levels brought about by different mechanisms. Previous experiments carried out on adipose tissue and heart[10,11] stated that drugs able to stimulate adenylcyclases act synergically with phosphodiesterase inhibitors. The potentiation found by us between salbutamol and theophylline extends such a statement to the bronchial smooth muscle and indirectly confirms that salbutamol behaves as an adenylcyclase stimulating agent.
On the other hand, the finding that potentiation also exists between salbutamol and dibutyrril-3'5'AMP suggests that the phosphodiesterase inhibiting properties of the latter compound play an important role. Thus, the assumption that it acts only as an easily permeable 3'5'AMP must be regarded with some criticism.

Riassunto. È stato dimostrato che la contemporanea stimolazione delle adenilciclasi, realizzata con salbutamolo, e la inibizione delle fosfodiesterasi prodotta da teofillina esercitano effetti broncodilatatori sinergici. Il potenziamento osservato anche fra salbutamolo e dibutirril-3'5'AMP è stato riferito alla inibizione delle fosfodiesterasi determinata da quest'ultima sostanza.

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[10] G. A. ROBISON, R. W. BUTCHER and E. W. SUTHERLAND, *Cyclic AMP* (Academic Press, New York 1971).
[11] P. GREENGARD and E. COSTA, *Role of Cyclic AMP in Cell Function* (Raven Press, New York 1970).
[12] Acknowledgments: We thank Mr. A. GIACOMELLI for the technical assistance.

Saturable Transport of Amphetamine Across the Blood-Brain Barrier[1]

The rapid onset of CNS stimulation after amphetamine administration is usually ascribed to free diffusion of its nonionized, lipophilic form across the blood-brain barrier (BBB)[2]. However, amphetamine has a pKa of 9.9[3]; at an arterial pH of 7.4, about 99.7% of the plasma amphetamine concentration would be protonated and lipophobic. This disparity between predicted effects (slow rate of barrier permeability) and observed effects (abrupt psychomotor changes and stereotyped behavior) suggests that some portion of the predominantly ionized form of amphetamine enters the brain from the circulation by a transport mechanism. This in vivo study describes some aspects of D-amphetamine uptake across the BBB which might explain the onset discrepancy, and demonstrates for amphetamine uptake two hallmarks of carrier-mediated transport: saturability and competitive inhibition.

Methods. The penetration of ^{14}C-D-amphetamine sulfate into brain was studied with the internal tritiated water technique described by OLDENDORF[4]. Male Sprague-Dawley rats, 350–400 g, were anesthetized with 45 mg/kg i.p. pentobarbital sodium and prepared for intracarotid injection. A single bolus (constant volume of 0.2 ml) was injected into the common carotid of each rat without impeding the arterial blood flow. The solution consisted of 0.25 μCi ^{14}C-D-amphetamine sulfate (s.a. 15.5 mC/m*M*, Schwarz/Mann), 0.25 μCi ^{3}H-water (s.a. 0.25 mC/g, New

[1] Supported in part by USPHS Research Grant No. NSO8884, NINDS.
[2] J. M. VAN ROSSUM, Int. Rev. Neurobiol. *12*, 307 (1970).
[3] T. B. VREE, A. Th. J. M. MUSKENS and J. M. VAN ROSSUM, J. Pharm. Pharmac. *21*, 774 (1969).
[4] W. H. OLDENDORF, Brain Res. *24*, 372 (1970).

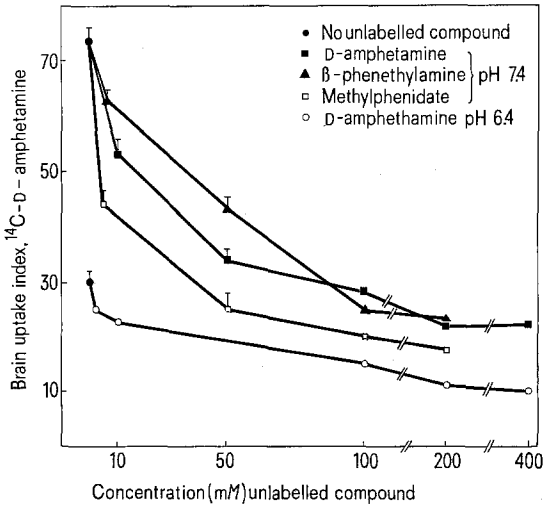

Fig. 1. The effects of increasing concentrations of various compounds on the uptake of ^{14}C-D-amphetamine sulfate into rat brain. All compounds were injected as a bolus (0.2 ml) into the carotid artery. Standard errors of the means are given for those values which represent 3 or more animals; other points are from individual rat brains.

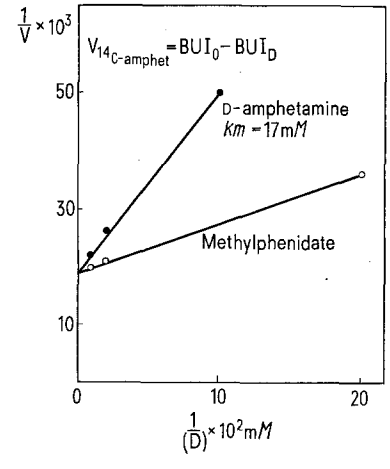

Fig. 2. Lineweaver-Burk plot of the effects of increasing concentrations of unlabelled D-amphetamine and methylphenidate on the uptake of a small dose (1 μg/kg) of ^{14}C-D-amphetamine into rat brain. Abscissa: reciprocal of the concentration of unlabelled drug (D). Ordinate: reciprocal of the differences in uptake of labelled amphetamine in the presence of specific concentrations of D.

England Nuclear), and varying concentrations of unlabelled compounds (vide infra) dissolved in lactated Ringer's solution buffered to pH 7.4.

Rats were decapitated 15 sec after the injection and the brains quickly removed from the calvaria. The cerebral hemispheres ipsilateral to the injection site and rostral to the midbrain were prepared for double isotope liquid scintillation counting by dissolving the macerated tissue in 1.5 ml NCS Solubilizer (Amersham/Searle), then adding 10 ml of a toluene-PPO scintillant. Samples of the injection mixtures were similarly prepared for scintillation counting. Standard quench curves were utilized to convert counts/min for each isotope (^{14}C and ^{3}H) to DPM[5]. A brain uptake index (BUI) for ^{14}C-D-amphetamine was obtained by dividing the ^{14}C-DPM/^{3}H-DPM ratio in the brain sample by the same ratio in the injection mixture[4]. This fraction multiplied by 100 yields a percent index of brain uptake of ^{14}C-D-amphetamine relative to tritiated water. BUI values thus represent the extent to which amphetamine enters the brain, during a 15 sec interval, compared to the freely permeable water.

Initially the brain uptake of a small fixed dose of ^{14}C-D-amphetamine (0.016 mM approximately 1.0 µg/kg) was determined, then measured amounts of unlabelled D- and L-amphetamine, β-phenethylamine, or methylphenidate were added to the injection mixture in a final volume of 0.2 ml to assess the degree of saturability, stereospecificity, and competition by compounds structurally related to D-amphetamine. A pharmacologic profile of the possible mechanisms by which amphetamine enters the brain was made by adding dopamine HCl, phenylephrine HCl, phenylalanine, or arginine to the injection mixture. The concentrations of all agents are reported as the free base. The effects of pH on amphetamine uptake were determined in several experiments by buffering the injections mixture to pH 6.4.

Results. The BUI (mean ± SE) for the fixed dose of ^{14}C-D-amphetamine was 73 ± 3% (Figure 1), which approximates the 67 ± 5% BUI which has been reported for ^{14}C-β-phenethylamine[6]. Unlabelled D-amphetamine, when added to the injection mixture, reduced the brain uptake of ^{14}C-D-amphetamine. At 100 mM concentrations of unlabelled compound the BUI for the labelled agent was approximately 29%. However, the uptake index of ^{14}C-D-amphetamine was not reduced to values less than 23%, even in the presence of amphetamine concentrations as high as 200 and 400 mM. Methylphenidate and β-phenethylamine, structural analogues of amphetamine, also depressed the BUI for ^{14}C-D-amphetamine (Figure 1). When the pH of the solution was lowered to 6.4, competition between labelled and unlabelled D-amphetamine reduced the BUI from an initial value of 30% to 10%.

Effects of phenethylamine derivatives and amino acids on the brain uptake index (BUI) of ^{14}C-D-amphetamine. Each value is the mean (± SE) BUI obtained from 3 rat brains

Compounds tested for inhibition of amphetamine uptake	^{14}C-D-amphetamine BUI (%)
Control[a]	73 ± 3
100 mM Dopamine HCl	74 ± 5
100 mM Phenylephrine HCl	54 ± 3
100 mM L-arginine	70 ± 2
100 mM L-phenylalanine	65 ± 3

[a] 0.016 mM ^{14}C-D-amphetamine sulfate (1.0 µg/kg).

The saturation data for amphetamine and methylphenidate are presented in Figure 2 in the form of a double reciprocal plot. A K_m of 17 mM was calculated for amphetamine from the slope and intercept of the amphetamine curve. The methylphenidate and amphetamine curves share the same intercept, providing evidence for competitive inhibition, i.e. the agents apparently compete for a common binding site in a critical step of transport.

When unlabelled L-amphetamine was substituted for the D-isomer, minimal differences were found between the compounds with respect to ^{14}C-D-amphetamine uptake. Dopamine, a 3,4-dihydroxy derivative of β-phenethylamine, did not inhibit amphetamine uptake (Table), while phenylephrine, a 3-hydroxy analogue, elicited only modest depression of the amphetamine BUI. Arginine and phenylalanine, representatives of the basic and neutral amino acids which are transported across the BBB[7], also failed to depress amphetamine uptake.

Discussion. Our observations on the saturability of amphetamine uptake into brain suggest that there is a carrier-mediated component in the passage of the compound across the BBB. The BUI for a compound entering solely by free diffusion would not be reduced by increasing the concentration of the agent in the microvasculature. On the other hand, if a compound traverses the barrier only by facilitated mechanisms, large concentrations of that agent should cause the BUI of a small labelled fraction to approach the background of the methodology, 1–2%[6]. Unlabelled amphetamine in concentrations up to 400 mM did not depress the BUI for ^{14}C-D-amphetamine beyond 23%, indicating that this considerable fraction of the drug enters the brain by a nonsaturable free diffusion process.

The effects of bolus pH on amphetamine uptake provide additional information about the free diffusion mechanism. Lowering the pH of the injection mixture from 7.4 to 6.4 increases the concentration of ionized drug only slightly from 99.7 to 99.9%, as calculated from the Henderson-Hasselbach relationship. The nonionized fraction, however, is reduced by an order of magnitude from 0.3 to 0.03%. Experimentally, changing the pH from 7.4 to 6.4 causes a reduction in the BUI for a small dose of ^{14}C-D-amphetamine from 73 to 30%. This change in barrier penetrability, consonant with the marked shift in the concentration of nonionized species, suggests that free diffusion predominates over facilitated transfer in their relative contributions to amphetamine uptake.

The transport mechanism for amphetamine is apparently not stereospecific, but is subject to saturation by methylphenidate, a compound with central stimulant properties similar to those of amphetamine. The extent of aromatic hydroxylation of phenethylamine appears to be an important determinant of carrier affinity. Thus, 100 mM concentrations of β-phenethylamine markedly depressed amphetamine uptake, whereas equivalent concentrations of phenylephrine and dopamine, mono- and dihydroxy analogues, respectively, elicited progressively less competition for the amphetamine carrier. The mechanism for facilitated uptake of amphetamine is apparently unrelated to the processes by which basic and neutral amino acids are transported across the BBB[6,7], since 100 mM arginine and phenylalanine failed to depress the amphetamine BUI.

[5] M. A. Williams, G. H. Cope, J. L. Jackson and P. Hill, Biochem. J. *118*, 379 (1970).
[6] W. H. Oldendorf, Am. J. Physiol. *221*, 1629 (1971).
[7] J. J. Richter and A. Wainer, J. Neurochem. *18*, 613 (1971).

In conclusion, our results indicate that amphetamine gains access to the brain by a combination of 2 independent mechanisms: free diffusion and a carrier-mediated process. The amphetamine carrier is saturable, pH dependent, and participates in the uptake of methylphenidate and β-phenethylamine. The rapid onset of action of amphetamine after intravenous administration may reflect the simultaneous operation of these uptake mechanisms.

Zusammenfassung. Untersuchungen über die die Penetration von Amphetaminen ins Gehirn bestimmenden Faktoren. Entwicklung neuer Aspekte im Hinblick auf den Transport von Amphetaminen über die Blut-Hirn-Schranke mittels eines Trägers.

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A Study of the Mechanism of Lowering the Sensitivity of Smooth Muscle to Noradrenalin with Sympathetic Hyperinnervation

Nerve growth factor (NGF) is known to induce the hypertrophy and hyperplasy of sympathetic and embryonal sensory cells[1–3]. The injection of NGF to new-born mice results in an increase in the amount and dimensions of the sympathetic ganglia neurons[2,3], a thickening of sympathetic trunks due to an increase of the fiber number, and also an augmentation of the density of sympathetic terminals in the effector organ[2,4,5]. As compared with the normal, the noradrenalin content of the peripheral organs is augmented in such animals[6]. Thus, by means of NGF, animals can be obtained in which organs are hyperinnervated by sympathetic terminals. An investigation of the intestine smooth muscle with sympathetic hyperinnervation revealed that the sensitivity of this muscle to noradrenalin is lowered as compared with the control[7]. It has been assumed that denervation may not only increase the sensitivity of the effector, according to the denervation law, but decrease at hyperinnervation[7].

Adrenergetic terminals are known for their capacity to uptake catecholamines from the environment. Therefore, some amount of the injected agent is uptaken by the terminals after noradrenalin injection and the noradrenalin concentration in the area of the smooth muscle membrane decreases. An augmented response of the smooth muscle to humoral stimulus may occur as a result of the destruction of sympathetic fibers, e.g. due to their degeneration, even if the sensitivity of the postsynaptic membrane has remained unchanged[8]. It is clear that the effect of lowering the sensitivity on account of the terminals will be more pronounced if the numbers of terminals on the periphery has increased. Against a cocaine background the terminals lose their capacity to take up-catecholamines[8–10]. Therefore, a comparative study of sensitivity in a normal and hyperinnervated smooth muscle in response to noradrenalin against a cocaine background permits us to determine whether the sensitivity of the effector organ cells to this agent differs or not.

Material and method. In the present work we used the NGF preparation from the Wellcome, Kent, England. The preparation was injected to young mice at a rate of 500 biological units per 1 g of body weight for 15 days, after which the animals were tested.

[1] S. COHEN and R. LEVI-MONTALCINI, Cancer Res. *17*, 15 (1957).
[2] R. LEVI-MONTALCINI, B. BOOKER, Proc. natn. Acad. Sci., USA *46*, 373 (1960).
[3] R. LEVI-MONTALCINI and S. COHEN, Ann. N. J. Acad. Sci. *85*, 324 (1960).
[4] W. HAMMER, K. A. NORBERG, L. OLSON, F. SJOGWIST. Acta physiol. scand. *69*, 127 (1967).
[5] L. OLSON, Z. Zellforsch. mikrosk. Anat. *81*, 155, 1967.
[6] D. C. EDWARDS, E. L. FENTON, S. KAKARI, B. J. LARGE, L. PAPADAKI, E. ZAMIS, J. Physiol. Lond. *186*, 10 (1966).
[7] N. A. KUZMICHEVA, A. S. ORLOVA, I. M. RODIONOV, V. M. RODIONOV T. D. BOLCHAKOVA, T. A. LUKICHEVA, V. V. MENSHIKOV, E. P. FROLOV, V. N. JARYGIN, Nature, Lond. *224*, 385 (1969).
[8] U. TRENDELENBURG, Pharmac. Rev. *18*, 629 (1966).
[9] R. F. FURCHGOTT, J. pharmac. exp. Ther. *142*, 39 (1963).
[10] W. H. MCMILLAN, Br. J. pharmac. exp. Ther. *142*, 39 (1963).

Mean noradrenalin doses ($M \pm m$; in μg) causing standard responses of the smooth muscle in normal and hypersympathetized animals in intact preparations and after cocaine treatment

Response	Norm	Hypersympathetization	Cocaine	
			Norm	Hypersympathetization
Threshold	0.865 + 0.097 − 0.088 $n = 27$	1.693 + 0.349 − 0.288 $n = 14$ $p < 0.01$	0.322 + 0.038 − 0.034 $n = 25$	0.473 + 0.075 − 0.065 $n = 16$ $p < 0.05$
50% of the maximal	1.914 + 0.375 − 0.311 $n = 22$	5.009 + 1.122 − 0.915 $n = 12$ $p < 0.01$	0.726 + 0.095 − 0.084 $n = 18$	1.446 + 0.269 − 0.227 $n = 10$ $p < 0.01$
Maximal	43.29 + 5.37 − 4.78 $n = 28$	149.0 + 39.0 − 30.9 $n = 13$ $p < 0.001$	11.11 + 1.91 − 1.62 $n = 25$	40.90 + 11.67 − 9.08 $n = 15$ $p < 0.001$