STUDIES ON THE INTERRELATIONSHIP BETWEEN THE BLOOD-BRAIN BARRIER AND ENTRY OF VIRUSES INTO THE CENTRAL NERVOUS SYSTEM

I. The Effect of Carbon Dioxide on Type II Poliovirus Infection in Mice*' ‡

By MARGRET I. SELLERS, Ph.D., and JOHN F. LAVENDER§

(From the Division of Virology, Department of Infectious Diseases School of Medicine, University of California, Los Angeles)

Plates 12 to 15

(Received for publication, September 5, 1961)

In 1885 Ehrlich (1) discovered that when coerulein-s, an acidic dye, was injected subcutaneously into experimental animals, various organs were stained but the brain was left entirely uncolored. In 1913 Goldman (2) performed a series of now classical experiments which established the concept of the blood-brain barrier. After injecting trypan blue intravenously, he found that vital staining occurred in all the tissues except the brain, which remained uncolored except for the choriod plexus.

In all probability, most substances penetrate the central nervous system to some degree. In addition, the blood-brain barrier is not uniform throughout the brain since well defined areas such as the hypophysis, infundibulum, area postrema, and others are readily stained by trypan blue from the circulation. However, the fact remains that many substances, *i.e.* electrolyte, colloid, vital dye, protein, etc., penetrate the central nervous system parenchyma with great difficulty, whereas the exchange of these substances between vessels and tissues in the rest of the body occurs much more freely. The blood-brain barrier is, in effect, a rate phenomenon and not a true barrier (3). Accumulative evidence indicates that it is a composite mechanism regulating the uptake of materials by the CNS; biochemical and physiological as well as anatomical factors all contribute to its function (*cf.* reference 4).

The role of the blood-brain barrier in the humoral spread of neurotropic viruses, and in particular poliovirus, to the CNS has long been a matter for speculation. In infected individuals, only rarely does poliovirus reach the CNS where it produces the paralysis that characterizes overt disease. The portal of entry is the oropharynx and lower intestinal tract; from these initial and/or

* Supported by a research grant from the National Institute for Allergy and Infectious Diseases, United States Public Health Service.

‡ A report on this work was presented at the 45th Annual Meeting of the Federation of America Societies for Experimental Biology, April 10, 1961.

§ Predoctoral Trainee, Mental Health Training Program, United States Public Health Service.

secondary sites of multiplication, virus may be shed into the blood stream and may circulate throughout the body (5). If the blood-brain barrier provides an important defense against entry of circulating virus into the CNS, events that impair its function should increase the incidence of CNS infection. To test this hypothesis, a study of the effect of blood-brain barrier impairment on the infectivity of Type II poliovirus for mice was initiated. Since virus was administered intravenously, it was postulated that it must traverse the blood-brain barrier before reaching the CNS with consequent infection and paralysis of the animals.

Exposure of animals to elevated concentrations of CO_2 results in an increase in the entry of a radioactive isotope (6), vital dye (7), and drugs (8) into the brain from the circulation. The reactivity of the cerebral vasculature to an increase in blood CO_2 tension includes dilatation and increase in rate of blood flow through the CNS (*cf.* reference 9); experiments were carried out to determine whether these changes would mediate an increase in viral infectivity. Our results reveal that inhalation of elevated concentrations of CO_2 has a profound effect on the infectivity of poliovirus inoculated intravenously into mice. However, the mechanism of action of CO_2 cannot be explained on the basis of an increased rate of blood flow alone; other factors which remain unidentified affect the net result.

Methods and Materials

A MEF-1 strain of Type II poliovirus which Krech (10) adapted to mice by the intravenous route was employed. Stock virus of relatively high infectivity was prepared in monkey kidney tissue cells and stored at −20°C. The virus inoculum consisted of tissue culture fluid in a volume of 0.25 ml for intravenous injection *via* the tail vein and 0.025 ml for intracerebral inoculation. The infectivity of stock virus was determined in mice by the intracerebral route and in HeLa cell cultures; $TCID_{50}$ titers of 10^{-6} for HeLa cells and LD_{50} titers of $10^{-4.5}$ for mice were obtained. Swiss mice from a random-bred, but closed colony originally derived from the Webster *Salmonella*-resistant strain were used. The majority were males, 5 to 7 weeks of age and weighing 20 to 25 gm. The animals were housed in stainless steel nesting boxes in groups of 7 with food and water available as desired.

The various CO_2–O_2 mixtures were supplied by the Ohio Chemical Company in standard gas cylinders.

For exposure to CO_2, or other gas mixtures, mice were placed in a large, air-tight, glass desiccator with an attachment containing a small glass inlet, and outlet; a gas mixture was allowed to flow through the jar at a constant rate for the specified time. Mice were then removed from the desiccator and returned to their cages. They were examined once each day postinfection for evidence of paralysis and the daily cumulative number of paralyzed animals as well as deaths were recorded. At the end of 14 days the number of survivors were recorded and the experiment terminated.

RESULTS

When first exposed to an atmosphere of 30 per cent CO_2, mice become hyperactive but within a few seconds are in a state of narcosis; breathing is shallow

and rapid. After exposures of 1 to 5 minutes, complete recovery occurs almost immediately after return to a normal atmosphere. Autopsy of the animals reveals a few or even many focal hemorrhages in the lungs; other gross pathology was not observed. After 15 minutes of exposure to 30 per cent CO_2 massive hemorrhages were found in the lungs. Upon removal from the CO_2 atmosphere, from 10 to 20 per cent of these mice die of an apparent hemorrhage from the lungs as evidenced by extrusion of bloody fluid from the nares. Since CO_2 raises blood pressure and increases cerebral blood flow without increasing cardiac

TABLE I

Effect of CO_2 on the Susceptibility of Mice to Intravenously Inoculated Type II Poliovirus

Virus dilution*	Procedure	No. of mice	Cumulative No. of paralyzed mice at indicated day postinfection													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Undiluted	Virus, CO_2‡	7	7													
	Virus	7	1	1	3	6	7									
1:5	Virus, CO_2	7	2	3	6	6	7									
	Virus	7	0	0	0	2	5	7								
1:15	Virus, CO_2	7	2	2	3	4	6	7								
	Virus	7	1	1	1	2	3	4	4	5	6	6	7			
1:20	Virus, CO_2	7§	1	2	2	3	6									
	Virus	7‖	0	0	0	0	0	0	1	2	2	2	2	3		

* The LD_{50} for mice when titrated by the intracerebral route was $10^{-4.5}$.

‡ Following virus inoculation, mice were placed in an atmosphere of 30 per cent CO_2–70 per cent O_2 for 2.5 minutes.

§ 1 mouse survived.

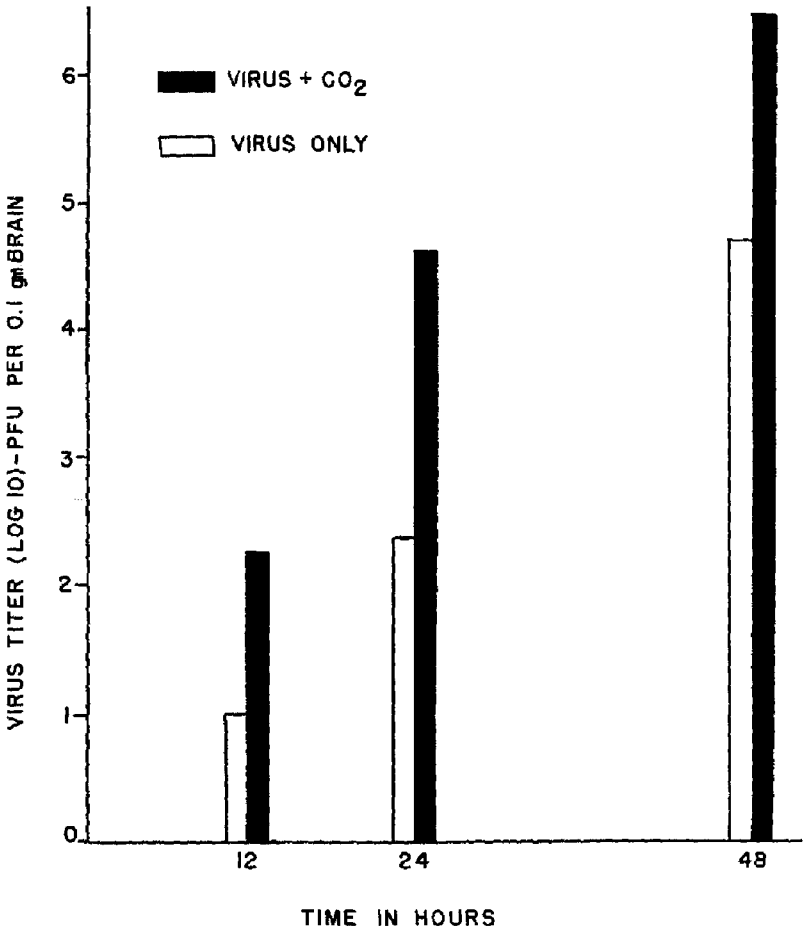
‖ 4 mice survived.

output (11), vasoconstriction must occur in the extracerebral vascular beds. The presence of hemorrhagic fluid in the respiratory passages implies that an increase in the permeability of the alveolar-capillary membrane has occurred. However, this may not necessarily be a hemodynamic effect but may be due to a direct toxic effect of the inhaled CO_2 on the alveolar epithelium.

Deaths resulting from CO_2 inhalation were acute, always occurring within a few minutes of exposure time. Damage to the lung was completely reversible in mice which survived; autopsy at 24 hours postexposure time failed to reveal gross lesions in the lungs, even in animals which had inhaled CO_2 for 15 minutes.

Effect of CO_2 Inhalation.—Data recorded in Table I show a titration of MEF-1 virus by the intravenous route and the influence of CO_2 inhalation on the results. The infectivity of a 1:20 dilution of virus was increased 2-fold by CO_2. Although

virus diluted up to 1:15 produced death in all the animals, the incubation period was considerably shortened among the groups placed in an atmosphere of 30 per cent CO_2–70 per cent O_2 immediately after virus inoculation, indicating that the amount of virus reaching susceptible cells in the CNS initially



TEXT-FIG. 1. The effect of CO_2 inhalation on the titer of Type II poliovirus in mouse brain during the first 48 hours after intravenous inoculation of virus. Titrations were carried out in HeLa cell monolayers.

was considerably increased under the influence of CO_2. Therefore, determinations of the amount of virus appearing in the brain during the earlier phases of infection were made. Virus titrations were carried out on brain from mice which had inhaled CO_2 following virus inoculation and from animals which had received the same dose of virus but were not treated with CO_2.

The mice were inoculated intravenously with 8×10^6 plaque forming units (PFU) of virus and divided into two groups. One group of animals was then placed in an atmosphere of 30 per cent CO_2–70 per cent O_2 for 5 minutes. At 12, 24, and 48 hour intervals following virus inoculation, 6 mice were chosen at random from each group; the animals were anesthetized with sodium pentobarbital and the thoracic and abdominal cavities opened. The abdominal aorta was severed and at the same time, saline was injected *via* the left ventricle of the heart until the brain was perfused free of circulating blood. Brains from each group were pooled (two pools of 3 brains per group) and a 10 per cent homogenate in normal saline prepared from each pool in a TenBroeck grinder. The homogenates were cleared by centrifugation, the supernatants removed and titrated for virus infectivity. The titrations were carried out in HeLa cells by Holland and McLaren's modification of Dulbecco's plaque technique (12); the results were expressed as PFU per 0.1 gm of brain.

TABLE II

Influence of CO_2 Concentration on Susceptibility of Mice to Intravenously Inoculated Type II Poliovirus

Experiment No.	Per cent CO_2	CO_2 exposure*	No. of mice	Survivors	
				No.	Per cent
		min.			
1	30	2.5	20	2	10
	20	2.5	10	5	50
	15	2.5	10	4	40
	10	5.0	10	4	40
	Controls	—	20	18	90
2	7	30	14	3	21
	5	300	60	29	48
	Controls	—	39	22	56

* Immediately following virus inoculation.

Data from two replicate experiments are summarized in Text-fig. 1. At the end of 12 hours, 10 times more virus was found in the brains of mice inhaling CO_2 than in brains from comparable animals which had not inhaled the gas. This differential had increased 100 times at the end of 24 and 48 hours. In these experiments, virus dosage was adjusted so that paralysis did not develop prior to 48 hours following virus inoculation; if large amounts of virus are employed then paralysis develops earlier and higher titers of virus are found in the brain. In the latter experiments, measurable amounts of virus can be found in the brains of animals inhaling CO_2 and sacrificed as early as 6 hours after infection. Titratable amounts of virus are not found in the brains of control animals at 6 hours postinoculation time. Thus, it is apparent that initially, under the influence of CO_2, large amounts of virus reach susceptible sites in the brain where

multiplication ensues. Within a relatively short time enough progeny is produced to infect an overwhelming number of available cells with early development of paralysis, followed by death, completing the cycle of events.

Threshold Concentrations of CO_2.—The effects of different concentrations of CO_2 on the susceptibility of mice to intravenously inoculated Type II poliovirus are recorded in Table II. Inhalation of 5 per cent CO_2 for as long as 5 hours failed to influence viral infectivity; these animals had essentially the same death rate as the controls. The lowest concentration of CO_2 found to significantly increase susceptibility to virus was 7 per cent with a minimum effective inhalation time of 30 minutes. The comparable inhalation time required for 10 per cent CO_2 was 5 minutes. An inhalation time of 2.5 minutes was highly effective for 15,

TABLE III

Rapidity of the CO_2 Effect on the Susceptibility of Mice to Intravenously Inoculated Type II Poliovirus

CO_2 exposure*	No. of mice	Cumulative No. of paralyzed mice at indicated day postinfection											Survivors
		1	2	3	4	5	6	7	8	9	10	14	
sec.													
150	10	1	2	6	9								1
30	10	0	0	8	10								0
15	10	0	1	4	6	6	9	9	10				0
5	10	0	0	5	6	9	9	9	10				0
Controls (virus only)	10	0	0	0	3	4	5						5

* Following virus inoculation, mice were placed in an atmosphere of 30 per cent CO_2–70 per cent O_2 for the specified number of seconds.

20, and 30 per cent CO_2; minimum times were not determined for 15 and 20 per cent concentrations but the data in Table III show that the effect of 30 per cent CO_2 is almost instantaneous. All mice receiving 1 LD_{50} of virus succumbed to the infection if placed in the CO_2 atmosphere for only 5 seconds following virus inoculation. This was the shortest interval tested since the animals could not be manipulated in and out of the CO_2 atmosphere in less time. The effects of both 5 and 15 seconds of inhalation were almost identical; increasing the time to 30 seconds shortens the incubation period while inhalation of the gas for 2.5 minutes leads to a further, though not significant, reduction in the incubation time. Subsequent experiments showed that 2.5 minutes inhalation of 30 per cent CO_2 was sufficient to produce maximally effective virus infectivity. The experiments with the lower concentrations of CO_2 clearly demonstrate that the effects are accumulative, the time required to increase susceptibility being proportional to the CO_2 concentration. Apparently, the threshold of activity lies at the 7 per

cent level. Although inhalation of 5 per cent CO_2 produces a significantly increased rate in blood flow in the brain (13) it does not increase the entry of poliovirus into this tissue.

Reversibility of the CO_2 Effect.—Carbon dioxide inhalation following virus inoculation is much more effective than is the reverse procedure; *i.e.*, gas inhalation followed by virus inoculation (Table IV). When gassing is continued for only 2.5 minutes and virus is then inoculated, infectivity is not enhanced; in fact, giving the gas for 2.5 minutes before, and again after, inoculation of virus does not increase the infectivity to any greater degree than the one gassing period after virus inoculation. When gassing is continued for 15 minutes prior

TABLE IV

Reversibility of the CO_2 Effect on the Susceptibility of Mice to Intravenously Inoculated Type II Poliovirus

Experiment No.	CO_2* inhaled:	Duration of CO_2 exposure	No. of mice	Survivors	
				No.	Per cent
		min.			
1	Before virus	2.5	25	15	60
	After virus	2.5	25	3	12
	Before and after virus	5	14	1	7
	Controls (virus only)	—	25	15	60
2	Before virus	15	12	5	42
	After virus	15	11	3	18
	Controls (virus only)	—	12	10	83

* 30 per cent CO_2–70 per cent O_2 mixture.

to virus inoculation, an enhancement of infectivity is found although not of the same order of magnitude as when gas is applied for the same length of time after virus inoculation. In these experiments the animals were inoculated immediately as they were removed from the desiccator jar through which CO_2 was passing; if a period as long as 2 minutes was allowed to elapse before virus inoculation, the enhancement effect was lost. The results indicate that virus must be present in the circulation when the CO_2 is inhaled if a significant enhancement of virus infectivity is to be demonstrated; the CO_2 effect is terminated almost immediately after removal of the animals from the CO_2 atmosphere.

A Comparison of the Effect of CO_2 on Virus Inoculated Intravenously and Intracerebrally.—Krech (10) found that detectable amounts of intravenously adapted MEF-1 virus remained in the blood stream for as long as 24 hours following intravenous injection into mice. We have found that significant titers

remain in the blood up to 8 to 12 hours following inoculation of large amounts of virus. Apparently, the virus is not rapidly cleared from the blood stream; confirmation of this is found in the experiments recorded in Table V. These results reveal that viral infectivity was significantly increased by CO_2 inhalation up to 8 hours following virus inoculation. Gassing the animals later than 8 hours postinoculation, including the late stages of the infectious process, did not influence viral infectivity. This indicates the CO_2 effect is concerned only with the initial spread of virus from the circulation to the CNS and that it does not influence the spread of virus within the CNS. This finding is substantiated by the results

TABLE V

Effect of CO_2 on the Susceptibility of Mice to Intravenously Inoculated Type II Poliovirus as Influenced by Time between Virus Inoculation and CO_2 Inhalation

CO_2* inhalation after:	No. of mice	Cumulative No. of paralyzed mice at indicated day postinfection														Survivors	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	No.	Per cent
Controls (virus only)	12	0	0	0	0	2										10	83
5 min.	12	0	0	0	2	8	12									0	0
1 hr.	12	0	0	0	2	6	10	10	12							0	0
2 hrs.	12	0	0	0	3	6	6	7	8							4	33
4 "	12	0	0	0	0	1	2	3	4	5						7	60
8 "	12	0	0	0	0	1	1	2	3	4	4	5				7	60
16 "	10	0	0	0	0	1	1	3								7	70
24 "	12	0	0	0	0	0	0	0	0	2	2	3				9	75
48 "	12	0	0	0	0	2	2	2	2	3						9	75
72 "	10	0	0	0	0	0	1									9	90

* Groups of mice were gassed with 30 per cent CO_2–70 per cent O_2 for 2.5 minutes at the specified time intervals following virus inoculation.

in Table VI; CO_2 inhalation at times ranging from 5 minutes to 6 days following intracerebral inoculation of virus, when very high titers of virus are present in the brain, produced no enhancement of viral infectivity. In fact, CO_2 inhalation at these times was an effective adjunct to specific antiserum therapy. Mice were inoculated with high titered specific Type II antiserum *via* the tail vein 48 hours postinfection and placed in an atmosphere of 15 per cent CO_2–85 per cent O_2 for 6 hours. The process was repeated at the end of 72 and 96 hours postinfection. Fifty per cent of the control animals infected with the same dose of virus and treated with antiserum but without the CO_2 treatment died whereas only half this number succumbed to the infection when the antiserum injection was followed by CO_2 inhalation. The entry of antibodies into the brain was ap-

parently facilitated by an impairment of the blood-brain barrier mediated by CO_2.

Effect of Anoxia and of an Elevated O_2 Concentration.—An experiment was performed to investigate the effect of anoxia on virus infectivity. Data recorded in Table VII, Experiment 1, show that inhalation of 10 per cent O_2–90 per cent N_2 did not influence the infectivity of poliovirus given by the intravenous route.

Further experiments to define the mechanism of action of CO_2 were carried out. Profound vasodilation of the cerebral vessels and a concomitant constriction of the peripheral vessels with increased flow of blood to the CNS are the prominent physiological features of increased CO_2 tension. The net effect of oxygen inhalation, in high concentrations, is in the opposite direction: vasoconstriction of the cerebral vessels and a reduction in cerebral blood flow of about

TABLE VI

The Effect of CO_2 on the Infectivity of Intracerebrally Inoculated Type II Poliovirus in Mice

CO_2* administered after:	No. of mice	Survivors	
		No.	Per cent
5 min.	14	7	50
72 hrs.	14	5	36
144 "	14	7	50
Controls (virus only)	14	6	43

* Groups of mice were gassed with 30 per cent CO_2–70 per cent O_2 for 2.5 minutes at the specified time intervals following virus inoculation.

10 per cent (11, *cf.* reference 4). Assuming that vasodilation of cerebral vessels together with an increased rate of blood flow to the CNS are factors contributing to an increase in the escape of virus from the circulation into the CNS, then inhalation of high concentrations of O_2 should theoretically have a sparing effect on the mice. To test this hypothesis, mice were placed in an atmosphere of 30 per cent O_2–70 per cent N_2 for 5 minutes immediately following virus inoculation; inhalation of this gas mixture conferred considerable protection against poliovirus inoculated intravenously (Table VII, Experiment 2).

Studies on Recent Type II Isolates.—In tropical areas where poliovirus is ubiquitous and the disease is endemic, predominantly as an inapparent infection, sharp outbreaks may occur. It has been postulated that these outbreaks are caused by virulent strains of virus suddenly appearing among the population (14).

We were interested to see how such strains of virus might behave with respect to intravenous inoculation and whether CO_2 inhalation might modify infectivity. Accordingly, two Type II poliovirus isolates which had been re-

cently recovered from an outbreak of poliomyelitis in Iquitos, Peru (15) were tested.

Virus was first isolated from fecal specimens and propagated in FL cell cultures. Undiluted tissue culture fluid from the third cell-culture passage having a $TCID_{50}$ titer of 10^{-4} was used; diluted virus was not lethal by either the intravenous or the intracerebral routes.

The MEF-1 strain used as routine in these experiments has an LD_{50} titer of $10^{-4.5}$ for mice when inoculated directly into the brain and a corresponding titer of $10^{-1.2}$ when given by the intravenous route; moreover, 10 times the volume of diluted virus was injected by the latter method. This tremendous

TABLE VII

The Effect of Low and High Oxygen Tension on Susceptibility of Mice to Intravenously Inoculated Type II Poliovirus

Experiment No.	Gas mixture*	No. of mice	Survivors	
			No.	Per cent
1	10 per cent O_2–90 per cent N_2	10	7	70
	Controls (virus only)	10	7	70
2	30 per cent O_2–70 per cent N_2	16	8	50
	Controls (virus only)	16	3	19

* Immediately after virus inoculation animals were gassed with the specified mixtures for 5 minutes.

differential between infectivity by the intracerebral and by the intravenous routes was not found for the Peruvian strains (Table VIII). Strain S-17, inoculated directly into the brain, produced death in 5 of 17 mice; following intravenous injection, 2 of 16 mice were infected and CO_2 inhalation substantially increased this infectivity. Intracerebral inoculation of strain S-8 resulted in the death of 1 of 16 mice; intravenously injected virus also produced death in 1 of 16 animals and here again, CO_2 inhalation increased the infectivity. In terms of reference to their infectivity by the intracerebral route, it appears as if the two, Type II strains recently recovered from paralyzed individuals have an increased capacity to invade the CNS from the circulation in the mouse.

Admittedly, observations on only two strains of virus are not sufficient to define viral characteristics or markers which may be correlated with virulence. These observations are reported in detail because information concerning

characteristics related to virulence of poliovirus is limited (16) and because opportunities to study Type II virus from an outbreak in a tropical area, have been rare. It may be of interest to add that with repeated passage in cell culture, the S-8 strain has almost completely lost its virulence for mice, while the S-17 strain has remained essentially unchanged. The S-17 strain proved to be virulent for monkeys by the intravenous route and CO_2 inhalation shortened the incubation period and increased the severity and extent of paralysis in these animals (17).

Effect of Environmental Temperatures on Viral Infectivity.—The epidemiology of poliomyelitis as influenced by climate has long been of interest to virologists. Although severe outbreaks have occurred in regions with temperatures below

TABLE VIII

Susceptibility of Mice to Recent Isolates of Type II Poliovirus

Virus strain	Route of virus inoculation	No. of mice	Deaths	
			No.	Per cent
Peru S-8	Intravenous	16	1	6
	Intravenous (plus CO_2*)	16	5	31
	Intracerebral	16	1	6
Peru S-17	Intravenous	16	2	13
	Intravenous (plus CO_2*)	15	9	53
	Intracerebral	17	5	29

* Mice were placed in an atmosphere of 30 per cent CO_2–70 per cent O_2 for 3 minutes following virus inoculation.

zero, paralytic disease is more prevalent in late summer and early fall in the temperate zones and occurs throughout the year in tropical regions. Therefore, experiments were carried out to investigate the influence of increased, as well as decreased, environmental temperatures on the infectivity of intravenously administered poliovirus. Concurrently the effect of CO_2 inhalation was also investigated.

Mice were separated into four groups and placed (*a*) in a cold room maintained at approximately 4°C with 10 per cent relative humidity; (*b*) in a room that was approximately 25°C and in which the humidity varied with atmospheric conditions; (*c*) in a warm room maintained at 30°C with a controlled relative humidity of 30 per cent; and (*d*) in a 37°C walk-in incubator with a controlled relative humidity of 30 per cent. The animals were placed in the indicated areas 2 days prior to virus inoculation to allow for a period of adaptation. Mice housed at 4°C responded by huddling and by increasing their intake of food; weight gain approximated that of the controls. A slight lowering of body temperature, 0.5 to 1 degree, was observed in these animals. Mice maintained at 37°C had a rise in body temperature of from 1 to 2 degrees and displayed rapid shallow breathing. Some weight loss occurred among these animals. The

same dose of virus was given to each mouse *via* the tail vein. In addition, half the mice in each group were placed in an atmosphere of 20 per cent CO_2–80 per cent O_2 for 5 minutes immediately after virus inoculation. The animals were kept in the regular nesting boxes in groups of 5 to 7 with food and water as desired.

Data from two replicate experiments are summarized in Table IX. Certainly there is no evidence of a potentiating effect of higher environmental temperature on the infectivity of poliovirus administered intravenously. In fact, the opposite effect was noted. The mice housed at higher temperatures were protected, whereas mice housed in the 4°C room were more susceptible.

A part of the physiological response of an animal to the stress of a cold environmental temperature, is an increase in adrenocortical secretion (18). In

TABLE IX

Effect of Temperature On the Susceptibility of Mice to Intravenously Inoculated Type II Poliovirus and the Influence of CO_2 on this Effect

Test temperature	Virus only			Virus followed by 5 minutes in 20 per cent CO_2		
	No. of mice	Survivors		No. of mice	Survivors	
		No.	Per cent		No.	Per cent
°C.						
4	27	14	52	29	4	14
25	37	26	70	37	0	0
31	15	12	80	15	3	20
37	35	31	89	35	9	26

an attempt to test directly the role that adrenocortical secretion might play in the increased viral susceptibility of mice maintained at 4°C, the effect of bilateral adrenalectomy on these animals was investigated. Although non-infected, adrenalectomized mice could be maintained at this temperature without loss for the duration of the experiment, poliovirus infection caused death in 70 per cent of these animals, whereas only 43 per cent of the intact, infected mice died. Approximately as many infected adrenalectomized mice maintained at room temperature survived as did intact, infected animals (33 and 31 per cent died, respectively). It appears that adrenocortical hypersecretions are not mediating the increase in susceptibility to intravenously injected poliovirus which is observed in mice maintained in an environmental temperature of 4°C. Walker and Boring (19) found that adult mice housed at 4°C succumbed to infection with the Conn-5 strain of Coxsackie B virus. On the other hand, when the animals were maintained at 36°C, a marked inhibition of viral invasion and multiplication was noted. These authors presented indirect evidence suggesting that the loss of resistance to Coxsackie

virus in mice housed at 4°C was not a consequence of adrenocortical hypersecretion.

Hyperventilation is among the responses noted in animals placed in environmental temperatures considerably higher than their normal habitat. By this process, large amounts of CO_2, free or fixed, stored in the blood and tissues, are rapidly washed out. The blood CO_2 tension may be substantially reduced with a consequent rise in the blood pH and resultant alkalosis; constriction of the cerebral vessels and reduced blood flow through the brain occurs (20, 21). It is postulated that the net effect of this physiological state on the animal's susceptibility to circulating poliovirus would be protective because of a decrease in the amounts of virus circulating in the CNS and a decrease in the entry of virus into the brain parenchyma resulting from an increase in the resistance of the blood-brain barrier. Recent experiments carried out by Goldberg *et al.* (8) show that the entry of urea and other compounds into the CNS is significantly reduced as a result of hypocapnea produced by passive hyperventilation.

If a reduced pCO_2 and alkalosis obtaining at high environmental temperatures results in the protection of mice, then conversely, an increase in the level of pCO_2 and resultant acidosis could be mediating the increase in virus susceptibility of mice maintained at 4°C. Animals in a state of hypothermia do indeed exhibit increased levels of pCO_2. Under this condition, minute volume decreases and pCO_2 increases as does the solubility of CO_2 in the blood. Despite decreased CO_2 production, a respiratory acidosis results (*cf.* reference 22). However, these data are not pertinent to our experiments since mice maintained at 4°C were not hypothermic; they maintained a body temperature not more than 1 degree lower than normal. We therefore attempted to determine whether pCO_2 levels of mice maintained for 2 or 3 days at 4°C were higher than in mice housed at 25°C.

The direct determination of blood gas tensions were made using the Astrup microchemical apparatus. For a satisfactory analysis, a minimum of 0.5 ml blood obtained anaerobically is required. Efforts were made to obtain a single specimen by heart puncture from a single animal. It proved to be very difficult to obtain an adequate amount of blood completely free of contaminating air bubbles; also, it was impossible to obtain arterial blood exclusively. Nevertheless, four different samples were considered to be more or less satisfactory for analysis from the group of mice at 4°C. The same number of samples from mice housed at 25°C were also analyzed.

The average pCO_2 value thus obtained was 31.9 mm Hg with a blood pH of 7.28 for animals housed at 4°C and the corresponding values for mice held at 25°C were a pH of 7.38 and pCO_2 of 27.0. The limitation of obtaining completely satisfactory blood samples precludes any conclusion concerning absolute values. However, it is interesting to find the average pCO_2 level of mice housed at 4°C to be significantly increased over that of animals housed at room temperature. It is possible that the increase in susceptibility to circulating polio-

virus in mice maintained at 4°C results from increased amounts of virus reaching the CNS from the circulation. This may be brought about by an increase in the permeability of the blood-brain barrier mediated by CO_2 which accumulates consequent to the lowered environmental temperature.

Data in Table IX also show the effects of CO_2 inhalation on the influence of environmental temperature on the susceptibility of mice to circulating poliovirus. When an increase in pCO_2 already exists, the response of the blood-brain barrier to CO_2 inhalation is somewhat diminished; 14 per cent of the mice housed at 4°C survived, whereas none of the animals maintained at 25°C survived. Likewise, a given reduction in pCO_2 produced by hyperventilation alters the net response to CO_2 inhalation in the same direction and to a greater degree; 20 and 26 per cent of the animals survived at 30° and 37°C respectively. In studies of the effect of CO_2 on cerebral vascular permeability, Noell and Schneider (23) found that CO_2 inhalation became less effective as the pCO_2 of the blood deviated from the normal physiological range.

Anatomical Studies of the Blood-Brain Barrier.—An anatomical location for the blood-brain barrier has not been definitely established. A concept held by many investigators places it in the capillary endothelium; others place it at the inner or basement membrane of the intracerebral vessels while other evidence indicates its location to be in the plasma membrane of the neural cells, particularly of the glial elements (*cf.* reference 24). Dempsey and Wislocki (25), studying the blood-brain barrier in the rat by electron microscopy, found that the neuroglial cells were separated from one another by a very narrow space filled with amorphous substance of moderate electron density. They concluded that either the glial plasma membrane or the amorphous ground substance acted as the hematoencephalic barrier which barred silver from entering the parenchymal cells of the brain; they were unable to determine which of the two structures was actually responsible for their results.

We attempted to locate a possible barrier site(s) in mice which might be altered by CO_2 inhalation. The animals were placed in an atmosphere of 30 per cent CO_2–70 per cent O_2 for various periods of time following intravenous injection of trypan blue. Examination of numerous preparations in the light microscope did not reveal staining of brain tissue in mice exposed to CO_2 beyond that found in the control animals. Although Clemedson *et al.* (7) employed trypan blue successfully in a study of the blood-brain barrier and the influence of CO_2 inhalation on its impairment in rabbits, guinea pigs, and cats, the dye does not appear to become concentrated to any substantial degree in the brains of mice. We therefore turned to India ink which has been widely used in studies of the RES system (26) as an indicator of increased permeability of the blood-brain barrier in mice. Relatively large amounts of this material are tolerated, and it had the additional advantage for our study of being electron-dense and similar in size to poliovirus which is 27 mμ in diameter (27).

Carbon suspensions were prepared from waterproof drawing ink supplied by Gunther Wagner, Hanover, Germany. The final preparation consisted of carbon particles suspended in physiological saline containing 0.5 per cent gelatin, 60 mg carbon/ml. The mean carbon particle size, as determined by electron microscopy, was 34 mμ. A total volume of 0.25 ml was injected into the tail vein; half of the mice were then exposed to an atmosphere of 30 per cent CO_2-70 per cent O_2 for 5 to 10 minutes. The animals were anesthetized with sodium pentobarbital and the brains perfused with saline followed by formalin. The brains were removed, and either examined directly or fixed in formalin before being examined for the presence of carbon.

All the animals showed some tremor following the injection of carbon, which, in the control mice, was of short duration. Following CO_2 exposure, immediate and profound CNS symptoms developed including circling, rigidity, and convulsions, with the animals finally becoming comatose or paralyzed. About half of these mice died within 30 minutes; some improved and at the end of an hour appeared normal but on twirling, again exhibited tremor and rigidity or convulsions.

Although there was considerable variation among both groups, there was no difficulty in distinguishing between brains from control mice and from those which had inhaled CO_2. In the gross, the former were not darkened to any significant extent while the latter were dark gray in appearance. Slicing the fixed brains revealed a darkly colored parenchyma in mice treated with CO_2 and a parenchyma of a relatively normal color in control animals. These preparations were made immediately after the mice were removed from the CO_2 atmosphere. When brains were examined from this group of mice which had been set aside for observation, the degree of discoloration was found to diminish with time. Even within 1 hour following CO_2 treatment, the brain appeared to have eliminated a significant amount of carbon. These results were confirmed by experiments in which the carbon suspensions was replaced with fluorescein. Scanning intact and sliced brains under an ultraviolet black light revealed that greatly increased amounts of fluorescein had traversed the blood-brain barrier under the influence of CO_2.

Apparently, significant numbers of carbon particles escaped from the circulation into the brain under the influence of CO_2. It was thought that a more definitive study might give some indication as to how and at what anatomical level the escape occurred. Therefore, examinations were made by electron microscopy to study possible morphological changes in brain architecture which could be related to the CO_2 effect and consequent leakage of carbon from the blood vessels.

Mice were prepared as indicated for the carbon experiments described above. After the animals were anesthetized, living biopsy specimens were removed at random from the cerebral cortex and fixed in 1 per cent buffered osmic acid containing sucrose. The tissue was dehydrated through graded alcohols and embedded in epon resin according to the method of Luft (28).

Ultrathin sections of tissue were stained with either lead hydroxide (29) or uranyl acetate (30) and examined in a Hitachi HU-10 electron microscope.

Features of normal brain, pertinent to this study, are the distinct, well defined, intact capillary endothelial cells and basement membrane and the sharply defined plasma membranes of the neural cells. In addition, the intracellular and amorphous ground substances are homogeneous (Figs. 1 to 3). Injection of carbon into the circulation had no discernible effect on these structures; only rarely did it escape from the cerebral vessels. When leakage did occur it was possible for the carbon to pass through the capillary endothelium and even beyond the basement membrane causing little, if any, alteration in the existing cellular components or boundaries (Figs. 4, 5). Also, no definitive anatomical changes in the brain could be related to the CO_2 effect alone but under the influence of CO_2 inhalation, there was a marked increase in the entry of carbon into some areas of the brain. The particles were not held in abeyance by the endothelial cells or by the capillary basement membrane; the glial elements did not contain a greater concentration than did other parenchymal cells (Fig. 7). The only loci in which carbon was not found were the nuclei of the neurons. However, carbon particles were found in the perikarya of the neurons. Concomitant with the migration of carbon into the brain parenchyma, boundaries of many cells became indistinct and the intracellular and amorphous ground substances were less dense than normal. In fact, in some areas of cortex where carbon was found after CO_2 inhalation, the architecture of the tissue was hardly recognizable; a kind of liquefication appeared to have taken place (Fig. 6). It is not difficult to see how these drastic alterations might follow the appearance of something so foreign as a carbon particle in such vital tissue as brain. Certainly it is not proposed that the appearance of poliovirus in the brain parenchyma brings about such drastic changes. However, it is conceivable that what has been so dramatically demonstrated with carbon under the influence of CO_2 obtains with poliovirus, but much less traumatically under the same conditions.

The administration of CO_2 did not result in a uniform distribution of carbon throughout the cortex; it is emphasized that a wide variation was found, even in sections from the same block of tissue. No attempt was made to determine which areas might be more vulnerable to CO_2; the biopsies were taken at random without reference to any specialized area.

No evidence bearing on the anatomical location of the blood-brain barrier was obtained. Perhaps the finding that carbon escaping from the circulation in brain from control mice occurred only in the extracellular spaces is of some significance. The extracellular amorphous ground-substance appears to form a continuous network throughout the brain and is in intimate contact with the vessels; it could provide a route by which carbon spreads throughout the

area (*cf.* Figs. 1 to 5). Thus, the transfer of carbon would take place from blood to an extracellular, extravascular space and from there gain entry into the CNS parenchyma as is the case in other organs. Cellular boundaries are too indistinct in brains from mice treated with CO_2 to permit confirmation or denial of this proposal.

Inhalation of CO_2 probably affects the net increase in the entry of carbon into the brain from the circulation at more than one level. One mechanism of action would be the increase in rate of blood flow resulting from an increase in pCO_2; this would bring more carbon into the CNS area, thus increasing the dosage. An additional and probably more fundamental effect of CO_2 is to facilitate the direct entry of carbon into the parenchymal cells. The mechanism of action for the latter effect is unknown.

DISCUSSION

Schmidt and his collaborators (20, 31, 32) found that CO_2 had a unique capacity to regulate cerebral blood flow; inhalation of increasing concentrations of CO_2 in O_2 for 1 minute by cats and rabbits resulted in increased vasodilatation and increased blood flow through the cerebral vessels. Similar results were obtained in limited experiments carried out on man. That increased functional activity produced increased blood flow in the nerve cells concerned, was indicated by the resultant increased blood flow in the visual cortex of the cat upon illumination of the eye. Because of the absence of demonstrable vasomotor innervation, it was suggested that these circulatory readjustments were probably accomplished through changes in the CO_2 tension in the tissue relative to changes in functional activity. The intrinsic regulation of cerebral blood flow by CO_2 was postulated "to subserve the needs of the nerve cells for changes in their blood supply in accordance with changes in their functional activity."

Recent experiments (8) have shown that CO_2 tension exerts a profound effect on the uptake by brain of various intravenously administered compounds such as phenobarbital, salicylic acid, acetazolamide, or urea. Exposure to CO_2 with a concomitant acidosis increased accumulation of the above compounds while hyperventilation with alkalosis reduced the amounts reaching the CNS. The increase in blood flow produced by CO_2 inhalation might be expected to significantly increase the entry of drugs into the brain. However, analysis of the results indicated that changes in cerebral blood flow did not exert a prominent effect on penetration of these drugs into the brain; the areas of greatest vascularity, *i.e.* the colliculi and geniculates (33), at no time showed the greatest concentration or the greatest relative change in drug concentration. While the degree of dissociation of the molecules as influenced by pH changes determined the rate of penetration to some degree, the exact mechanism of the CO_2 effect was not identified.

Undoubtedly, the mechanism(s) whereby CO_2 inhalation increases the

infectivity of intravenously administered poliovirus is primarily one of increasing the initial dose of virus traversing the blood-brain barrier. On a purely statistical basis, a significant increase in the rate of blood flow through the CNS would serve to increase the amount of virus reaching the brain. In other words, the more virus traveling through the cerebral vessels, the greater would be the probability of access to susceptible CNS centers. This is reflected in ordinary virus titrations; greater amounts of injected virus not only infect more mice but the incubation period is shortened as well. Perhaps of some significance is the fact that gray matter, which is more highly vascularized than white matter, and has a higher rate of perfusion normally (33–35), shows the greatest percentage increase in blood flow under the influence of CO_2 (36). The capacity of poliovirus to proliferate in gray matter is well documented (37, 38). However, of all the structures in the gray matter, blood flow is the lowest in the reticular substance (0.59 ml/gm minutes) (34) and this is one of the brain-stem centers which is involved initially and most severely in poliomyelitis (39). This probably reflects a high proportion of competent sites in the reticular substance which adsorb and support proliferation of virus.

Certain centers in the brain are not susceptible to poliovirus. Other centers are rarely infected, apparently because they are seldom reached by infective amounts of virus. Structures in the former category are the lateral geniculates and the visual cortex which fail to support virus growth even when inoculated directly. The caudate nucleus and putamen fall into the latter category and are rarely infected except by inoculation of virus into the frontal lobe of the cortex (37, 38). Whether CO_2, in addition to causing a greater amount of virus to reach the CNS initially, also produces a wider dissemination of the primary dose of virus, particularly into such loci as the caudate nucleus and putamen, remains to be determined. This problem must await analysis of histopathological changes in CNS tissues from both mice and monkeys treated with CO_2 after intravenous virus inoculation (17). The question of the distribution of poliovirus lesions in the brain and the factors controlling distribution and proliferation of virus continues to be one of the most interesting facets of the study of the pathogenesis of poliomyelitis.

Additional evidence that dilatation of the vessels as well as an increase in the rate of blood flow in the CNS are factors influencing virus dosage is found in the effect of inhalation of 30 per cent O_2–70 per cent N_2 which produces a constriction of cerebral vessels and a reduction in rate of blood flow through the CNS. This effect, although not of equal magnitude, is in the opposite direction to that produced by high concentrations of CO_2 and the results were also in the opposite direction; namely a protective effect was elicited.

Nevertheless, the extreme rapidity with which the CO_2 effect is mediated indicates that something more than dilatation of the vessels and increase in rate of blood flow through the CNS is responsible for the total increase in virus infectivity. The fact that a significant result of CO_2 inhalation can be

demonstrated after only 5 seconds' inhalation time indicates that some intrinsic mechanism for the transport of materials into the CNS is operating. The effects of CO_2 were so consistent and the reversibility of the reaction so complete that one is led to suspect that the mechanisms involved are of outstanding physiological significance. We are unable to offer a satisfactory theory for this mechanism of action of CO_2. None of the theories which have been advanced, including a change in bioelectric potentials, changes in hydrogen ion concentration, lipid solubility changes, enzyme activation or inhibition, ionization effects or selective binding, offers a satisfactory explanation of the data (*cf.* references 4, 8, 9).

Bodian has shown that one of the important characteristics of paralytogenic strains of poliovirus is the capacity to gain a foothold systemically and to produce a high viremia titer (40). Apparently, massive numbers of circulating viral particles comprise a threshold dose and only when this occurs does a break in the blood-brain barrier serve to admit infective amounts of virus from the circulation into the CNS (5, 40). Such events as irritating injections, peripheral trauma, and violent exercise significantly increase the likelihood of CNS involvement in poliomyelitis (*cf.* references 5, 40), in all probability by genesis of localized breaks in the blood-brain barrier. There is a correlation between the sites of trauma or muscle groups undergoing strenuous exercise and initial paralysis indicating that primary virus proliferation in the CNS occurs in the motor areas segmental to paralyzed extremities. A possible relationship between the mechanism of action of CO_2 and the effect of these provoking events in facilitating the entry of poliovirus into the CNS is postulated. It may well be that Bodian's concept of "a reflex increase of penetrability to circulating virus of the blood vessels in the motor centers corresponding to the injured peripheral muscle" (41) is related to an increased CO_2 production in motor areas segmental to the muscle groups involved. In fact there is considerable evidence for the occurrence of an altered permeability of the blood-brain barrier in localized areas in the CNS in response to injury or exercise. Intramuscular injections of either croton oil or 10 per cent formalin causes increased permeability of vessels to intravenously injected dyes in the cord segmental to the muscle group injected (42, 43). Animals which have been exercised by swimming show an increased number of patent blood vessels in the spinal cord (43). Additional evidence indicates that vasodilatation accompanying muscular activity is localized to the active neural segments. Peripheral nerve stimulation usually causes vasodilation localized to the activated cord segment; response to peripheral leg stimulation was obtained only in the lumbar cord (44). Because inhalation of CO_2 causes vasodilation and increased blood blood flow and since regional increases in both vasodilation and blood flow can be elicited by neuronal activity (32, 44), it is tempting to postulate that activities of given neurons result in an increased regional production of CO_2. The latter, in turn, causes a localized increase in the permeability of the blood-brain

barrier which, according to Schmidt's original concept, facilitates passage of needed nutriments from the blood to the activated neurons. The mechanism(s) which would permit increased passage of nutrients across the blood-brain barrier into localized areas undergoing increased activity might also facilitate passage of blood-bourne virus into the same area secondarily. Thus, the primary localization of poliovirus in neurons segmental to muscle groups involved in trauma or violent exercise can be explained. A predominating factor in this set of events would, of course, be the simultaneous occurrence of a viremia.

The question of the role of vascularity in influencing sites of poliovirus infection in the spinal cord in naturally acquired infections is of interest. In paralysis of the extremities, the proximal muscles are more commonly affected than the distal muscles (45). Most of the blood supply in the cord is supplied through the central arteries, while most of the blood is drained by the peripheral veins. Thus, a gradient of flow is established from the ventral median fissure in a lateral direction. The importance of this gradient can be seen by examining the somatotropic organization of the pyramidal cells. Those cells innervating the trunk region of the body lie most medially. The cells innervating the arms or legs, forearms or calves, hands or feet, and digits, lie respectively, more laterally. Therefore, the afferent blood supply will, to a large extent reach the cells associated with the trunk first, flowing on through the cells innervating the muscle groups of the limbs in the order named, finally reaching the veins peripherally (46, 47). In the face of a pronounced viremia, this arrangement could act as a filtering mechanism; the more lateral a neuron was situated the less might be the likelihood of its picking up infective amounts of virus. Another factor to be considered here is the occurrence of initial leg paralysis two times more frequently than arm paralysis. This result may be associated with a provoking effect produced by the relatively greater amount of activity that leg muscles undergo.

The increase in poliovirus infectivity produced by CO_2 inhalation is not without precedent. An influence of increased CO_2 tension on the infectivity of *sigma* virus in *Drosophilia* has been reported (48). Infected flies are found in abundance in nature but remain completely asymptomatic until placed in an increased atmosphere of CO_2; in high concentrations, an exposure time of 15 seconds is sufficient to produce disease. The virus localizes in the motor neurons causing paralysis and death. Also pertinent was the finding that high environmental temperatures could, under certain conditions, rid the flies of virus.

SUMMARY

Inhalation of elevated concentrations of CO_2 produces a significant increase in the susceptibility of mice to intravenously inoculated Type II poliovirus. The CO_2 effect is directly proportional to the concentration; 2.5 minutes inhalation of a mixture of 30 per cent CO_2–70 per cent O_2 produces maximal effects, while lower concentrations of CO_2 require correspondingly longer

periods. The threshold level is 7 per cent; inhalation of lower concentrations, even for long periods of time, fails to enhance virus infectivity. Placing the animals in the CO_2 atmosphere before injection of virus does not influence susceptibility; virus must be in the circulation at the time CO_2 is inhaled if enhancement of infectivity is to be elicited. The effect is completely reversible, disappearing almost immediately upon withdrawal of the animals from the CO_2 atmosphere. CO_2 mediates an increase in the entry of virus into the CNS from the circulation but does not affect the spread of virus within the CNS; susceptibility of mice to intracerebrally inoculated poliovirus is not influenced by CO_2 inhalation. The mechanism(s) of action of CO_2 can be explained, in part, by the dilatation of cerebral blood vessels and increased rate of blood flow through the CNS produced by the CO_2. However, other factors, which remain unidentified, contribute to the net effect of CO_2.

The relationship between the mechanism of action of CO_2 and the provoking effects of trauma and violent exercise in poliomyelitis is discussed. Also, a relationship between the CO_2 tension of the blood and environmental temperatures on poliovirus susceptibility is proposed.

We wish to thank Mr. Yoshimi Nishi and Mr. Zane Price for making the electronmicrographs and Dr. Daniel H. Simmons of the Department of Medicine for help with the pCO_2 determinations.

BIBLIOGRAPHY

1. Ehrlich, P., Das Sauerstüfbedurfnis des Organismus, Eine Farbenanalytische Studie, Berlin, A. Hirschwald Verlag, 1885.
2. Goldman, E. E., Vitalfarbung am Zentralnervensystem, *Abhandl. Königl. preuss. Akad. Wiss.*, 1913, **1**, 1.
3. Tschirgi, R. D., Blood-brain barrier, *in* Biology of Mental Health and Diseases, New York, Paul B. Hoeber, Inc. 1952.
4. Roth, L. J., and Barlow, C. F., Drugs in the brain, *Science*, 1961, **134**, 22.
5. Bodian, D., Mechanisms of infection with polioviruses, *in* Cellular Biology Nucleic Acids and Viruses, (O. V. St. Whitelock, editor), New York, New York Academy of Sciences, 1957.
6. Brierly, J. B., The penetration of ^{32}P into the nervous tissue of the rabbit, *J. Physiol.*, 1951, **116**, 24P.
7. Clemedson, C. J., Hartelius, H., and Holmberg, G., The influence of carbon dioxide inhalation on the cerebral vascular permeability to trypan blue, *Acta Path. et. Microbiol. Scand.*, 1958, **42**, 137.
8. Goldberg, M. A., Barlow, C. F., and Roth, R. J., The effects of carbon dioxide on the entry and accumulation of drugs in the central nervous system, *J. Pharmacol. and Exp. Therap.*, 1961, **131**, 308.
9. Sokoloff, L., The action of drugs on the cerebral circulation, *Pharmacol. Rev.*, 1959, **11**, 1.
10. Krech, U., Intravenous infectivity of Type 2 poliomyelitis virus in mice, *Proc. Soc. Exp. Biol. and Med.*, 1954, **86**, 488.

11. Kety, S. S., and Schmidt, C. F., The effects of altered arterial tensions of carbon dioxide and oxygen on cerebral blood flow and cerebral oxygen consumption of normal young men, *J. Clin. Inv.*, 1948, **27,** 484.
12. Holland, J. J., and McLaren, L. C., Improved method for staining cell monolayers for virus plaque counts, *J. Bact.*, 1959, **78,** 596.
13. Kety, S. S., Blood flow and metabolism of the human brain in health and disease, *in* Neurochemistry, (K. A. C. Elliott, I. H. Page, and J. Quastel, editors) Springfield, Illinois, Charles C. Thomas, 1955.
14. Sabin, A. B., Paralytic consequences of poliomyelitis infection in different parts of the world and in different population groups, *Am J. Publ. Health*, 1951, **41,** 1215.
15. Sellers, M. I., Recuperacion de virus de poliomielitis de una epidemia en Iquitos y de casos en el Hospital del Nino de Lima, *Anal. Fac. Med.*, 1958, **41,** 703.
16. Evans, C. A., Factors influencing the occurrence of illness during naturally acquired poliomyelitis virus infections, *Bact. Rev.*, 1960, **24,** 341.
17. Stone, R. S., Lavender, J. F., and Sellers, M. I., unpublished experiments.
18. Sayers, G., and Sayers, M. A., Regulation of pituitary adrenocorticotrophic activity during the response of the rat to acute stress, *Endocrinology*, 1947, **40,** 265.
19. Walker, D. L., and Boring, W. D., Factors influencing host-virus interactions. III. Further studies on the alteration of Coxsackie virus infection in adult mice by environmental temperature, *J. Immunol.*, 1957, **80,** 39.
20. Schmidt, C. F., The intrinsic regulation of the circulation in the hypothalamus of the cat, *Am. J. Physiol.*, 1934, **110,** 137.
21. Irving, L., Welch, M. S., The effect of the composition of the inspired air on the circulation through brain, *Quart. J. Exp. Physiol.*, 1935, **25,** 121.
22. Segar, W. E., Ripley, P. A., Jr., and Barilla, T. G., Urinary composition during hypothermia, *Am. J. Physiol.*, 1956, **185,** 528.
23. Noell, W., and Schneider, M., Über die Durchblutung und die Sauerstoffversorgung des Gehirns, IV. Die Rolle der Kohlensaure, *Arch. ges Physiol.* (Pflüger's), 1944, **247,** 514.
24. Bakay, L., Blood Brain Barrier, Springfield, Illinois, Charles C. Thomas, 1956.
25. Dempsey, E. W., and Wislocki, G. B., An electron microscopic study of the blood-brain barrier in the rat, employing silver nitrate as a vital stain, *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 245.
26. Wistar, R., Jr., Treadwell, P. E., and Rasmussen, A. F., Jr., The role of the reticuloendothelial system in mouse anaphylaxis as tested with homologous antibody-antigen complexes, *J. Exp. Med.*, 1960, **111,** 631.
27. Schwerdt, C. E., and Schaffer, F. L., Some physical and chemical properties of purified poliomyelitis virus preparations, *Ann. New York Acad. Sc.*, 1955, **61,** 740.
28. Luft, J., personal communication, 1960.
29. Watson, M. L., Staining of tissue sections on electron microscopy with heavy metals. II. Application of solutions containing lead and barium, *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 727.
30. Watson, M. L., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 475.

31. Schmidt, C. F., and Pearson, J. C., The intrinsic regulation of the blood vessels of the medulla oblongata, *Am. J. Physiol.*, 1934, **108,** 241.
32. Schmidt, C. F., and Hendrix, J. P., The action of chemical substances on cerebral vessels, *Res. Publ. Assn. Nev. and Ment. Dis.*, 1937, **18,** 229.
33. Barlow, C. F., Schoolar, J. C., Domels, N. S., Benson, D. W., and Roth, L. J., Observations on isotope labeled drugs in the central nervous system, *in* United Nations Peaceful Uses of Atomic Energy, Proceedings of the 2nd International Congress, Geneva, United Nations Publication, **24,** 1958.
34. Sokaloff, L., Local blood flow in neural tissues, *in* New Research Techniques of Neuroanatomy, (W. W. Windel, editor) Springfield, Illinois, Charles C. Thomas, 1957.
35. Kety, S. S., Measurement of local blood flow by the exchange of an inert, diffusible substance, *Methods Med. Research*, 1960, **8,** 228.
36. Hanson, D. B., Sultzer, M. R., Freygang, W. H., and Sokaloff, L., Effect of low O_2 and high CO_2 concentrations in inspired air on local cerebral circulation, *Fed. Proc.*, 1957, **16,** 54.
37. Bodian, D., and Howe, H. A., An experimental study of the role of neurones in the dessemination of poliomyelitis virus in the nervous system, *Brain*, 1940, **63,** 135.
38. Bodian, D., and Howe, H. A., Neurotropism and the genesis of cerebral lesions in poliomyelitis: An experimental study, *Johns Hopkins Hosp. Bull.*, 1941, **68,** 58.
39. Bodian, D., Poliomyelitis: Pathologic anatomy, *in* Poliomyelitis, Papers and Discussions Presented at the 1st International Conference, Philadelphia, J. B. Lippincott, 1949.
40. Bodian, D., Viremia in experimental Poliomyelitis. I. General aspects of infection after intravascular inoculation with strains of high and of low invasiveness. *Am. J. Hyg.*, 1954, **60,** 339.
41. Bodian, D., A reconsideration of the pathogenesis of poliomyelitis, *Amer. J. Hyg.*, 1952, **55,** 414.
42. Zakaraja, E. P., Zur Frage des Mechanismus de segmentären Affektion des Newensystems und deren Falgen, *Z. ges. Exp. Med.*, 1932, **80,** 683.
43. Trueta, J., Physiological mechanisms involved in the localization of paralysis, *Ann. New York Acad. Sc.*, 1955, **61,** 883.
44. Field, E. J., Grayson, J., and Rogers, A. F., Observations on the blood flow in the spinal cord of the rabbit, *J. Physiol.*, 1951, **114,** 56.
45. Buchthal, F., Problems of the pathologic physiology of poliomyelitis, *Am. J. Med.*, 1949, **6,** 579.
46. Craigie, H. E., Vascular supply of the spinal cord, *in* The Spinal Cord, Basic Aspects and Surgical Considerations, (G. Austin, editor), Springfield, Illinois, Charles C. Thomas, 1961.
47. Chambers, W., Anatomy of the spinal cord, *in* The Spinal Cord, Basic Aspects and Surgical Considerations, (G. Austin, editor), Springfield, Illinois, Charles C. Thomas, 1961.
48. L'Heritier, P., Sensitivity to CO_2 in *Drosophilia*. A review, *Heredity*, 1948, **2,** 325.

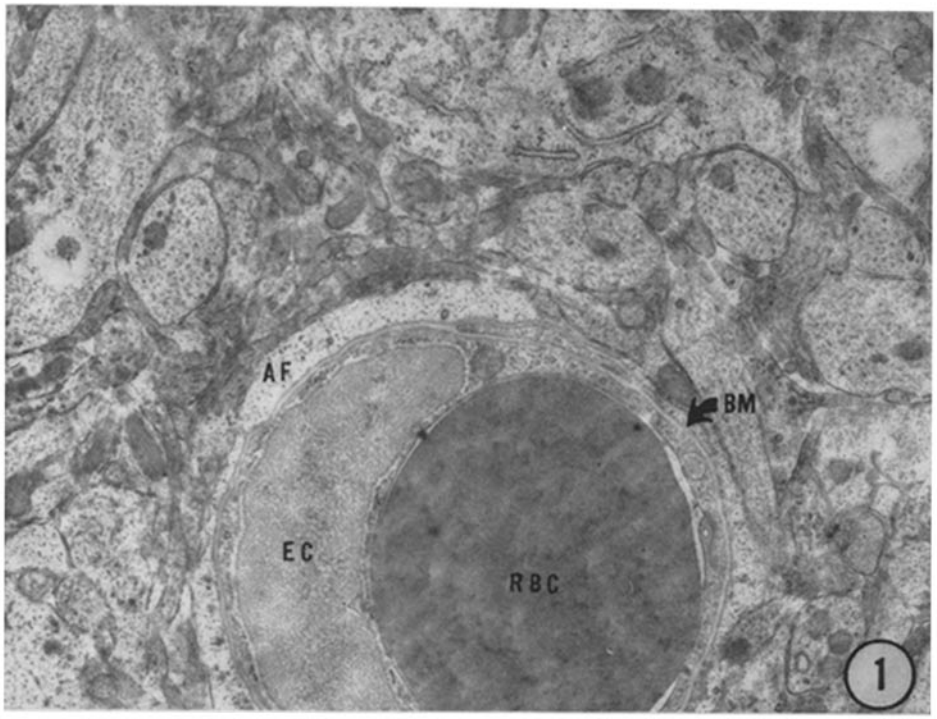
EXPLANATION OF PLATES

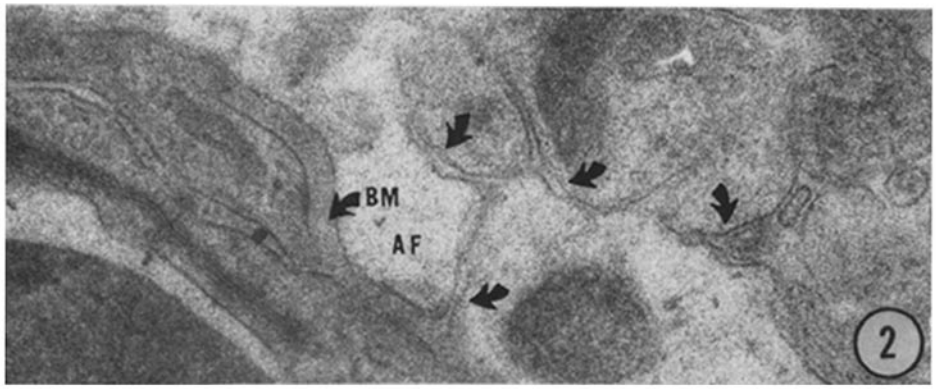
PLATE 12

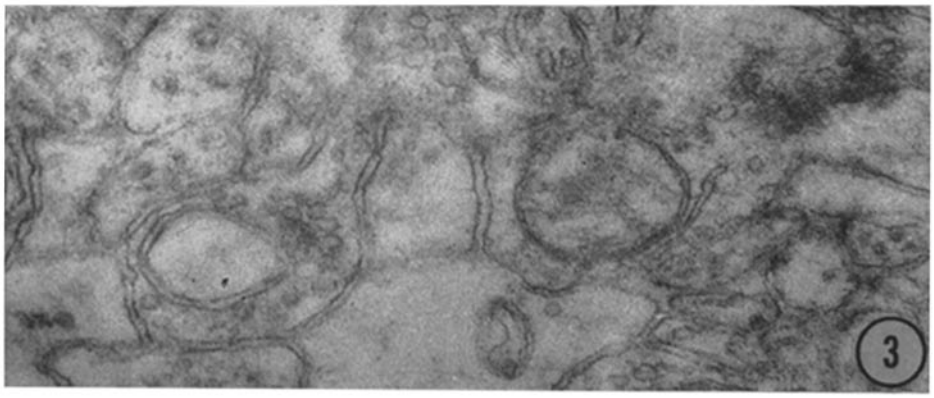
FIG. 1. Section through parenchyma and capillary containing a red blood cell (*RBC*). The endothelial cell (*EC*) and the capillary basement membrane (*BM*) are distinct. An astrocytic food process (*AF*) lies adjacent to the capillary on the left. The plasma membranes of the parenchymal cells are distinct and the intracellular and extracellular ground substances are homogenous. Normal mouse brain cortex. × 15,000.

FIG. 2. Photomicrograph showing continuity of the extracellular ground substance between the parenchymal cells with the ground substance lying within the capillary basement membrane (arrows). Normal mouse brain cortex. × 35,500.

FIG. 3. Higher magnification of parenchyma showing continuous extracellular ground substance. Normal mouse brain cortex. × 34,000.



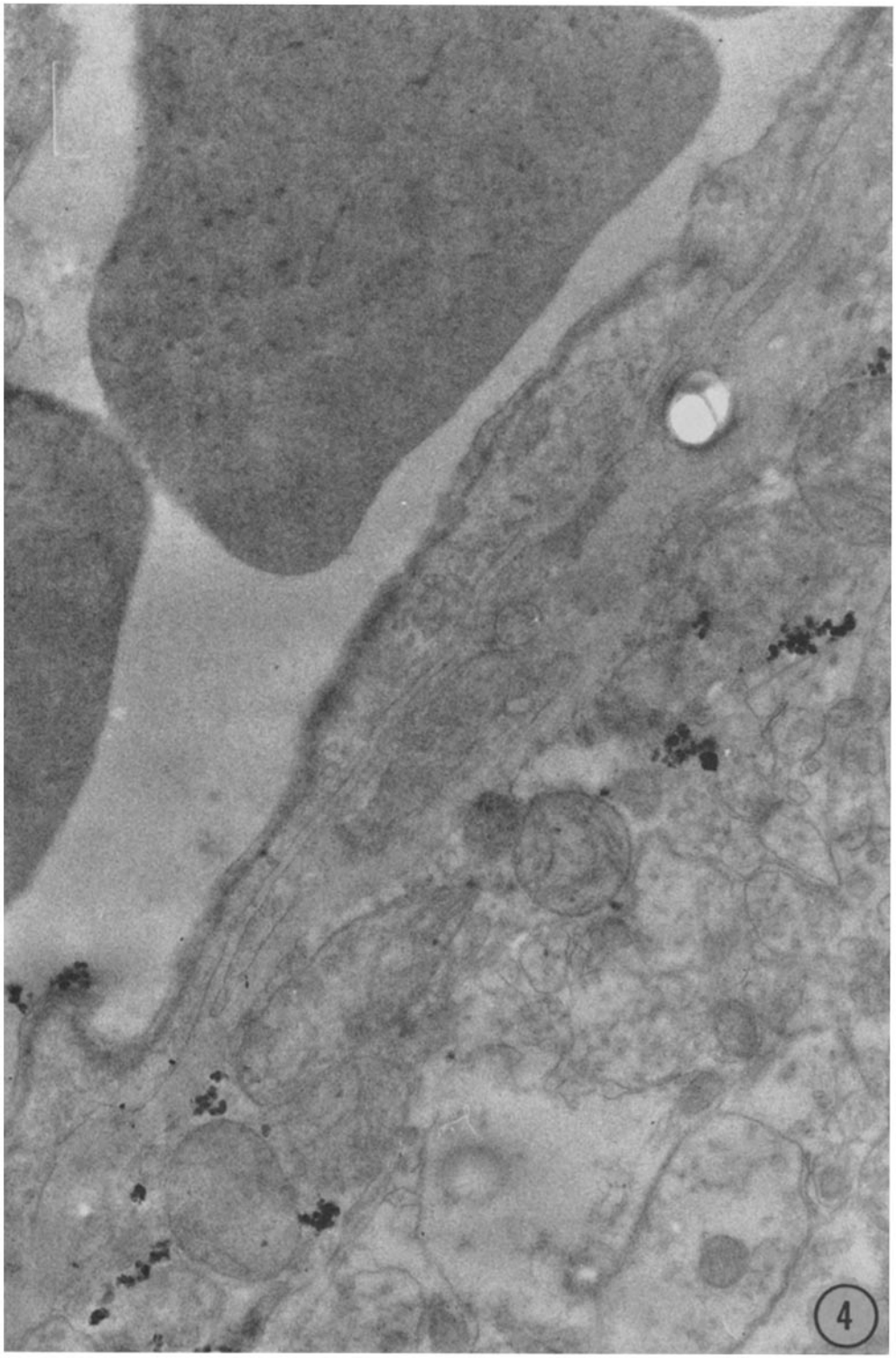




(Sellers and Lavender: Blood-brain barrier and entry of viruses. I)

PLATE 13

FIG. 4. Section of cortex from control mouse brain showing carbon particles lying within the extracellular spaces. Capillary with *RBC* above and to the left. × 26,000.

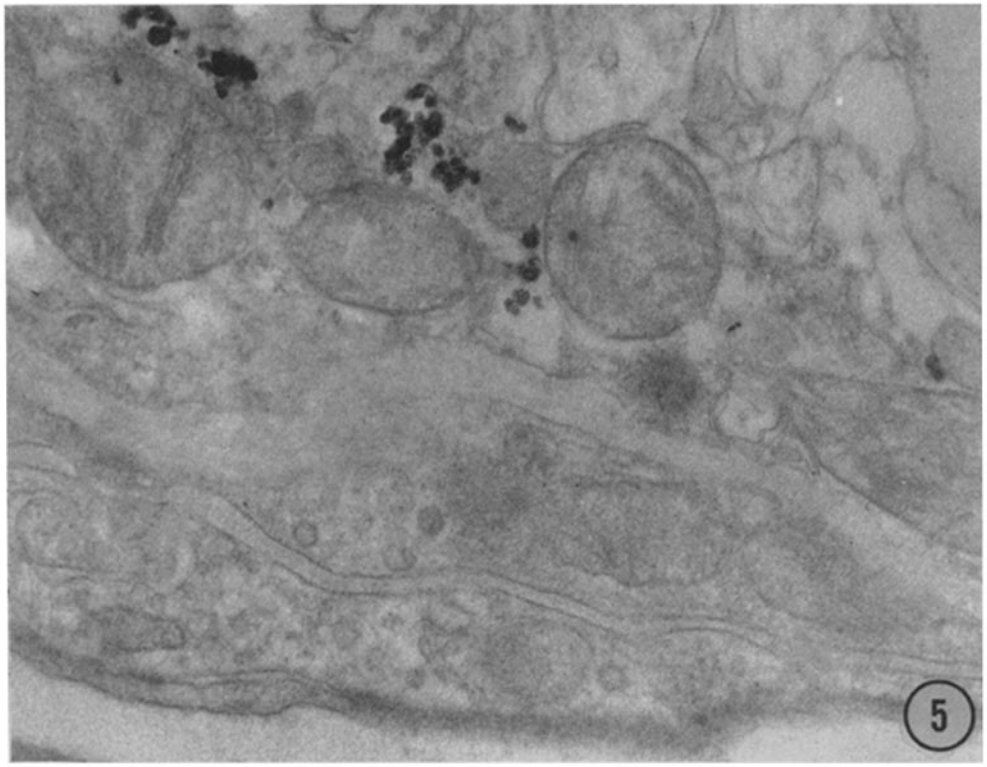


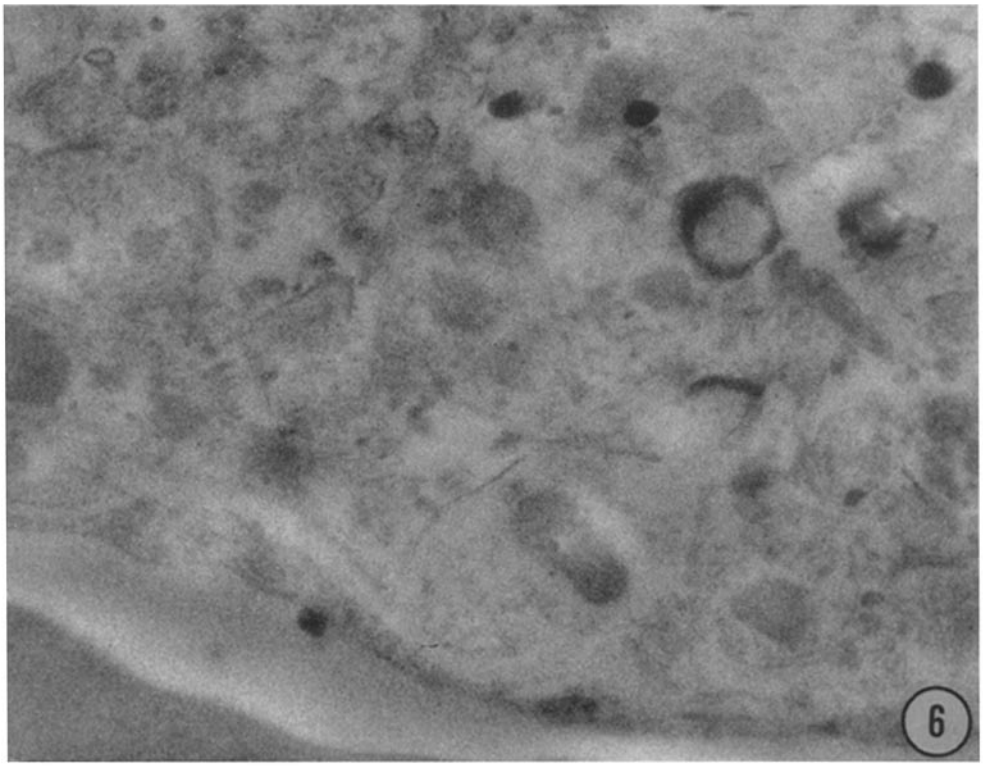
(Sellers and Lavender: Blood-brain barrier and entry of viruses. I)

PLATE 14

FIG. 5. Section from the same area of cortex as the preceding section but slightly deeper in the tissue showing that carbon particles are distributed in two planes in this particular area of parenchyma. × 42,000.

FIG. 6. Higher magnification of a section from the same brain as in Fig. 7. Note the indistinct portion of the capillary basement membrane and indistinct plasma membranes of the parenchymal cells. A kind of "liquefaction" of the tissue is observed. × 26,500.

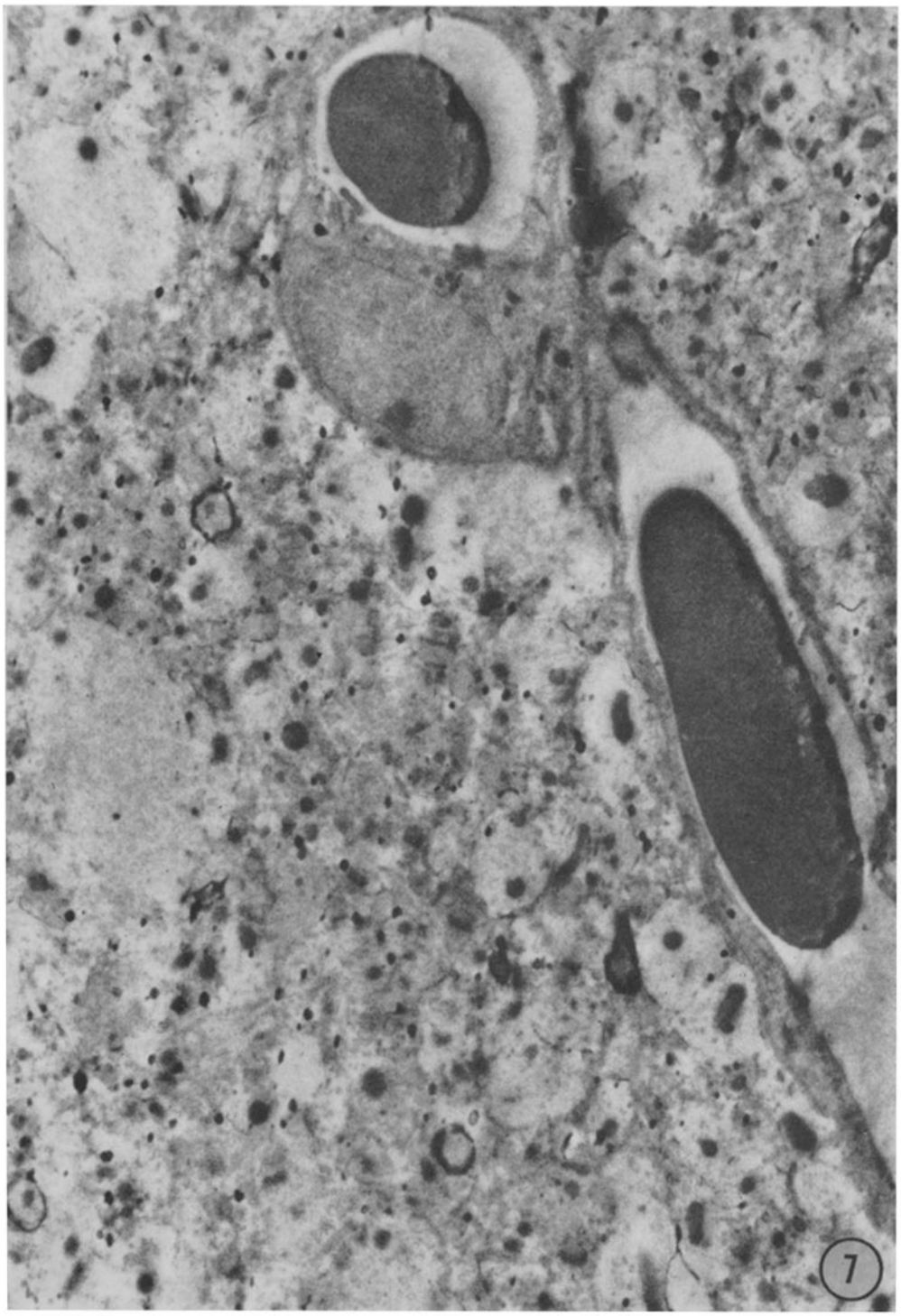




(Sellers and Lavender: Blood-brain barrier and entry of viruses. I)

PLATE 15

FIG. 7. Section of cortex from a mouse injected with carbon and treated with CO_2. A longitudinal and cross-section of capillary containing red blood cells and surrounding brain parenchyma studded with carbon particles. × 8,400.



(Sellers and Lavender: Blood-brain barrier and entry of viruses. I)