methamine by sulfamethoxazole for inhibition of the *E. coli* dihydrofolate reductase, a 2 m*M* concentration of sulfamethoxazole is required. High concentrations of sulfamethoxazole, for example, mean values of 1.2 m*M* free and 2.0 m*M* total, are attained in the urines of patients treated with normal doses of trimethoprim plus sulfamethoxazole (*22*), although concentrations in the serum are lower, near 0.2 m*M* (*23*).

Direct experimental confirmation of the ability of *E. coli* MB 1428 dihydrofolate reductase to bind simultaneously pyrimethamine and 4,4′-diaminodiphenylsulfone (dapsone) has been made by an ultraviolet difference spectrum titration (*24*).

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16. The inhibition constant, K_I, was calculated as follows. Enzymatic inhibition index [Table 1; see also (*4*)] set equal to $(K_I/K_M)[1 - (K_M/S)]$ where K_M, K_I, and S are the Michaelis constant for *p*-aminobenzoate ($2.5 \times 10^{-6} M$) (*5*), inhibition constant, and the *p*-aminobenzoate concentration, respectively.
17. See equation 10-2 in Webb (*10*); this equation is also discussed by Segal (*18*). To calculate the curve in Fig. 1, $\alpha = 1$, and $\beta = \gamma = \infty$.
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25. I thank K. Hoogsteen for useful discussions.

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Cholinergic Stimulation of the Rat Hypothalamus: Effects on Liver Glycogen Synthesis

Abstract. *Cholinergic stimulation of the lateral hypothalamic neurons with intrahypothalamic microinjections of acetylcholine or carbachol caused a marked increase in the content of the active form of glycogen (starch) synthase in the liver. Total activity of the enzyme (active plus inactive forms) was not increased significantly. The lowest effective dose of acetylcholine was 5×10^{-10} mole, and the optimum dose was 5×10^{-9} mole. Similar applications of other neurotransmitters, such as norepinephrine, dopamine, serotonin, and γ-aminobutyric acid, did not affect the enzyme's activity.*

The hypothalamus and its autonomic nervous connections aid in regulating enzymic activities implicated in carbohydrate metabolism in the liver, and the ventromedial (VMH) and the lateral hypothalamic nucleus (LH) act reciprocally in that regulation (*1–3*). Electrical stimulation of the VMH causes glycogenolysis and enhances gluconeogenesis in the liver to produce hyperglycemia (*1, 2*). Stimulation of the LH in rabbits and rats, however, can induce glycogenesis and inhibit gluconeogenesis in the liver (*1, 3*). In contrast to VMH stimulation, electrical stimulation of the LH generally produces effects on the metabolic alterations that are less pronounced; such stimulation sometimes results in obscure changes, presumably because neuronal organization of the LH is complex.

Since the fundamental work of Grossman (*4*), the roles of neurotransmitters in normal feeding, drinking, and thermoregulation have often been studied by their intracranial injection. However, there are few studies in which metabolic changes have been observed in the extracerebral organs after neurotransmitters have been injected into the hypothalamus. In order to gain further insight into the regulatory function of the LH in liver metabolism, we selectively stimulated neurons of the lateral hypothalamus with local applications of the different neurotransmitters and studied changes in the activity of liver glycogen synthase (E.C. 2.4.1.11), an enzyme catalyzing the rate-limiting step in glycogen biosynthesis.

Male Wistar rats weighing 250 to 300 g were housed in individual cages and given free access to laboratory chow and water. A double-walled cannula, which had outer and inner diameters of 0.65 mm and 0.30 mm, respectively, was stereotaxically implanted into the LH of each rat under pentobarbital anesthesia. The coordinates used for implantation have been described (*1*). The cannula was permanently anchored to the skull by screws and dental cement. Correct placement of the tip of the cannula was verified microscopically in brain sections made after the experiments were completed. Two weeks after the implantation, intrahypothalamic injections of different neurotransmitters were given in single doses (usually 5×10^{-8} mole) through the inner cannula. Transmitters were dissolved in 0.9 percent saline solution, and the injection volume was 1 μl. To minimize the effects of circadian variation of glycogen synthase activity in the liver, all the experiments were started at 1:00 p.m., and the animals were not fed during the 4 hours before and during the experiments. At the time indicated after administration of transmitters, the animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg per kilogram of body weight), the abdominal wall was cut open, and a portion of the liver was quickly removed and immersed in liquid nitrogen. The activity of glycogen synthase was then assayed (Table 1). Intrahypothalamic injection of norepinephrine or dopamine at the experimental dose sometimes elicited sniffing and grooming behavior of the animals, but that of serotonin or γ-aminobutyric acid (GABA) generally produced little change in behavior. Administration of acetylcholine at doses less than 1×10^{-8} mole also produced little behavioral change, but at higher doses it induced salivation, analgesia, and cataleptic reactions.

Cholinergic stimulation of the LH neurons by intrahypothalamic administration of acetylcholine plus neostigmine greatly increased the amount of the active form (I form) of liver glycogen synthase 1 hour after its administration (Table 1). Total activity (T) of the enzyme showed little change. Consequently, the I/T ratio of the enzyme increased as a result of hypothalamic stimulation with acetylcholine. It is therefore likely that the effect is due largely to conversion of the inactive form (D form) of glycogen synthase into the active form. Control injections of equally minute amounts of neostigmine alone did not have a significant effect on glycogen synthase, which suggests that cholinesterase was not significantly inhibited by these small doses; in consequence, acetyl-

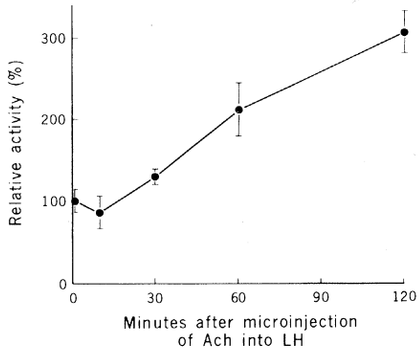

Fig. 1. Time course of changes in the activity of liver glycogen synthase-I produced by a microinjection of acetylcholine (Ach; 5×10^{-8} mole) into the lateral hypothalamic nucleus (LH). Enzyme activity in control rats (saline group) was taken as 100, and that obtained from rats with acetylcholine treatment was expressed as a percentage of the activity of the control group. Each point is the mean ± the standard error of between four and eight determinations.

choline did not accumulate in concentrations sufficient to elicit the response. The administration of carbachol (carbamylcholine chloride), a cholinergic agonist that is not hydrolzed by cholinesterase, resulted in similar but somewhat smaller effects on liver glycogen synthase than those produced by the same molecular amounts of acetylcholine.

Noradrenergic, dopaminergic, and serotonergic stimulations of the LH neurons, however, had no significant effects on glycogen synthase activity. Microinjection of GABA into the LH tended to cause decreases in the content of the I form of liver glycogen synthase and also in the I/T ratio, but these were not statistically significant ($P > .05$).

The response of glycogen synthase became evident 30 minutes after acetylcholine application ($P < .05$) (Fig. 1). The magnitude of the response increased progressively to beyond 2 hours, an approximately threefold increase.

The dose-response curve for acetylcholine-induced activation of liver glycogen synthase (Fig. 2) shows that the I form (and the I/T ratio) of the enzyme rose progressively as the dose increased, a significant increase being observed at the lowest dose of 5×10^{-10} mole ($P < .05$). The maximum effects on enzyme activity at 1 hour occurred with a dose of acetylcholine of 5×10^{-9} mole. Higher doses resulted in a submaximum increase in enzyme activity. The concentration of acetylcholine in the rat hypothalamus has been reported to be 3.4×10^{-8} mole per gram (*5*). The dose used in this study does not greatly exceed the amount in the brain. In addition, judging from the results of studies on the regional distribution of the rate-limiting enzyme for acetylcholine synthesis, that is, choline acetyltransferase (*6*), the LH seems to contain a relatively high concentration of acetylcholine, compared with most other hypothalamic nuclei.

The specific activation of liver glycogen synthase produced by the stimulation of LH with cholinergic agents suggests that, among other neurons, cholinergic neurons in the hypothalamus are particularly active in regulating glycogen biosynthesis in the liver. Electrical stimulation of the vagus nerve, parasympathetic nerves innervating the liver, causes a marked increase in the activity of glycogen synthase followed by an accumulation of glycogen in the liver (*3*).

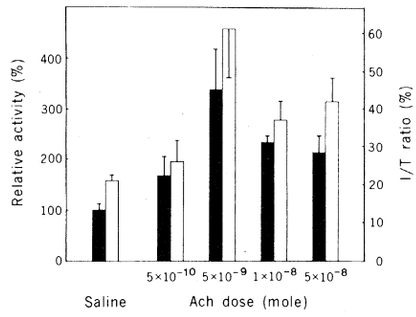

Fig. 2. Effects of various doses of acetylcholine (Ach) on liver glycogen synthase 1 hour after intrahypothalamic application. Glycogen synthase-I activity (black bars) is expressed as a percentage of that of the saline control group. The I/T ratio (white bars) denotes the value of I-activity relative to the total activity. Each column is the mean ± the standard error of between four and eight determinations.

Table 1. Effects of microinjections of the different neurotransmitters into the lateral hypothalamic nucleus on the activity of glycogen synthase in rat liver. Animals were killed 1 hour after the neurotransmitters were administered. The doses of transmitters were 5×10^{-8} mole, except for the dose of neostigmine of 2×10^{-8} mole. For the enzyme assay, a portion of the liver that had been frozen in liquid nitrogen was homogenized with five volumes of cold 50 m*M* imidazole-HCl buffer (*p*H 7.4) containing 50 m*M* NaF, 5 m*M* EDTA, and 0.25*M* sucrose. The homogenate was centrifuged for 10 minutes at 600*g* and 0°C. The supernatant was immediately analyzed for glycogen synthase activity with (for total activity) and without (for I-form activity) glucose 6-phosphate added (*8*). One unit of the enzyme is the amount which catalyzes the incorporation of 1 μmole of glucose into glycogen per minute. Results are means ± standard errors of the means for the number of animals in each group. Activities are given as 10^{-3} unit per milligram of protein. Values significantly different from the group treated with saline at $P < .05$ are in boldface type.

Neurotransmitter	*N*	Total activity (T)	I-form activity (I)	I/T ratio (%)
Saline (control)	7	4.84 ± 0.18	0.96 ± 0.11	21.0 ± 1.5
Acetylcholine + neostigmine	8	5.02 ± 0.33	**2.05 ± 0.33**	**42.1 ± 6.3**
Neostigmine	4	5.30 ± 0.42	0.96 ± 0.24	17.6 ± 3.7
Carbachol	8	4.85 ± 0.31	1.82 ± 0.21	**35.6 ± 2.5**
Serotonin	4	4.61 ± 0.39	0.94 ± 0.16	19.9 ± 2.1
Dopamine	4	5.52 ± 0.20	0.97 ± 0.12	17.4 ± 2.0
Norepinephrine	4	5.19 ± 0.60	1.30 ± 0.23	25.0 ± 3.4
γ-Aminobutyric acid	4	4.67 ± 0.09	0.73 ± 0.12	15.7 ± 2.7

Also, acetylcholine can stimulate glycogen synthase in isolated, perfused, rat liver and in isolated hepatocytes (*7*). The neural regulation of liver glycogen synthesis therefore appears to be mediated by the cholinergic system centrally as well as peripherally.

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