and isolated, weighed, and then transferred to a small test tube containing 0.001 N HCl. The midguts were macerated in the acid with a glass rod, allowed to leach for 15 min at 37 °C, and then centrifuged for 5 min at room temperature at 2000 × g. The supernate was used for chymotrypsin, trypsin, and protein assays as described elsewhere[5].

Results and discussion. The results are given in tables 1 and 2. Midgut weights of house bees are larger than those of forager bees in 15 of the 16 samples, reflecting the total body weight difference reported by DeGroot[4]. Both chymotrypsin and trypsin are typically more abundant in house than in forager bees, whether expressed as units per midgut or as units per mg midgut protein. However, the ratio of chymotrypsin to trypsin is greater for forager than for house bees in 15 of the 16 samples, with decreased trypsin activity being the primary component of the increased ratio. The range of increase in the 15 ratios is from 1.42 to 5.21 ($\bar{X} = 2.32 \pm 0.97$) times greater. The data for chymotrypsin show an apparent seasonal pattern, with lowest activity values recorded on September 12; no similar seasonal pattern is apparent in the trypsin data.

Chymotrypsin is the most abundant protease in both honeybee worker midguts and in 14 surveyed pollens[1]. In our earlier study[1] we presented correlative evidence linking levels of chymotrypsin activity in honeybee midguts with the presence of that enzymic activity in pollen; no correlation could be similarly made for trypsin. The present data are similar to the earlier study in that they show an apparent seasonal pattern in chymotrypsin activity but not in trypsin activity. The consistency with which the age correlated decrease in trypsin activity is greater than that for chymotrypsin activity in these studies is thus noteworthy. An age correlated decrease in chymotrypsin activity in honeybee worker midguts could be due, at least in part, to a decrease in pollen consumption. The consistency of the greater decrease in trypsin activity is less apparently attributable to decreased pollen consumption. Question has existed as to whether proteases for digestion of pollen in honeybee midguts are endogenous, derived from pollen, or microfloral in origin[6]. That question remains unresolved.

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The effect of physostigmine on $(Na^+ + K^+)$-ATPase activity in different rat brain regions[1]

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Summary. The activity of $(Na^+ + K^+)$-ATPase and acetylcholine esterase were followed in rat brain cerebral cortex, caudate, thalamus, hippocampus and medulla after i.v. administration of physostigmine. Both enzymes were found to be inhibited in a dose-dependent manner. The most pronounced inhibition of $(Na^+ + K^+)$-ATPase was found in caudate, where the highest activity of acetylcholine esterase is found.

The relationship between neurotransmitters, including acetylcholine, and $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) has been extensively investigated in vitro[3-6]. $(Na^+ + K^+)$-ATPase has been implicated in active ion transport across the cell membrane and this enzyme is considered to be synonymous with the sodium pump[5]. Certain neurotransmitters, such as dopamine and norepinephrine, have been shown to stimulate the $(Na^+ + K^+)$-ATPase activity in vitro in a dose-dependent manner[3,5,6,8]. With respect to acetylcholine (ACh), it has been shown that in vitro this neurotransmitter is a potent inhibitor of $(Na^+ + K^+)$-ATPase[3-5]. However, to our knowledge, there are no data regarding the in vivo influence of ACh on $(Na^+ + K^+)$-ATPase activity in the brain. Inhibition of acetylcholine esterase (AChE, EC 3.1.1.8) is followed by an increased ACh level in the brain tissue[9,10]. Among the numerous AChE inhibitors, physostigmine per se does not affect $(Na^+ + K^+)$-ATPase activity in vitro[4], and does not interfere with the inhibitory effect of ACh on the $(Na^+ + K^+)$-ATPase activity[4,5]. Therefore, it appears that the effects of physostigmine on the $(Na^+ + K^+)$-ATPase activity in the brain are to be solely attributed to ACh.

Materials and methods. Male Wistar rats (200 ± 10 g b.wt) were used in the study; animals had free access to food and water ad libitum.

Physostigmine salicylate (50, 100 and 200 µg/kg b.wt) was injected in a total volume of 0.1 ml into the tail vein 30 min prior to decapitation. Animals were sacrificed between 10.00 and 11.00 h in order to avoid possible diurnal variations in ACh content. In the pilot study, doses lower than 50 µg showed no significant effects on the brain AChE, while doses higher than 200 µg caused incipient toxic effects (tremor, convulsions, incotinence).

5 brain structures (cerebral cortex, caudate, thalamus, hippocampus and medulla) were dissected out, in the cold, immediately after decapitation according to Glowinski and Iversen[11]. Tissue samples were homogenized in ice-cold 0.32 M sucrose, Triton X-100 (0.5% v/v, final concentration) was added, and after 30 min standing in the cold, the pellet was removed by centrifugation (30,000 × g, 0–4 °C, 60 min). The resulting supernatant was the enzyme source.

Acetylcholine esterase and $(Na^+ + K^+)$-ATPase activities in different rat brain regions

Brain region	$(Na^+ + K^+)$-ATPase	AChE
Cerebral cortex	168.7 ± 5.7	18.5 ± 0.4
Caudate	234.3 ± 8.0	52.4 ± 1.8
Thalamus	207.0 ± 6.5	44.7 ± 2.0
Hippocampus	232.7 ± 4.0	42.3 ± 1.9
Medulla	234.5 ± 3.6	31.2 ± 0.9

Numbers indicate the mean value ± SEM nmoles of respective substrate hydrolyzed/mg protein/min. There were 6 animals in each experimental group.

Acetylcholine esterase activity was measured at 37 °C using Ellman's[12] kinetic assay with acetylthiocholine iodide as substrate and 0.1 mM tetraisopropylpyrophosphoamide (iso-OMPA) as the inhibitor of non-specific esterases. Reaction was started by adding the substrate, after 15 min preincubation of the sample in the presence of inhibitor.

$(Na^+ + K^+ + Mg^{2+})$-ATPase was assayed at 37 °C in an assay mixture composed as follows (final concentrations): 45 mM Tris-HCl buffer, pH 7.4; 3 mM ATP; 100 mM NaCl; 20 mM KCl; 3 mM $MgCl_2$. Reaction was started by addition of ATP and stopped with 10% TCA after 10 min incubation. Liberated phosphorus was estimated according to Lowry and Lopez[13]. Mg^{2+}-ATPase activity was measured in presence of 0.5 mM ouabain and NaCl and KCl were also omitted. $(Na^+ + K^+)$-ATPase activity was calculated as the difference of the activities between $(Na^+ + K^+ + Mg^{2+})$-ATPase and Mg^{2+}-ATPase. Enzyme activities are expressed on the basis of protein[14]. Student's t-test was used for the estimation of statistical significance.

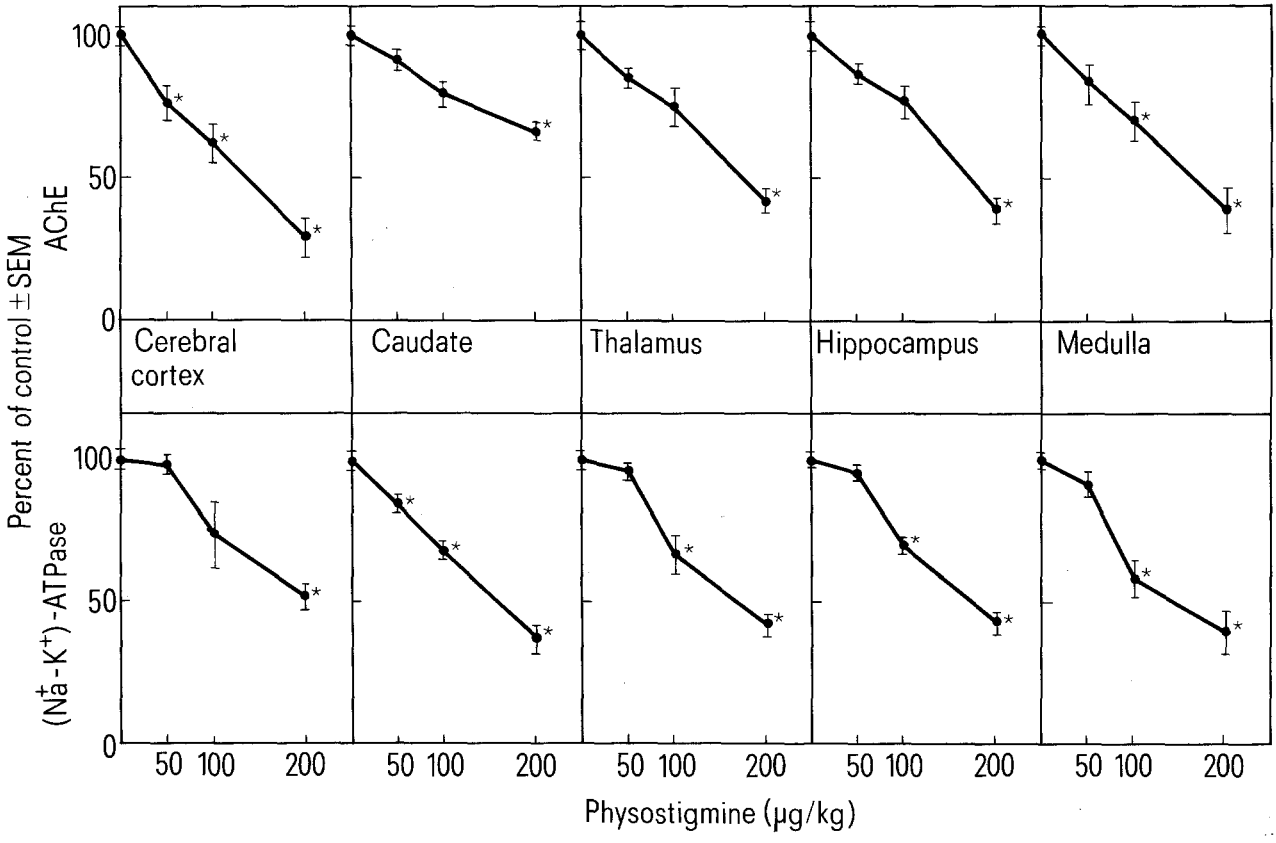
Results. The baseline AChE and $(Na^+ + K^+)$-ATPase activities are similary distributed in rat brain regions; low AChE activity in general runs parallel to a low $(Na^+ + K^+)$-ATPase activity (table). The lowest AChE activity is found in the cerebral cortex; it is significantly lower than in the other brain regions investigated ($p < 0.05$). The highest enzyme activity is found in caudate and it exceeds that in other parts of the brain. Similarly, the lowest $(Na^+ + K^+)$-ATPase activity among the brain regions investigated is found in the cerebral cortex and the difference is significant ($p < 0.005$).

Physostigmine at a dose of 50 µg/kg b.wt significantly ($p < 0.05$) inhibited AChE activity in cerebral cortex, thalamus, hippocampus and medulla, but not in caudate (figure). On the contrary, $(Na^+ + K^+)$-ATPase activity is inhibited only in caudate ($p < 0.01$). In other brain regions $(Na^+ + K^+)$-ATPase activity remained essentially unchanged (figure).

Physostigmine at a dose of 100 µg/kg b.wt significantly reduced AChE activity in all brain regions; above 200 µg/kg b.wt the drug inhibition is more profound ($p < 0.01$). Essentially, enzyme response to the increase in physostigmine concentration is linear, with a slight difference among the brain regions (figure).

It should be pointed out that the greatest AChE inhibition is observed in cerebral cortex (for about 70% of control values), while $(Na^+ + K^+)$-ATPase inhibition is smallest in this particular brain region (to about 50% of initial values). On the other hand, the structure where AChE is least inhibited, namely caudate, show the highest degree of $(Na^+ + K^+)$-ATPase activity inhibition (figure).

Discussion. $(Na^+ + K^+)$-ATPase is an enzyme present in most animal cells, it shows particularly high activity in organs which transport large amounts of sodium and potassium; most specifically, with reference to mammals, the major organs involved are the kidney and the brain[15]. Our results show a) that the amount of $(Na^+ + K^+)$-ATPase differs from region to region, and b) that the baseline AChE and $(Na^+ + K^+)$-ATPase activities are similarly distributed in rat brain regions. Both AChE and $(Na^+ + K^+)$-ATPase are localized in the plasma membranes of the cell[16,17]; also, it has been shown that the interaction between AChE and $(Na^+ + K^+)$-ATPase is accomplished in such a way that the substrate of each of the systems inhibits the function of the other system, namely ACh inhibits $(Na^+ + K^+)$-ATPase, while ATP inhibits AChE activity[4,5].



The effects of physostigmine on the activity of AChE and $(Na^+ + K^+)$-ATPase in different rat brain regions. Each point represents the mean value for 6 animals. * $p < 0.01$.

Regional distribution of AChE activity found in this study follows the regional distribution of ACh in the rat brain[18]; the structures with high AChE activity and ACh content are assumed to represent cholinergic structures[19]. We have found a slight discrepancy between the degree of AChE and $(Na^+ + K^+)$-ATPase inhibition by means of physostigmine in cerebral cortex and caudate; this is presumably due to the fact that the former brain structure represents one of the least cholinergic structures of the brain, and vice versa. Namely, $(Na^+ + K^+)$-ATPase inhibition occurred to a greater degree in the structures with high amounts of AChE activity.

There are some similarities between the effect of ACh application to the neuron membrane and the effect of the specific $(Na^+ + K^+)$-ATPase inhibitor ouabain[20] with respect to membrane permeability and sodium flux. Both ACh and ouabain application on the neuron surface resulted in increased membrane permeability, increased intracellular sodium, and depolarization[21-23]. Increased intracellular sodium concentration, caused by ACh, stimulates the sodium pump, which can be inhibited by addition of ouabain[21]. We assume that ACh, increased after inhibition of AChE, inhibits $(Na^+ + K^+)$-ATPase activity; this is followed by increased intracellular sodium, due to stimulation of passive transport, and depolarization of the postsynaptic membrane. It is worthwhile mentioning that the inhibitory action of ACh on $(Na^+ + K^+)$-ATPase in vitro is specific and observed only with synaptosomal membranes, not with other membranes of any subcellular structures[5]. Hence, having in mind the above mentioned data and the results of this study, we suggest the hypothesis that the physiological action of ACh is mediated through its inhibitory influence on postsynaptic $(Na^+ + K^+)$-ATPase.

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A possible method for improving the efficacy of dapsone

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Summary. The antileprosy drug dapsone is unable to penetrate intact *Mycobacterium leprae* in vitro, as determined by its effect on o-diphenoloxidase in the bacilli. When combined with the peptide polylysine, the sulfone drug passes through the bacterial cell membranes, and penetrates the enzyme protein, resulting in a 100% inhibition of its activity.

Dapsone, 4-4′diaminodiphenyl sulfone (DDS) is the most widely used drug in the treatment of leprosy. Even after years of treatment with dapsone, viable leprosy bacilli persist in the tissues of lepromatous cases. DDS has been reported to inhibit folic acid synthesis in other bacteria, but the mechanism of action of dapsone in leprosy remains unknown. Our studies show that the drug does not penetrate intact *M. leprae* in vitro. Making an antibacterial agent to permeate its target organisms should enhance its effectiveness. o-Diphenoloxidase is the only enzyme proven to be present in the leprosy organisms[1-3]. We have reported earlier that diethyldithiocarbamate (DDC), which penetrates the bacilli and inhibits the enzyme, is bactericidal to the leprosy organism[4-6]. However, the compound is unstable under acid conditions. In this communication, we present evidence to show that, when combined with the peptide polylysine, dapsone penetrates *M. leprae* and produces complete inhibition of its o-diphenoloxidase.

Materials and methods. There are as yet no authenticated procedures for culturing *M. leprae* in vitro. Purified suspensions of the organisms were prepared from the spleen tissue of experimentally infected armadillos[7], as described before[4]. If precautions are not taken to prevent enzyme denaturation and if the bacterial preparations are contaminated with host-tissue elements, little o-diphenoloxidase activity would be detected in them[8]. The bacilli were disrupted by ultrasonic oscillation in a Sonifier-Cell Disruptor, coupled with a Time-Temperature Control Module, which prevents heat build-up and denaturation of proteins. DDS powder was purchased from Sigma Chemical Co., polylysine HCl (mol.wt 27,000) from Miles Laboratories, and D-DOPA from ICN Nutritional Biochemicals.

DDS was suspended in water, or dissolved in 50% ethanol at a concentration equivalent to 0.02 M. Polylysine was added to the DDS suspension or solution at 5 mg/ml. o-Diphenoloxidase was assayed spectrophotometrically, as reported earlier[2,4]. The reaction mixture contained: D-DOPA (final concentration), 0.02M; DDS (with or without polylysine), 0.04M; and bacilli, 5×10^9. The volume was 3 ml, pH 6.8, temperature 37 °C and incubation time 60 min. After centrifugation of the reaction mixtures, absorbance maximum of the quinone (dopachrome) formed from DOPA was determined in the supernatant fraction. The readings were corrected for any absorbance