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PERFUSION OF CEREBRAL VENTRICLES: ASSAY OF PHARMACOLOGICALLY ACTIVE SUBSTANCES IN THE EFFLUENT FROM THE CISTERNA AND THE AQUEDUCT

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In cats under chloralose anaesthesia the cerebral ventricles were perfused from a cannula in the lateral ventricle. The effluent was collected either from the cisterna or from the aqueduct. When acetylcholine was added to the perfusion fluid and its destruction was prevented by the addition of an anticholinesterase, its recovery in the internal effluent was incomplete and irregular. This could be explained mainly by the devious route the perfusion fluid had to take in order to reach the cisterna and the unavoidable mixture with the cerebrospinal fluid of the subarachnoidal space. When collection was from the aqueduct 90% or more of the acetylcholine added to the perfusion fluid was recovered. In the presence of an anticholinesterase in the perfusion fluid, acetylcholine appeared in the effluent. Neostigmine was more effective than eserine or dyflos. The acetylcholine originated mainly from structures lining the lateral and third ventricle because the amounts in the effluent from the aqueduct were only a little less than those in the cisternal effluent. When injected intravenously, eserine was found to pass more readily into the perfusion fluid than neostigmine, probably because eserine passes the blood-brain barrier more readily than neostigmine. The method provides a quantitative approach for the study of the blood-brain barrier for pharmacologically active substances.

Experiments in which the cerebral ventricles of the anaesthetized cat were perfused from the lateral ventricles and the outflow was collected either from the cannulated cisterna magna or from the cannulated aqueduct were described by Bhattacharya and Feldberg (1958b). Perfusate collected from the cisterna had traversed the lateral, third and fourth ventricles and part of the subarachnoidal space; perfusate collected from the aqueduct had passed through the lateral and third ventricles only. Drugs injected intravenously affected the outflow from the two points of collection in different ways. In the present experiments the two methods of perfusion have been used to examine the following problems. (1) What happens to substances added to the perfusion fluid? Can they be fully recovered in the effluent? The two substances examined were acetylcholine and histamine. (2) Does acetylcholine appear in the effluent when the ventricles are perfused with Locke solution containing an anticholinesterase or when an anticholinesterase is injected into the blood stream? (3) Do anticholinesterases injected into the blood stream pass into the perfusion fluid?

METHODS

The experiments were performed on cats anaesthetized intravenously with chloralose. The method of perfusion of the cerebral ventricles and the collection of the effluent from the cisterna or aqueduct was that described by Bhattacharya and Feldberg (1958b).

In experiments in which acetylcholine or histamine was added to the perfusion fluid in order to determine their recovery in the effluent, perfusion with the substances was allowed to proceed for about 20 min. before the first sample for assay was collected, in order to wash out the fluid present in the ventricular space. The effluent was then collected in successive 20 min. samples which were assayed either for acetylcholine on the frog rectus muscle or for histamine on the atropinized guinea-pig ileum.

In those experiments in which the appearance of endogenous acetylcholine in the effluent was examined, an anticholinesterase (eserine, neostigmine or dyflos) was injected into the circulation or added

to the perfusion fluid and the effluent was collected for successive 20 min. periods. These were assayed for acetylcholine on the eserinized leech muscle preparation. When the anticholinesterases were injected into the circulation by the intravenous (eserine or neostigmine) or by the intracarotid (dyflos) routes, it was necessary to counteract the peripheral actions by atropine and tubocurarine. Intravenous injections of eserine or neostigmine were given every 20 min., together with atropine and tubocurarine; the first dose was 1 mg. eserine or 1 mg. neostigmine together with 0.5 mg. atropine and 0.5 mg. tubocurarine and subsequent doses 0.25 mg. of each. In experiments with intracarotid injections of dyflos, 1 mg./kg. was injected into each carotid before the collection of the first sample was begun. As soon as there were signs of salivation and muscle twitching, 0.5 mg. of atropine and 0.5 mg. of tubocurarine were injected intravenously and sometimes again after 1 or 2 hr. In the experiments in which the anticholinesterases were added to the perfusion fluid it was necessary to inject atropine and tubocurarine only when concentrations as high as 1:50,000 were used. In such experiments, particularly with neostigmine and dyflos, salivation and frequent muscular twitching occurred during prolonged perfusion. When this happened 0.5 mg. of both atropine and tubocurarine were injected intravenously. In experiments in which the anticholinesterases were added to the perfusion fluid and injected intravenously as well, an initial period of perfusion varying from 1 to 4 hr. usually preceded the intravenous injections. The amounts of eserine or neostigmine injected intravenously were 1 mg. for the first and 0.25 mg. for all subsequent injections given at 20 min. intervals. At the same time atropine and tubocurarine were injected, the procedure being the same as described for the experiments with intravenous injections of eserine or neostigmine alone.

In experiments in which the passage of anticholinesterases from the blood stream into the perfusion fluid was measured, plain Locke solution was used for perfusion, and eserine, neostigmine or dyflos were injected intravenously. In some experiments with dyflos the injections were made into the carotid arteries. Intravenous injections were given when perfusion had gone on for 20 to 30 min. and before the first sample of effluent was collected. The anticholinesterase was injected together with tubocurarine and atropine; injections were repeated at 20 min. intervals. The amounts of anticholinesterase for the first injections were for eserine and neostigmine 1 mg. and for dyflos 1.75 mg., and a quarter of these amounts for all subsequent injections. The amounts of tubocurarine and atropine were 0.5 mg. for the first and 0.25 mg. for all subsequent injections. When dyflos was given into the carotid arteries, injections of the same amount were made once in each carotid. The effluent was collected for consecutive 40 min. periods and samples were assayed for their content of anticholinesterase as described by Bhattacharya and Feldberg (1958a).

In all experiments in which tubocurarine was injected intravenously artificial ventilation was applied through the cannulated trachea.

Atropine was used as sulphate, tubocurarine as hydrochloride, eserine as sulphate, neostigmine as methylsulphate, and acetylcholine as chloride. All values refer to the salts. Histamine was used as acid phosphate, and the values refer to the base.

RESULTS

Recovery of Histamine or Acetylcholine Added to the Perfusion Fluid

Recovery from the Cisterna.—In experiments (Bhattacharya and Feldberg, 1958b) in which the cerebral ventricles were perfused with plain Locke solution, the effluent collected from the cannulated cisterna magna at the beginning of the perfusion always exceeded the inflow and was greater than 2 ml. in 20 min. As perfusion continued, the volume of the outflow approximated to that of the inflow within 1 or 2 hr. When the perfusion fluid contained acetylcholine 1:200,000 together with an anticholinesterase, or histamine 1:200,000, the outflow almost always exceeded the inflow (Table I). In one experiment (No. 5) it approximated to the inflow within the first hr., in another experiment (No. 3) it remained high although perfusion was continued for over 3 hr. In all other experiments the effluent was collected from the cisterna for up to 2 hr., and although the outflow had decreased by this time it remained greater than the inflow. The finding that the volume of fluid recovered was equal to the volume infused, or even in excess of it, does not mean that substances like acetylcholine or histamine added to the perfusion fluid were fully recovered in the outflowing fluid. The recovery of these substances varied from experiment to experiment independent of the volume of the effluent collected.

In Table I the 7 experiments in which the perfusion fluid contained acetylcholine 1:200,000 together with an anticholinesterase are arranged in such a way as to illustrate the recovery in ascending order. In experiment 1 recovery was between 15 and 20% and in experiment 7 between 95 and 100%. In each experiment the degree of recovery varied only slightly from sample to sample; when recovery was low it remained low, when it was high it remained high. In two short experiments in which histamine 1:200,000 was added to the perfusion fluid the recovery was 71% in one and in the other reached 100% after the first sample.

TABLE I

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA
Recovery in the effluent from the cisterna or aqueduct of acetylcholine (Expts. 1 to 7) or histamine (Expts. 8 and 9) added to the perfusion fluid in a concentration 1: 200,000. In experiments 1, 3, 4, 5, and 6 the perfusion fluid contained eserine 1 : 100,000, in experiments 2 and 7 neostigmine 1: 50,000 as well. The values in **bold type** indicate that the outflow was no longer collected from the cisterna but from the aqueduct. The underlined values were obtained after death in experiments 3 and 4 from the cisterna and in experiments 5 and 6 from the aqueduct.

Volume in ml. and Recovery of Acetylcholine or Histamine as % in Consecutive 20 min. Samples

Expt. 1		Expt. 2		Expt. 3		Expt. 4		Expt. 5		Expt. 6		Expt. 7		Expt. 8		Expt. 9	
ml.	%	ml.	%	ml.	%	ml.	%	ml.	%	ml.	%	ml.	%	ml.	%	ml.	%
2·3	15	2·4	50	2·65	74	2·45	80	2·2	85	2·6	90	2·45	95	2·4	76	2·15	71
2·3	15	2·5	60	2·65	78	2·45	82	2·15	80	2·5	90	2·5	97	2·4	100	2·15	71
2·3	20	2·4	58	2·35	70	2·3	82	2·05	75	2·4	85	2·4	100	2·4	100		
2·2	15	2·25	56	2·45	78	2·2	80	2·05	80	2·4	93	2·45	100				
2·3	20	**2.15**	**90**	2·4	80	2·15	80	2·0	85	2·4	90						
		2.0	**93**	2·45	78	2.0	78	2·15	80	**2.1**	**90**						
		2.05	**93**	2·55	78	2.0	98	2·15	80	**2.2**	**93**						
				2·4	78	1.95	100	**2.05**	**95**	**2.05**	**95**						
				2·3	78			**2.15**	**90**	**2.1**	**97**						
				2·45	75			**2.15**	**95**	1.95	100						
				—	—			**2.05**	100								
				1.95	100												
				1.95	98												

Recovery from the Aqueduct.—In experiments in which perfusion was carried out with Locke solution the outflow from the aqueduct was either equal to the inflow or exceeded it by 0.05 to 0.15 ml. in 20 min. (Bhattacharya and Feldberg, 1958b). The same result was obtained when the ventricles were perfused with Locke solution containing acetylcholine and an anticholinesterase. This is shown in experiments 2, 5, and 6 in Table I. In these experiments the effluent was first collected from the cisterna but later from the aqueduct. This switch-over increased the recovery of acetylcholine to between 90 and 95%. Experiment No. 2 is particularly striking. The recovery during the collection from the cisterna was 50 to 60%. When collection was from the aqueduct, the rate of outflow decreased, but in spite of this reduction the recovery rose to 93%.

Recovery of Acetylcholine after Death.—In some experiments the cat was killed with intravenous pentobarbitone sodium and the effluent was collected from the cannulated cisterna. The precaution was taken to make certain that the free opening of the cannula from which the fluid dripped lay below the level of the spinal cord. When the opening was above the level of the spinal cord the outflow not only stopped on killing the cat but the fluid in the cannula was drawn in and it took several minutes before the cannula filled again and the outflow started. The rate was then equal to the inflow. The initial stoppage and backflow is easily explained by the increased capacity of the subarachnoidal space when the filled vessels collapse after death. When the outflow was from a level below the spinal canal this stoppage and backflow did not occur and the outflow became almost equal to the inflow as illustrated in experiments 3 and 4 of Table I. When collection was from the aqueduct, the level at which the opening of the outflow cannula was kept was immaterial; the outflow became almost equal to the inflow, and changed little or not at all as illustrated in experiments 5 and 6 of Table I.

When the perfusion with acetylcholine was continued after death the recovery rose either at once, or after one sample, to 100% or to nearly 100% whether the collection was from the cisterna or from the aqueduct. This is illustrated in the experiments 3, 4, 5 and 6 of Table I.

Appearance of Acetylcholine in the Effluent

When the ventricular spaces were perfused with Locke solution and no anticholinesterase was injected into the blood stream, the effluent contained no detectable amounts of acetylcholine when tested on the eserinized leech muscle preparation which responded to acetylcholine 1×10^{-9}.

Effect of Anticholinesterases Injected into the Blood Stream.—When eserine or neostigmine were injected intravenously, or dyflos into both carotid arteries, but no anticholinesterase was added to the perfusion fluid, minute amounts of acetylcholine appeared in the effluent collected from the cisterna. This is shown in Table II. In experiment 7 (Table II), perfusion was continued for over 4 hr., and although the dyflos was injected only once, at the beginning of the perfusion, the output of acetylcholine rose steadily.

TABLE II

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA. APPEARANCE OF ACETYLCHOLINE IN CISTERNAL EFFLUENT AFTER INTRAVENOUS INJECTIONS OF ESERINE OR NEOSTIGMINE OR AFTER INTRACAROTID INJECTIONS OF DYFLOS

	Acetylcholine (ng./min.) in Successive 20 min. Samples of Effluent						
Expt. 1	2	3	4	5	6	7	
	Eserine		Neostigmine		Dyflos		
Nil	0·3	0·4	0·1	0·2	<0·3	0·3	
,,	0·3	0·2	0·2	0·2	<0·3	0·4	
<0·4	0·3	0·5	0·1	0·2	<0·3	0·4	
<0·4	0·3	0·5	0·1	0·2	<0·3	0·6	
			0·1	0·2	<0·3	0·6	
				0·3		1·0	
				0·5		0·8	
				0·6		1·0	
				0·3		1·3	
						1·6	
						1·6	
						1·6	
						2·0	
						1·7	

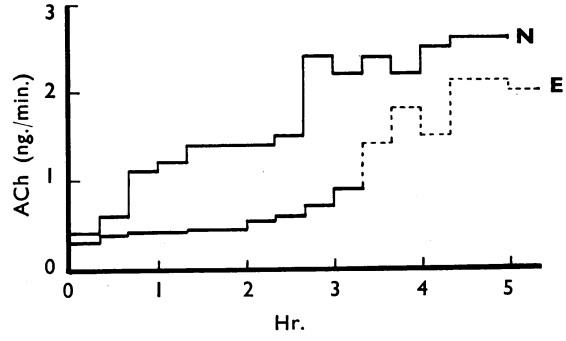

FIG. 1.—Perfusion from the lateral cerebral ventricle in two cats under chloralose anaesthesia. Appearance of acetylcholine in the cisternal effluent on perfusion with eserine (E) or neostigmine (N) in a concentration of 1: 200,000. The dotted line in experiment E indicates that after 200 min. perfusion eserine was injected intravenously every 20 min., 1 mg. being the first dose and 0.5 mg. at all subsequent injections whilst perfusion with eserine continued. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

Effect of Anticholinesterases Added to the Perfusion Fluid.—When an anticholinesterase was added to the perfusion fluid acetylcholine appeared in the effluent from the cisterna, and the amounts varied with the anticholinesterase and with the concentration used for perfusion.

The amounts of acetylcholine which appeared in successive 20 min. samples during perfusion with eserine 1:200,000 expressed in ng./min. are shown in Fig. 1. The output did not rise to more than 0.4 ng./min. during the first 2 hr. of perfusion. It was therefore of the same order as that obtained after intravenous injections of eserine. When perfusion was continued for over 3 hr. the acetylcholine output rose to 0.9 ng./min. In experiments in which the concentration of eserine in the perfusion fluid was 1:50,000, the amounts of acetylcholine in the cisternal effluent were only slightly greater (see experiment E in Fig. 2 and Table III).

The output of acetylcholine was greater when neostigmine instead of eserine was added to the perfusion fluid. Fig. 1 shows the difference between the effects of the two anticholinesterases when perfused at a concentration of 1:200,000, The output after 4 hr. perfusion with neostigmine reached about 2.5 ng./min. This high output of acetylcholine was never obtained when perfusing with eserine 1:200,000 or 1:50,000; it was also greater than the output which occurred when neostigmine was injected intravenously.

TABLE III

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance of acetylcholine (ng./min.) in successive 20 min. samples of cisternal effluent on perfusion with either eserine or neostigmine 1: 50,000.

	Eserine						Average	Neostigmine																	Average
Expt. 1	2	3	4	5	6		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
<0·4	0·6	0·6	0·4	<0·3	0·3	0·4	1·0	0·6	0·4	0·6	0·4	0·4	0·9	0·4	0·4	1·1	0·5	1·0	0·4	0·5	0·6	1·0	2·2	0·8	0·6	0·7
<0·4	0·8	0·6	0·7	0·4	0·4	0·5	1·0	1·2	1·2	1·1	2·0	1·2	2·0	0·7	1·2	2·0	1·4	2·0	2·3	1·3	1·4	2·3	—	1·2	2·0	1·5
<0·4	1·4	0·5	0·6	0·4	0·7	0·7	1·1	1·8	1·7	1·6	5·1	1·4	2·0	1·0	2·0	2·2	2·2	2·2	2·2	1·6	3·7	4·3	4·0	1·9	2·1	2·4
0·4	1·4	0·5	0·7	0·7	0·8	0·7		1·3	1·8	1·6	4·4	2·0	2·2	1·2	1·8	2·0	3·5	2·9	3·2	2·5	3·9	7·1	5·0	2·8	2·2	3·0
0·5	1·5	0·5	0·7	0·5	0·7	0·7				1·8	4·2	2·3	3·1	1·6	3·3	2·4	2·8	4·2	3·7	2·5	4·2	5·8	6·2	3·7	2·2	3·4
0·8	1·5	0·5	0·8	0·7	1·1	0·9				2·3	4·0	2·7	2·5	1·7	3·7	3·6	3·3	6·0	3·2	2·7	4·0	5·2	7·1	4·0	2·3	3·6
0·8	1·2	0·5	1·0	—	1·0	0·9										4·0	3·0	5·0	3·0	3·0	4·7	4·3	7·6	5·0	2·9	4·3
0·6	1·7	0·5	1·3	0·9	1·0	1·0											2·8	5·1	3·8	3·3	4·0	3·8	6·1	7·1	3·2	4·4
		0·7	1·5	0·7	1·1	1·0												6·0	6·1	4·7	4·4	4·3	6·6	7·3	4·9	5·5
				0·7	1·0	0·8															4·5	3·8	7·1	7·6	4·8	5·6
				1·0	0·8	0·9																5·8	8·7	7·0	5·2	6·7
				0·8	0·9	0·8																	8·4	6·2	6·1	6·9
					1·2	1·2																	8·5	6·8	6·5	7·3
																								6·2	5·5	5·9
																								6·2	5·0	5·6
																								6·6	5·8	6·2
																								6·2		6·2

When neostigmine 1:50,000 instead of 1:200,000 was perfused the output of acetylcholine in the cisternal effluent increased further (Fig. 2; Table III). From the average output of acetylcholine in successive 20 min. samples obtained in the 6 experiments with eserine and in the 19 with neostigmine recorded in Table III, it can be seen that, after 1 to 2 hr. perfusion with neostigmine 1:50,000, the output was more than 4 times greater and after longer perfusion even 7 to 8 times greater than after perfusion with eserine 1:50,000. Thus the difference between neostigmine and eserine became more pronounced as the perfusion continued. The greater efficacy of neostigmine is shown in the same experiment. For instance in an experiment in which perfusion during the first 4 hr. was with eserine 1:50,000 (Fig. 3), the output reached about 1 ng./min. during the second hour and then remained relatively steady, a value of 1.2 ng./min. being obtained during the last 20 min. of the 4 hr. period. The perfusion fluid was then changed to one containing neostigmine 1:50,000 and the output rose steeply and reached a value of 5.5 ng./min. within 2 hr.

The amounts of acetylcholine which appeared in successive 20 min. samples of cisternal effluent on perfusion with dyflos 1:50,000 were of the same order as after perfusion with eserine 1:50,000 as illustrated in Fig. 2, although on a weight for weight basis eserine is about 35 times more potent in inhibiting true cholinesterase than dyflos, whereas dyflos is about four times more potent in inhibiting pseudocholinesterase than eserine (Bhattacharya and Feldberg, 1958a).

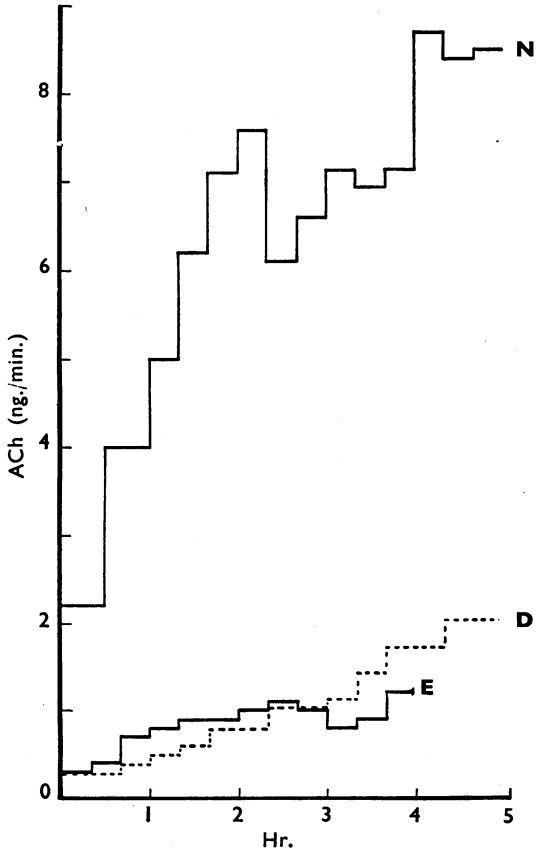

FIG. 2.—Perfusion from the lateral cerebral ventricle in three cats under chloralose anaesthesia. Appearance of acetylcholine in the cisternal effluent on perfusion with eserine (E), with neostigmine (N) and with dyflos (D) in a concentration 1:50,000. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

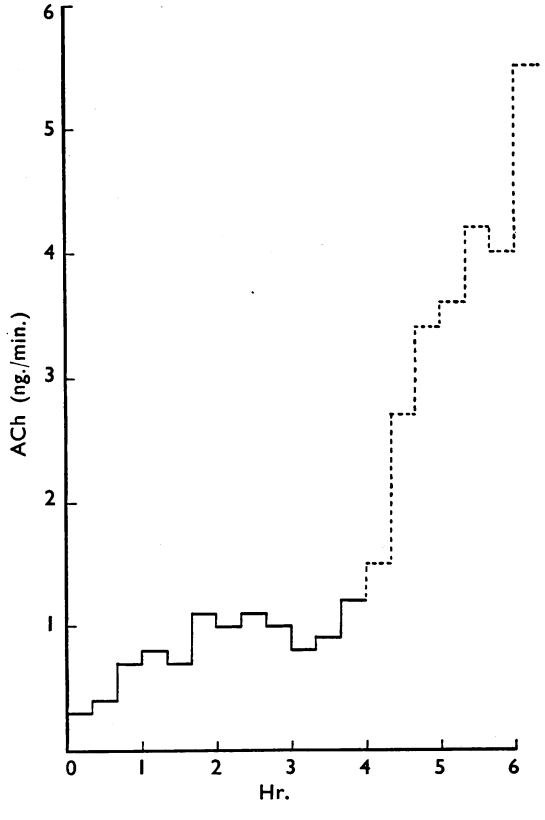

FIG. 3.—Perfusion from the lateral cerebral ventricle in a cat under chloralose anaesthesia. Perfusion during the first 4 hr. was with eserine 1:50,000 (continuous line) and afterwards with neostigmine 1:50,000 (broken line). Appearance of acetylcholine in cisternal effluent. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

Effect of Anticholinesterases Added to the Perfusion Fluid and Injected into the Blood Stream.—If acetylcholine were to diffuse into the ventricles and into the subarachnoidal space from the tissue bordering these spaces only, no increase in the output of acetylcholine by additional intravenous injection would be expected, particularly in experiments in which a strong concentration of neostigmine is used for perfusion, because the cholinesterase of the structures in immediate contact with the ventricular spaces must be assumed to be fully inhibited. Thus the additional intravenous injection of an anticholinesterase would not be able to improve the conditions for the escape of acetylcholine into the ventricular or subarachnoidal spaces. On the other hand, if acetylcholine were able to diffuse into the perfusing fluid from more distant regions, the additional intravenous injection of an anticholinesterase might allow this acetylcholine to reach the fluid undestroyed, thereby increasing the output beyond that obtained when the anticholinesterase is added to the perfusion fluid only. The results obtained varied according to whether eserine or neostigmine was used for intravenous injection. Eserine increased the output of acetylcholine; the effect of neostigmine was doubtful.

The experiment illustrated in Fig. 1 shows the increased output of acetylcholine which occurred on intravenous injection of eserine during perfusion with eserine 1:200,000. Table IV gives the results of experiments in which the effects of intravenous injections of eserine or neostigmine were studied during perfusion with either anticholinesterase in a concentration of 1:50,000. In the first four experiments of Table IV, perfusion was continued for several hours with eserine 1:50,000 before intravenous injections of either eserine or neostigmine were given. The output of acetylcholine, which had reached a relatively steady level before these injections, rose in all four experiments. Nevertheless the output remained lower than that obtained in many experiments in which neostigmine 1:50,000 was used for perfusion and no additional intravenous injections were given. In experiments 5 to 12 of Table IV perfusion was with neostigmine 1:50,000. As the output of acetylcholine usually increased on prolonged perfusion with this concentration of neostigmine it was necessary to find out whether the increases which occurred after the additional intravenous injections of neostigmine or eserine were the outcome of these injections. The result is shown in Fig. 4, in which the continuous line represents the mean output of acetylcholine for each successive 20 min. sample from the 19 experiments of Table III in which perfusion was with neostigmine 1:50,000. The individual values obtained from experiments 5 to 12 of Table IV after intravenous neostigmine are shown as solid circles, those obtained after intravenous eserine as squares. It will be seen that the solid circles are grouped around the continuous line, about half of them above and half of them below. This shows that the intravenous injection of neostigmine had not significantly changed the output of acetylcholine. On the other hand, most of the squares lie well above the continuous line, which shows that the intravenous injection of eserine had increased the output of acetylcholine in the effluent.

TABLE IV

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance of acetylcholine in cisternal effluent during perfusion with eserine or neostigmine 1: 50,000. The values in **bold type** were obtained when either eserine or neostigmine was given intravenously as well. In Expt. 13 collection was from the aqueduct.

Expt. No.	Anticholinesterase Used for		Acetylcholine (ng./min.) in Successive 20 min. Samples of Effluent																	
	Perfusion	Intravenous Injection																		
1	Eserine	Eserine	<0·3	0·4	0·4	0·7	0·5	0·7	—	0·9	0·7	0·7	1·0	0·8	1.9	2.0	1.6	1.5	1.7	1.7
2	,,	,,	0·6	0·6	0·5	0·5	0·5	0·5	0·5	0·5	0·7	2.3								
3	,,	,,	0·6	0·8	1·4	1·4	1·5	1·5	1·2	1·7	2.9	2.4	4.2	4.7	3.8	4 3	4.4			
4	,,	Neostigmine	0·4	0·7	0·6	0·7	0·7	0·8	1·0	1·3	1.5	2.2	2.2	2.7	4.0	3.6	3.6			
5	Neostigmine	,,	0·6	1·1	1·6	1·6	1·8	2·3	2.5	4.4	5.5	5.7	6.1	5.0	5.2					
6	,,	,,	0·4	2·0	5·1	4·4	4·2	4·0	4.5	4.6	4.6	5.2	6.5	4.8	7.4	6.5	6.6			
7	,,	,,	0·4	1·2	1·4	2·0	2·3	2·7	3.4	5.5	6.6	6.6	6.6	8.9	8.5	9.6	10.0			
8	,,	Eserine	1·0	1·0	1·1	3.1	4.9	5.9	5.3	7.6	6.9									
9	,,	,,	0·6	1·2	1·8	1·3	—	3·9	7.0	7.1	8.3	10.5	11.3	11.0						
10	,,	,,	0·4	1·2	1·7	1·8	2.3	6.5	7.8	7.0	7.5	8.4	7.9	7.3						
11	,,	,,	0·5	1·4	2·2	3·5	2·8	3·3	3·0	2·8	9.8	12.0	12.0	11.6	11.6	11.6	11.6	14.5		
12	,,	,,	2.0	3.0	4.4	4.7	6.9	7.6	7.7	8.5	8.1	7.1	7.5	7.1	7.6	10.0	10.3			
13	,,	,,	0·4	0·8	1·0	1·4	1·1	1·6	1·5	1·9	6.8	7.1	8.4	9.1	7.0					

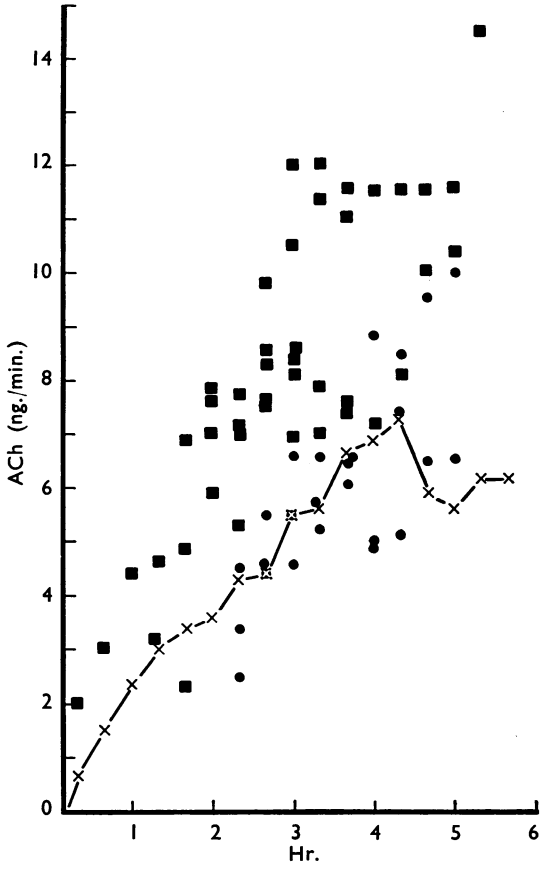

FIG. 4.—Perfusion from the lateral cerebral ventricle in cats under chloralose anaesthesia. Appearance of acetylcholine in the cisternal effluent in successive 20 min. samples during perfusion with neostigmine 1: 50,000. Continuous line: mean output of the 19 experiments recorded in Table III. Individual values for each 20 min. sample after intravenous neostigmine (●) or intravenous eserine (■) are shown. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

Collection from the Aqueduct.—When the collection was from the aqueduct, acetylcholine continued to appear in the effluent. This is shown in the experiments of Table V in which neostigmine 1: 50,000 was added to the perfusion fluid. The output of acetylcholine increased in successive samples, but the increase, particularly during prolonged perfusion, was smaller than that in most of the corresponding experiments of Table III with the collection from the cisterna. It would therefore seem that a relatively large portion of the acetylcholine which appears in the effluent collected from the cisterna does not enter the perfusion fluid in the lateral and third ventricles. However, the results of the following three experiments led to a somewhat different conclusion. In two experiments in which perfusion was with neostigmine 1: 50,000, collection was first from the cisterna and later from the aqueduct. The results are given in Table VI. The change of collection did not decrease the output of acetylcholine which had gradually risen in the course of 2 hr. of perfusion; the output continued to rise, although the rise may have been more gradual than it would have been if collection had continued from the cisterna. In another experiment (Expt. 13 of Table IV) in which perfusion was also with neostigmine 1: 50,000, collection was from the beginning from the aqueduct. The output of acetylcholine had risen to less than 2 ng./min. within 3 hr., but when eserine was then injected intravenously the output rose quickly to values between 7 and 9 ng./min.

TABLE V

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance of acetylcholine in effluent from the aqueduct during perfusion with neostigmine 1: 50,000.

Expt. No.	Acetylcholine (ng./min.) in Successive 20 min. Samples of Effluent														
1	0·7	1·8	2·6	2·1	4·2	2·5	3·5	3·7	2·4	2·6	2·9	3·1			
2	1·1	1·7	1·8	2·4	2·3	2·4	1·6	4·4	5·0	4·4	5·8				
3	0·7	0·9	0·9	0·8	1·0	1·4	1·3	1·3	1·1	1·3	1·5	2·4	2·5	3·3	

TABLE VI

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance of acetylcholine in effluent from the cisterna and later from the aqueduct (numerals in **bold type**) during perfusion with neostigmine 1: 50,000.

Expt. No.	Acetylcholine (ng./min.) in Successive 20 min. Samples of Effluent												
1	1·1	2·0	2·2	2·0	2·4	3·6	4·0	**4.3**	**4.3**	**5.0**	**4.8**	**4.3**	**5.1**
2	0·4	1·2	2·0	1·8	3·3	3·7	**4.1**	**5.9**	**5.1**	**5.9**	**5.0**	**6.3**	**6.2**

Output after Death.—A few cats were killed by intravenous injection of pentobarbitone sodium several hours after the perfusion with neostigmine 1: 50,000 when the acetylcholine in the effluent from the cisterna had reached a high level. Perfusion was continued. Killing the cat resulted in a progressive steep decrease of the acetylcholine output from sample to sample. This is illustrated by two typical experiments in Fig. 5. In experiment A the cat was killed after 3 hr. perfusion when the acetylcholine output had reached a level of 4.7 ng./min. In this experiment there was a further slight rise to 5.2 ng./min. in the first 20 min. sample collected after the pentobarbitone injection, then a fall to 2.6 and 0.6 ng./min. in the following two samples and finally a fall to 0.3 ng./min. Usually the fall occurred in the first

sample collected after killing the cat as in experiment B of Fig. 5. In this experiment neostigmine was injected intravenously after 2 hr. perfusion and the acetylcholine output in the following 200 min. reached a value of 10.6 ng./min. before the cat was killed; the output then fell to 2.3 ng./min. within 1 hr.

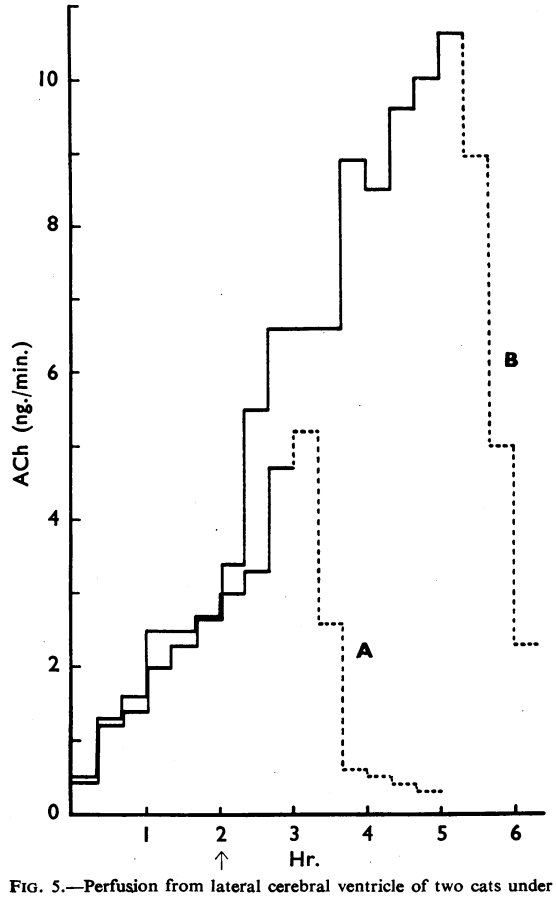

FIG. 5.—Perfusion from lateral cerebral ventricle of two cats under chloralose anaesthesia. Appearance of acetylcholine in the cisternal effluent during perfusion with neostigmine 1: 50,000. Continuous line perfusion in living cat: broken line perfusion after death due to intravenous pentobarbitone sodium. The arrow refers to the upper tracing and indicates the beginning of intravenous injections of neostigmine at 20 min. intervals. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

Passage of Anticholinesterases from the Blood Stream into the Perfusion Fluid

When the anticholinesterases were injected into the blood stream tubocurarine was injected as well. Since the assay of the effluent for anticholinesterase involved measuring acetylcholine contractions on the frog rectus muscle, and since tubocurarine is known to depress these contractions, it was necessary to find out if some of the injected tubocurarine appeared in the perfusion fluid and if it would interfere with the assay.

The contractions produced by 1 μg. acetylcholine in the frog rectus muscle suspended in a 5 ml. bath were slightly depressed by 0.1 μg. tubocurarine. However, no depression occurred if the acetylcholine was given with 4 ml. perfusate collected during 40 min. after an intravenous injection of 1 to 10 mg./kg. tubocurarine. This means that tubocurarine had not entered the perfusion fluid, or at least at a rate of less than 2.5 ng./min. which would not interfere with the assay.

Eserine and Neostigmine.—In Table VII the appearance of eserine and neostigmine in the cisternal effluent collected in successive 40 min.

TABLE VII

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance in the effluent from the cisterna or from the aqueduct (values in **bold type**) of anticholinesterases after their intravenous injection.

Anticholinesterase (ng./min.) in Successive 40 min. Samples of Effluent												
Expt. No.												
1	2	3	4	5	6	7	8	9	10	11	12	13
Eserine								Neostigmine			Dyflos	
24	40	10	36	53	28	25	25	0·5	1·1	2·6	2·1	1·8
48	26	43	52	28	29	—	21	0·4	1·0	2·5	3·1	2·5
61	20	35	83	34	37	30	24	0 4	1·0	3·4	3·1	4·0
75	18	27	92	**21**	**16**	**15**	**10**	0 8	2·2	6·4	3·1	3·7
	20	28	92	**20**	**18**	—	**10**		1·8	6·7		
		31	63	**20**	**16**	**17**	**10**					
					17	**16**						

samples is shown. In all experiments the anticholinesterases were injected intravenously at the beginning of the collection and again at 20 min. intervals. First 1 mg. and thereafter 0.25 mg. were injected. It will be seen that eserine appeared in much larger amounts in the effluent than neostigmine. The mean value of all samples collected from the cisterna in the experiments of Table VII was 39 ng./min. for eserine and 2.2 ng./min. for neostigmine. In four of the eserine experiments the effluent was later collected from the aqueduct. This decreased the content of eserine in the effluent to about half its previous value; therefore about half of the eserine assayed in the cisternal effluent has entered the perfusion fluid on its passage through the lateral and third ventricles.

Dyflos.—The last two experiments of Table VII show the amounts of dyflos found in the cisternal effluent when the drug was injected intravenously

in amounts (first 1.75 mg. and thereafter a quarter of this dose) somewhat higher than the amounts of eserine and neostigmine used in the corresponding experiments. The passage of dyflos into the perfusion fluid is of about the same order as that of neostigmine and much lower than that of eserine. Since dyflos is known to be an irreversible inhibitor of cholinesterase, it was thought that when injected intravenously part of it might have entered into a more or less irreversible combination with the pseudocholinesterase of the blood before reaching the brain. Therefore in three experiments 1 mg./kg. dyflos was injected into each carotid artery. In the first two experiments the injections were made immediately before collection of the first sample was begun. In the third experiment the injections were made before perfusion was started; about 10 min. later 1 ml. of cerebrospinal fluid was collected, perfusion was then started and the first sample collected. The results of these three experiments are given in Table VIII. The amounts of dyflos which

TABLE VIII

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance in cisternal effluent of dyflos after its injection into the carotid artery. At the start of the collection of sample 5 a second injection of 2 mg./kg. dyflos was made into each carotid.

Sample	Time of Collection in min.	Dyflos (ng./min.) in Effluent		
		Expt. 1	Expt. 2	Expt. 3
1	20	4	2·5	3
2	20	1	2	3
3	40	0·2	0·8	2
4	40	0·05	0·3	1
5	20			9
6	40			5

passed into the cisternal effluent during the first 20 min. after the carotid injections were between 2.5 and 4 ng./min., and were of the same order as those found in the experiments with intravenous dyflos. The 1 ml. of cerebrospinal fluid collected at the beginning of the third experiment contained 50 ng. which corresponds to an output of 5 ng./min. The amounts of dyflos diminished in each successive sample, but when, as in experiment 3, the drug was injected a second time and in a larger amount (2 mg./kg. into each carotid artery), the output increased in the next sample to 9 ng./min. and then decreased again.

DISCUSSION

Recovery of Substances Added to the Perfusion Fluid.—Acetylcholine added to the fluid perfusing the cerebral ventricles and protected by an anticholinesterase was incompletely recovered in the cisternal effluent in spite of the fact that the outflow was greater than the inflow. Therefore an outflow that exceeds the inflow should not be taken as an indication of full recovery of substances dissolved in the perfusion fluid, or even of the fluid itself. The incomplete recovery could be explained in different ways. Substances could either be absorbed from the choroid plexuses, be taken up by the ependyma, could diffuse into the brain substance or could escape into more distant regions of the subarachnoidal space where they would mix with the cerebrospinal fluid and be gradually absorbed into the blood stream.

The finding that the recovery of acetylcholine in the effluent from the aqueduct was always 90% or more renders it unlikely that selective absorption from the choroid plexuses is the main cause for the low and irregular recovery from the cisternal effluent, because the third and lateral ventricles with their choroid plexuses are still included when collection is from the aqueduct. For similar reasons it is unlikely that the unrecovered acetylcholine has been taken up by the ependyma. It is also unlikely that the incomplete and irregular recovery is mainly the result of diffusion into the brain substance because this would imply that diffusion varies greatly from brain to brain. It seems therefore more likely that acetylcholine escapes into distant parts of the subarachnoidal space. The absence of a foramen of Magendie in cats means that the fluid leaving the ventricle through the foramina of Luschka has to traverse the subarachnoidal space surrounding the brain stem before reaching the cisterna. Some mixing with the cerebrospinal fluid is thus inevitable and some of this mixed fluid is probably carried away into the subarachnoidal space which covers the cerebrum and is absorbed into the blood stream. The devious route the perfusion fluid has to take and the fact that on its way to the cisterna it comes into contact with the main current of cerebrospinal fluid make it understandable why the loss of acetylcholine in the cisternal effluent varies so much from experiment to experiment. After death, when the circulation of cerebrospinal fluid has ceased, the only outlet for the perfusion fluid is through the cannulated cisterna and this would explain why there is then 100% recovery of acetylcholine. The finding that, in the living cat, the recovery in the effluent from the aqueduct is not complete, although it is over 90%, means that some acetylcholine is lost when the fluid passes the ventricular spaces and therefore the possibility of some absorption by the choroid plexus or removal by the ependymal cells cannot be

excluded. There is some evidence that substances present in the cerebrospinal fluid can be taken up by the ependyma and the nervous tissue surrounding the ventricles and the subarachnoidal space (Wallace and Brodie, 1940; Bakay and Lindberg, 1949; Lindberg and Ernster, 1950; Sacks and Culbreth, 1951; Rodriguez, 1955).

Appearance of Acetylcholine in the Perfusion Fluid.—The acetylcholine detected in the effluent cannot have originated from acetylcholine circulating in the blood stream, because it appeared not only when anticholinesterases were injected intravenously but also, and in even greater amounts, when they were added to the perfusion fluid. Therefore the acetylcholine must derive from the brain. Further, the finding that after death there was a steep fall in the output of acetylcholine excludes a simple process of leakage from brain tissue and suggests central cholinergic neuronal activity as the cause for the appearance of acetylcholine in the effluent.

There are a few previous reports on the appearance of acetylcholine in the cerebrospinal fluid collected from the cisterna (Feldberg and Schriever, 1936; Chang, Hsieh, Li and Lim, 1938; Adam, McKail, Obrador, and Wilson, 1938). In these experiments also anticholinesterases were given either by injection into the blood stream or by addition to the fluid with which the cerebral ventricles were perfused or by a combination of both. Further, MacIntosh and Oborin (1953) showed that acetylcholine diffuses out of an eserinized surface in the brain of the cat.

Since the anticholinesterases present in the perfusion fluid are unlikely to penetrate deeply into the brain substance the acetylcholine of the effluent must originate from structures close to the surface over which this fluid passes. On prolonged perfusion the layer of brain tissue into which the anticholinesterases penetrate may, however, increase and the acetylcholine may diffuse into the fluid from deeper layers. This would explain why the acetylcholine content of the effluent increases on prolonged perfusion. The further increase obtained when eserine was injected intravenously during perfusion with neostigmine could be explained by diffusion from even more distant layers, because eserine given intravenously would naturally cause inhibition, or at least partial inhibition, of the cholinesterase in the whole brain.

The acetylcholine appearing in the effluent from the aqueduct must have entered the perfusion fluid on its passage through the lateral and third ventricles. The structures of origin are most likely to be the superficial layers of the caudate nucleus, the thalamus and some of the hypothalamic structures which are known to be rich in acetylcholine and choline acetylase, and which form part of the walls of the ventricles. Acetylcholine, however, is added to the cisternal effluent when the fluid passes through the fourth ventricle, or through the subarachnoidal space around the brain stem. In some experiments this contribution was only small.

The finding that greater amounts of acetylcholine appear in the perfusion fluid when it contains neostigmine instead of eserine cannot be explained by a greater potency of neostigmine as an anticholinesterase. If the greater output of acetylcholine nevertheless signifies greater inhibition of brain cholinesterase, it must mean that, when the blood-brain barrier is circumvented, neostigmine has a greater ability to penetrate to the actual sites of cholinesterase, and once having penetrated to these sites becomes more firmly attached than eserine. The greater ability of neostigmine to penetrate to these sites could be explained by a greater effect of neostigmine on the permeability of the nervous tissue. In this connexion it is of interest that neostigmine greatly reduces the excitability of the sciatic nerve whereas eserine does so to a lesser extent. This effect is attributed not to inhibition of cholinesterase but to interference with the permeability changes during nerve activity (Zaimis, 1953, and personal communication). Easier penetration or firmer attachment of neostigmine would also explain why the acetylcholine output rises more steeply on prolonged perfusion with neostigmine than with eserine.

Desmedt and La Grutta (1957) suggested that the arousal reaction of the sleeping brain produced in the *encéphale isolé* by carotid injections of anticholinesterases was related to inactivation of pseudocholinesterase rather than to inactivation of true cholinesterase in the brain, because selective inhibitors of pseudocholinesterase were more effective than those of true cholinesterase. The finding that on perfusion with neostigmine instead of eserine the output of acetylcholine was greater cannot be explained on these lines because neostigmine is a less potent inhibitor of pseudocholinesterase than eserine. Further, if the output of acetylcholine were related to inactivation of brain pseudocholinesterase it should be greater with dyflos than with neostigmine, because dyflos is a selective inhibitor of pseudocholinesterase and is active on this enzyme in smaller amounts than neostigmine (Bhattacharya and Feldberg, 1958a).

However, the output of acetylcholine on perfusion with dyflos was less than on perfusion with neostigmine.

The greater output of acetylcholine on perfusion with neostigmine instead of eserine emphasizes the fact that the potency of an anticholinesterase on cholinesterase preparation *in vitro* is not a reliable index for its pharmacological actions or its ability to inhibit the destruction of acetylcholine *in vivo*. Similar conclusions were reached when discussing the action of these two anticholinesterases on the leech muscle (Bhattacharya and Feldberg, 1958a).

Passage into the Perfusion Fluid of Substances Injected into the Blood Stream.—The finding that anticholinesterases injected into the blood stream pass into the perfusion fluid, although in varying amounts, and can be assayed in the cisternal effluent could be the result either of passage through the choroid plexuses or of diffusion from the brain tissue. If the anticholinesterases passed into the perfusion fluid from the choroid plexuses the amounts assayed in the effluent from the aqueduct should be only a little less than those assayed in the cisternal effluent. In both conditions the choroid plexuses of the third and lateral ventricles are included in the perfusion, the only difference being that the contribution from the choroid plexus of the fourth ventricle is lacking when collection is from the aqueduct. However, when in the course of an experiment collection from the cisterna was changed to that from the aqueduct the amounts of eserine assayed in the effluent decreased to about half. This suggests an extrachoroidal origin, and diffusion from the brain substance into the perfusion fluid is probably the main mechanism responsible for the appearance of the anticholinesterases in the effluent. From the experiments of MacIntosh and Oborin (1953) we know that such diffusion occurs ; in cats 0.02 ml. fluid/min./cm.^{2} was found to transude from the exposed cerebral cortex into a saline pool created at its surface. For substances present in the blood stream to transude in this way into the ventricular and subarachnoidal spaces they must first have passed the blood-brain barrier. The finding, therefore, that substances injected into the blood stream appear in different amounts in the perfusion fluid is likely to provide a basis for studying quantitatively the passage of pharmacologically active substances through the blood-brain barrier. From the present experiments it would appear that tubocurarine does not pass the blood-brain barrier, and that eserine passes this barrier about 20 times more readily than neostigmine. One should expect therefore that central effects are more prominent with eserine than with neostigmine when these anticholinesterases of almost equal potency are injected into the blood stream. This is in accordance with the following known facts.

Bülbring and Burn (1941) found that eserine was effective on the knee jerk in much smaller doses than neostigmine when these anticholinesterases were injected into the blood perfusing the spinal cord of a dog. Later Eccles, Fatt and Koketsu (1954) and Eccles, Eccles and Fatt (1956) showed the same difference between intravenous eserine and neostigmine on the discharge of the Renshaw cells in the spinal cord. Intravenous eserine greatly prolonged the repetitive discharge evoked by an antidromic volley in the motor neurones and induced spontaneous discharge, whereas intravenous neostigmine was either inactive or had only a slight effect on the evoked discharge. On the other hand, when injected through the recording cannula in the environment of the Renshaw cells, neostigmine was at least as active as eserine. The difference in effectiveness on intravenous injection was attributed to the ability of eserine to pass the blood-brain barrier more readily than neostigmine.

The same explanation could be advanced for the observations that intravenous eserine has a strong, and intravenous neostigmine a weak, action on respiration in anaesthetized and decerebrate cats (Erdmann, Kempe and Lühning, 1955), and that intravenous eserine produces in unanaesthetized cats and monkeys, low-voltage irregular, fast activity in the electroencephalogram similar to the activity pattern of the arousal reaction, whereas intravenous neostigmine does not alter the pattern even when given in doses which cause profound peripheral effects (Funderburk and Case, 1951 ; Bradley, 1953 ; Bradley and Elkes, 1953, 1957). There are also observations of Funderburk and Case (1947) on the effect of anticholinesterases on behaviour in cats ; an intravenous injection of eserine, but not of neostigmine, abolished the conditioned response for as long as 2 hr. In rats, intravenous eserine produces a rise in arterial blood pressure of central origin which is rarely seen with intravenous neostigmine (Varagić, 1955 ; Hornykiewicz and Kobinger, 1956 ; Medaković and Varagić, 1957), but on cisternal injection both anticholinesterases have a strong pressor effect as shown by Hornykiewicz and Kobinger.

Funderburk and Case (1951) quote an unpublished observation by Du Bois, Erway and Byerrum that intravenous eserine increases the

acetylcholine content of the brain of the rat, whereas intravenous neostigmine does not. This observation again is in line with our finding that in experiments in which neostigmine was present in the perfusion fluid, intravenous eserine definitely increased the output of acetylcholine in the effluent whereas the effect of intravenous neostigmine was doubtful. The difference in the ability of the two anticholinesterases to pass the blood-brain barrier would account for these results as well as for those of Du Bois, Erway and Byerrum.

The results obtained on the appearance in the perfusion fluid of intravenously injected dyflos resembled those obtained with neostigmine rather than those with eserine. Since the potency of dyflos in inhibiting true or pseudocholinesterase activity is different from the potency of neostigmine or eserine, its effectiveness in eliciting central reactions on intravenous injection cannot directly be compared with that of the other two anticholinesterases.

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