thiopental penetration and have found that the levels in the cerebral cortex at these early times after injection exceed the levels in plasma by a factor of 2, while a surprisingly low concentration was found in the lipoid-rich white matter. Equilibrium between gray matter, white matter, and plasma is not reached for approximately 30 minutes.

As with phenobarbital, the amount of thiopental in the cerebrospinal fluid is comparable to the amount in an ultrafiltrate of plasma. The finding of rapid accumulation or binding of an unusual amount of thiopental in gray matter at 1 minute after injection is in sharp contrast to our findings for phenobarbital. At 30 minutes after injection these distinctions are no longer apparent. The ultrashort action of thiopental (that is, early onset and early recovery) may not be due exclusively to its rapid depletion from brain but may also be related to a rapid fall from unusually high early levels in such gray matter structures as cortex, geniculate bodies, and colliculi. Assay of whole-brain homogenates have obscured these findings, which are clearly demonstrated by autoradiography and regional radioassay. We have detected only unchanged thiopental in brain at 1 and 5 minutes after injection; however, thiopental metabolites may appear in brain at later times.

Acetazolamide (Diamox), a drug designed to produce diuresis by inhibition of the enzymatic conversion of carbon dioxide and water into carbonic acid in the kidney, has also been found to be useful in the treatment of epilepsy. How this drug functions in suppressing epileptic seizures is not known at present, although inhibition of carbonic anhydrase activity in the brain with increase in localized concentrations of carbon dioxide has been suggested. Elucidation of this problem has been hampered by lack of sensitive analytical procedures and further complicated by the fact that this drug is found in brain parenchyma in microgram quantities. Attempts to assay the brain for acetazolamide have been limited to determinations on extracts and homogenates of whole brain—a procedure which does not permit analysis of significant relationships between enzyme and drug in discrete anatomical structures. We found $S^{35}$-labeled acetazolamide to be a convenient tool in our studies, since acetazolamide is not metabolized by the body (*40*). Autoradiography and radioassay reveal a surprising pattern of entry into the brain (*41*). Acetazolamide was found to enter by two distinct routes—the cerebrospinal fluid and the capillary circulation (Fig. 11). A high concentration develops first in the cerebrospinal fluid, with the ventricular walls acting as the site from which the drug diffuses into paraventricular areas. At 4 hours after injection this pattern is further developed. The areas of greatest concentration are still those in close proximity to the cerebrospinal fluid, and considerably more diffusion into periventricular and periaqueductal tis-

sues has by this time taken place. However, a second pattern becomes evident as the deeper structures are entered from the capillary circulation.

Eight hours after injection, when the level of the circulating drug has fallen, diffusion from the cerebrospinal fluid is no longer apparent. However, three distinct areas of the brain have retained the drug in relatively high concentration—caudate nucleus, hypothalamus, and hippocampus. Although the hypothalamus and thalamus are similarly exposed to the high concentration of acetazolamide in the cerebrospinal fluid, the hypothalamus retains the drug in high concentration at 8 hours, whereas the thalamus does not. Whatever the significance of the double mode of access in the case of acetazolamide, the regional differences in penetration and retention are more conspicuous with this drug than with any other we have studied. The development of high concentrations in discrete and widely separated structural units not only serves to emphasize in compelling fashion the relevance of cerebral architecture in neuropharmacology, but also strongly suggests some sort of localized and active biochemical binding.

The behavior of $C^{14}$-labeled diphenylhydantoin (Dilantin) is illustrative of an unusually high degree of tissue binding (*42*). This drug has been found to concentrate in the brain at levels that are two to three times the level in the circulating plasma and that persist for 24 hours after a single pharmacologic dose. If one compares levels in the brain with the non-bound, diffusible drug level in plasma, one finds that the brain levels exceed the plasma level by a factor of 10.

We have discussed the manner in which certain drugs, urea, and sulfate have been found to enter the brains of physiologically normal animals. We have noted that diversity is the rule, and that anatomic loci, structure, maturity, and vascularity play unpredictable roles that differ with the substance under investigation. It appears certain that heterogeneity will become increasingly evident as additional drugs are tested. Many more compounds possessing diverse physical and chemical properties will require investigation before valid general rules can be laid down. The role of infection and of the species, state of health, and nutritional, pathological, and physiological state of the animal also bear investigation.

Waddell and Butler (*32*) studied the effect of acidosis and alkalosis on the rate of urinary excretion of phenobarbital and noted that respiratory acidosis increased the concentration of phenobarbital in the total brain. Metabolic acidosis was shown by Gray *et al.* (*43*) to shorten the time required for acetazolamide to reach maximum anticonvulsant activity.

Considering these results, we undertook an investigation of the regional penetration characteristics of phenobarbital and acetazolamide under conditions of respiratory acidosis and alkalosis (*34*). We also included salicylic acid and urea for comparative purposes, since salicylic acid ($pK_a$ 3.0) is a stronger acid than phenobarbital or acetazolamide, while urea is a nonlipoid-soluble neutral solute. Under conditions of hypercapnia (plasma *p*H 6.81) and hypocapnia (plasma *p*H 7.86), marked alterations were found in the penetration of all brain areas by these compounds; penetration increased with hypercapnia and decreased with hypocapnia (Table 1). Furthermore, each of the three ionizable drugs showed proportionately higher concentrations in white matter than in gray under conditions of hypercapnic acidosis. This may be related to suppression of ionization, with presentation to lipoid membranes of a higher concentration of the nonionized, fat-soluble form of the test compound, white matter being particularly sensitive because of the multiplicity of myelin lamellae. Conversely, we find that white matter is poorly penetrated under conditions of hypocapnic alkalosis. The greatest effect is evident with salicylic acid, whose degree of ionization is most strongly affected by variations from normal physiological *p*H. These data are qualitatively compatible with a fourfold increase in the number of un-ionized salicylate molecules calculated for this acidotic state as com-

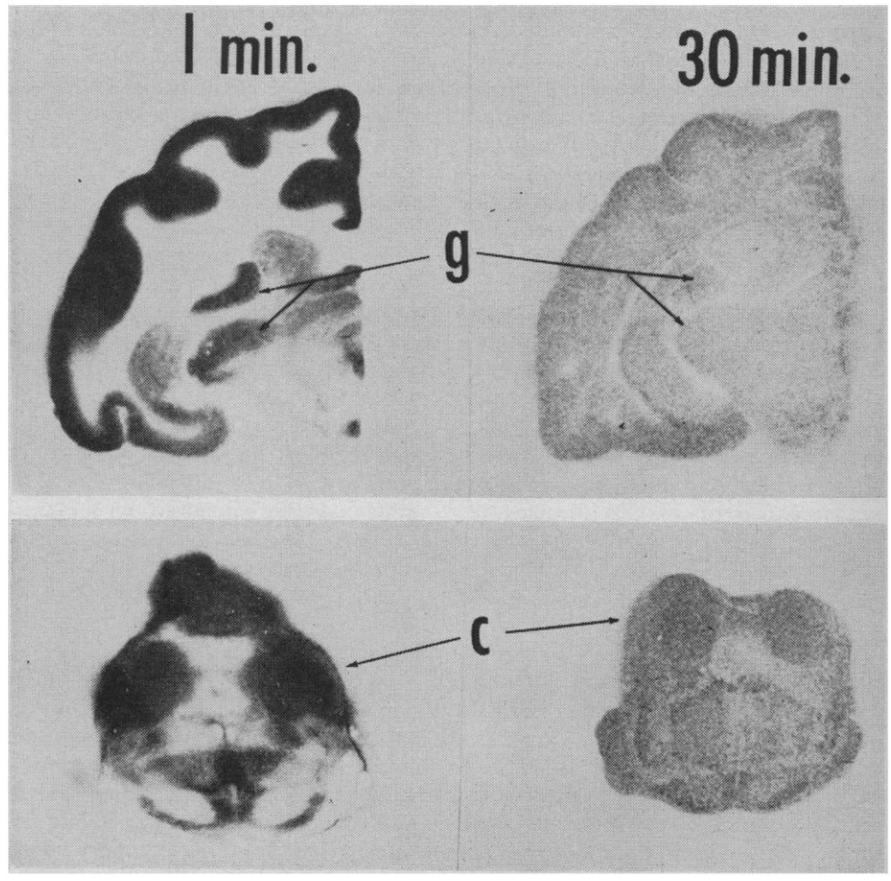



Fig. 10. Autoradiograms recorded after injection of $C^{14}$-labeled thiopental. Animals were sacrificed 1 minute and 30 minutes, respectively, after injection. At 1 minute after injection there is high radiodensity of cortex, geniculate bodies (*g*), and inferior colliculi (*c*), indicating high concentrations of the drug. The pattern is reminiscent of that of blood flow (Fig. 2). At 30 minutes after injection all areas are of almost equal radiodensity.

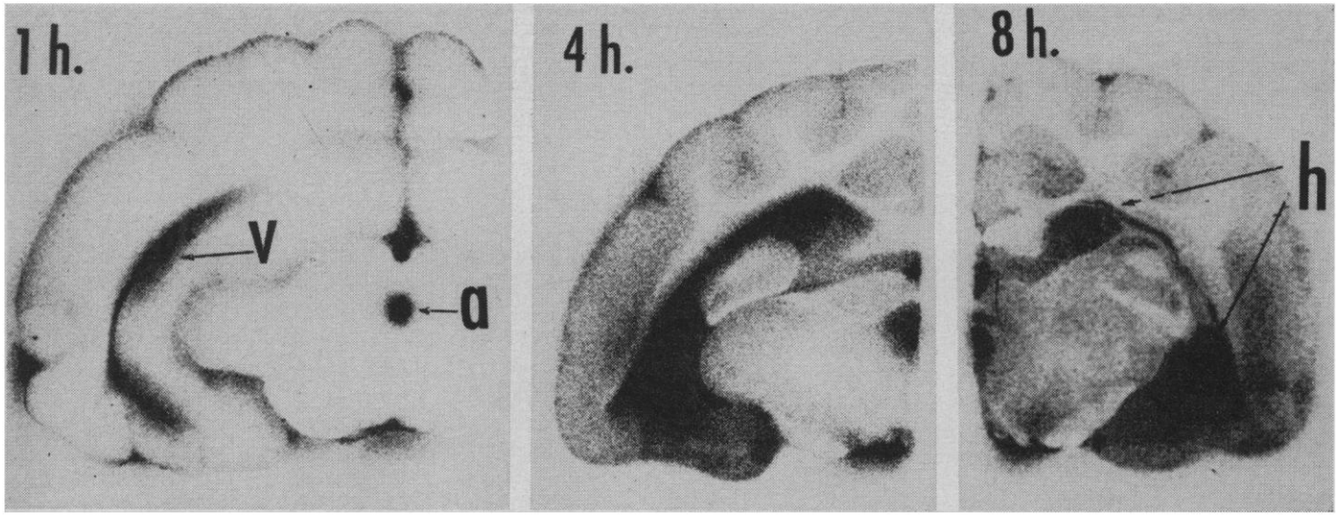



Fig. 11. Autoradiograms recorded at 1, 4, and 8 hours, respectively, after injection of $S^{35}$-labeled acetazolamide. At 1 hour after injection there is high radiodensity of the cerebrospinal-fluid pathways, especially prominent in the lateral ventricle (*v*) and the aqueduct (*a*). However, the line of demarcation between cerebrospinal fluid and brain is not sharply defined, as it is after injection of sulfate (Fig. 4), and a diffusion front is apparent. The autoradiogram recorded 4 hours after injection shows extension of penetration of the drug from the ventricle and aqueduct into adjacent areas of the brain. However, an anatomical pattern distinguishing the cortex from the underlying white matter has appeared, suggesting the more usual blood-to-brain entry pattern. At 8 hours after injection the major point of interest is the heavy radiodensity confined to the hippocampus (*h*).

pared to the normal number, and with a tenfold increase for the acidotic state as compared to the alkalotic state (*34*).

Comparison of the effects of hypercapnia and hypocapnia on the penetration of phenobarbital and salicylic acid, on the one hand, and of urea, on the other, reveals an important difference. Although hypercapnia facilitates and hypocapnia hinders the entry of urea, according to the general pattern, the entry is nonselective, and concentrations in all areas are proportionately increased with acidosis or decreased with alkalosis. This lack of difference in the penetration pattern occurs only with the nonelectrolyte urea. Changes in penetration of urea with administration of carbon dioxide demonstrate that a significant portion of the effects with carbon dioxide are due to factors other than ionic dissociation.

No direct correlation can be demonstrated to exist between penetration and vascularity or blood flow, although it is well known that blood flow is increased by hypercapnia and decreased by hypocapnia. If blood flow were a dominant factor, gray-matter areas should be markedly affected, since carbon dioxide increases blood flow in gray matter more than in white (*44*). Blood flow as a lesser role is shown by our finding that cats, on inhalation of carbon dioxide (5 percent), failed to demonstrate significant variations in brain concentrations of phenobarbital as compared to normal animals, although Kety (*45*) has found that inhalation of carbon dioxide at this level produces a marked increase in blood flow. The highly vascular colliculi and geniculate bodies also failed to demonstrate an increased penetration of drugs even at the highest carbon dioxide levels used.

The exact mechanism of the change induced by carbon dioxide is not known, but as we have indicated, carbon dioxide has a specific and perhaps unique effect on the entry of compounds into the brain—that is, on the blood-brain barrier—above and beyond its effect on dissociation. Further study of a larger series of divergent compounds, with analysis of the contribution of such factors as blood flow and tissue binding, will be necessary.

## Summary

In our studies on the entry of drugs into the central nervous system we have found the technique of autoradiography combined with radioassay to be a valuable research tool. It has disclosed such unsuspected phenomena as the dual routes of entry into the brain of acetazolamide. Although many factors controlling drug entry remain to be studied, we propose certain general conclusions.

1) The anatomical boundaries of brain are clearly reflected by the penetration and accumulation of all compounds we have studied—a finding that confirms the original proposition that whole-brain homogenates are inadequate for the study of drug and brain relationships.

2) Circulation, expressed as regional blood flow or volume of capillary blood, was seldom decisive in influencing entry or accumulation of exogenous substances in the brain. To date, the only compounds demonstrated to be circulation-dependent are trifluoroiodomethane and thiopental. Both are extremely fat-soluble. Tissue binding appears to be an additional factor in the case of thiopental.

3) Penetration is retarded by myelin. All substances we have studied have shown a relatively slower rate of entry into this tissue. In immature brain, before myelinization has taken place, the primordial white matter is readily penetrated. We have suggested that entry into mature white matter is retarded by the lamellated membranes of the myelin sheath, which should be regarded, therefore, as a component of the blood-brain barrier. The small interstitial space indicated by the limited entry of sulfate ion is an additional hindrance to dispersal of exogenous substances into brain parenchyma. The blood-brain barrier is a complex anatomical, physiological, and biochemical phenomenon, and no unitary hy-

pothesis is adequate to embrace all the observed events.

4) Accumulation of a drug in the brain implies some form of binding or interaction between drug and tissue. Findings on injection of phenobarbital, thiopental, or diphenylhydantoin illustrate such an accumulation. These binding interactions may be nonspecific, as is probable in the case of drugs bound to plasma protein. However, a more fundamental significance is suggested when a drug is found to bind, react with, or accumulate in, a specific anatomical structure of the brain. We have made reference to this possibility in connection with the localization of isonicotinic acid hydrazide or its metabolites in the hippocampus (*46*), and we have also reported the striking accumulation of acetazolamide in hippocampus, caudate nucleus, and hypothalamus. Although the binding process is poorly understood, further investigation of these phenomena should lead to a clearer understanding of regional variations in brain chemistry. While one should not assume that the demonstration of a focal concentration of a drug implies site of action, correlation between pharmacological action, electrophysiological events, biochemical changes, and temporal and regional drug concentrations may indeed exist (*47*).

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12. Mongrel cats were injected intravenously with a single dose, in amounts adequate for autoradiography but not exceeding accepted therapeutic levels. Administration of urea in trace doses produced no measurable increase in the total amount of circulating urea, and administration of $S^{35}$-labeled sulfate in trace doses produced no observable pharmacological effect. Radioactively labeled phenobarbital, isoniazid, thiopental, diphenylhydantoin, and acetazolamide were synthesized in our laboratories by Leon Clark. Labeled urea, salicylic acid, and $S^{35}$-labeled sodium sulfate were obtained from commercial sources. The radiochemical purity of each was established by standard chemical methods, including chromatography. The animal was sacrificed at selected times after injection; the brain was removed and cut into sections, which were apposed to photographic film, an autoradiographic picture of isotope localization thus being produced. The autoradiographic findings were supported by dissection and radioassay of 16 anatomically defined areas of cat brain, some as small as the medullary pyramids (*41*); the presence or absence of metabolites was determined by standard techniques. Care must be taken in such studies to secure radiochemical purity of the injected material and to analyze for metabolites in order not to misinterpret the meaning of the assay data or the autoradiogram. It should also be pointed out that the autoradiogram is not quantitative under these circumstances. Artifacts may be introduced by differences in self-absorption of tissues [S. Ullberg, *Acta Radiol. Suppl.* **118** (1954)]. White matter, with its high lipoid content, tends to have a somewhat higher self-absorption. Autoradiograms, therefore, must be evaluated in terms of radioassay of dissected structures.
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