normally used in Krebs-Ringer bicarbonate buffer, then both acetylcholine (with eserine for protection) and norepinephrine clearly had stimulatory effects on glucose oxidation to carbon dioxide.

The variable stimulation found when eserine was added without acetylcholine, but with calcium, might have been caused by variations in the endogenous concentrations of acetylcholine in the glands.

It is not established how acetylcholine and norepinephrine exert their stimulation, but their mechanisms of action appear not to be completely independent, since their effects were not additive.

Deutsch and Raper[1] reported that acetylcholine has a stimulatory effect on respiration in the submaxillary gland of the cat, but only when a suitable carbohydrate substrate, such as glucose, is present. Our findings indicate, however, that the primary action of acetylcholine is not simply to cause an increase in glucose influx into the tissue, since insulin stimulated glucose 'uptake' more than did acetylcholine, but had little or no effect on glucose oxidation.

Acetylcholine, epinephrine, and norepinephrine each had been found previously to increase the oxidation of glucose to carbon dioxide in thyroid gland slices.[6] Subsequently, the stimulation of glucose oxidation in the thyroid by acetylcholine, as well as by menadione, thyroid-stimulating hormone, epinephrine, or serotonin, was reported to be associated with an increased tissue concentration of triphosphopyridine nucleotide (NADP) by Pastan *et al.*[7] However, even if the acetylcholine or neopinephrine were responsible for the accumulation of NADP in the submaxillary, more data are required before one can say how this phenomenon would affect the glucose metabolism.

In whatever way acetylcholine and norepinephrine effect their stimulation of glucose oxidation, calcium appears to have an important role in the mechanism. In their paper concerning the secretory response of the submaxillary gland of the cat to acetylcholine or to noradrenaline, Douglas and Poisner[3] expressed the opinion that calcium probably has its effects by influencing the membrane events associated with the extrusion of electrolytes and proteins during the process of secretion, but they also suggested the possibility that calcium may be rate-limiting in the system that supplies energy for secretion. Our findings support the latter view that calcium exerts its effect in supplying energy from glucose oxidation, but the specific step or steps in which it is involved are not yet established.

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Department of Physiology and Pharmacology,
University of Nebraska College of Medicine,
Omaha, Nebraska, U.S.A.

RANDHIR S. SANDHU
C. F. GESSERT
A. R. MCINTYRE

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The inhibition of dopamine-β-hydroxylase by tropolone and other chelating agents*

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THE ENZYME dopamine-β-hydroxylase, which catalyzes the last step in the biosynthesis of norepinephrine, has been shown to catalyze the β-hydroxylation of phenylethylamines and phenylpropylamines.[1–3] It was shown that EDTA inhibits the enyzmatic norepinephrine formation and that the

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inhibition is reversed by addition of Co^{2+} ions.[4] In the present study several other chelating agents were investigated as possible inhibitors of dopamine-β-hydroxylase. Since it was shown that the inhibition of catechol methyl transferase by tropolones is due to the chelating properties of the latter,[5] the inhibition of dopamine-β-hydroxylase by tropolones was explored.

Dopamine-β-hydroxylase was purified from beef adrenal glands according to the method of Levin *et al.*[6] The enzyme obtained at the gel eluate stage was used. The incubation mixture contained, unless otherwise stated, the following components in micromoles: potassium phosphate buffer, pH 6·4, 100; ascorbic acid, 10; fumaric acid, 10; dopamine, 4; ATP, 10; and catalase, 100 units, and 25–50 μg of purified enzyme. The mixture was incubated at 37° for 20 min and the reaction stopped by addition of 1 ml of 3% trichloroacetic acid. The amount of norepinephrine formed was determined by a modification of the fluorometric method.[7]

The effects of various chelating agents on dopamine-β-hydroxylase activity are shown in Table 1. It is evident that EDTA inhibits the enzymatic activity at a concentration of 10^{-4} M, and at a concentration of 10^{-5} M it even slightly stimulates activity. The stimulation may be due to the removal of some traces of inhibiting metals from the incubation mixture. It is noteworthy that such chelating agents as O-phenanthroline and α,α′-dipyridyl, which are known to be potent Fe^{2+} chelators, are also potent dopamine-β-hydroxylase inhibitors. The inhibition of the enzymatic activity by chelating agents can be reversed by addition of Fe^{2+} or Co^{2+} ions (Table 1). The inhibition is also reversed by 24-hr dialysis against phosphate buffer, pH 6·4, which indicates that the metalloenzyme–chelator complex is dissociable. In separate experiments the effects of Fe^{2+} and Co^{2+} ions on the enzymatic β-hydroxylase activity were investigated. At a concentration of 2×10^{-4} M, Co^{2+} ions stimulate 15–25% the enzymatic activity in the entire investigated pH range (pH 4·5–6·5). Dopamine-β-hydroxylase activity is optimal between pH 5·5 and 5. However, in presence of 2×10^{-4} M Fe^{2+}, a broad pH optimum ranging from 5·5 to 4 was observed. The stimulation of the enzymatic activity below pH 5 is due to the protection by Fe^{2+} of the enzyme from inactivation by H_2O_2. Ferrous ions act in the same manner as catalase and, especially below pH 5 where catalase activity is diminished, Fe^{2+} stimulates the enzymatic norepinephrine formation.[8]

TABLE 1. INHIBITION OF DOPAMINE-β-HYDROXYLASE WITH CHELATING AGENTS AND RECONSTITUTION OF ACTIVITY BY ADDITION OF METAL IONS

Chelating agent	% Activity*	Metal ion added†	% Activity
None	100	Fe^{2+}, 2×10^{-4} M	80
		Co^{2+}, 2×10^{-4} M	125
EDTA, 10^{-5} M	120		
EDTA, 10^{-4} M	60		
O-Phenanthroline, 10^{-5} M	30	Fe^{2+}, 2×10^{-4} M	80
		Co^{2+}, 2×10^{-4} M	125
α,α′-Dipyridyl, 10^{-5} M	40		
4-Isopropyltropolone, 10^{-5} M	25	Fe^{2+}, 2×10^{-4} M	80
		Co^{2+}, 2×10^{-4} M	120
Colchiceine, 10^{-4} M	0		
Colchicine, 10^{-4} M	100		

* The results represent averages of 3 experiments with a standard deviation from the mean of ±10%. In the control experiments 1·8 μmoles of norepinephrine was formed, which is represented as 100%.

† Enzyme was preincubated at room temperature with the inhibitor for 20 min, and then for 10 min with the metal ion.

Table 1 also shows that tropolones are potent inhibitors of dopamine-β-hydroxylase. The restoration of activity upon addition of Fe^{2+} or Co^{2+} ions, and the inhibition by colchiceine which has the acyloin structure, but not by colchicine which does not have the acyloin structure, suggests that the enzymatic inhibition by tropolones is due to their metal-chelating properties.

The effective inhibition of dopamine-β-hydroxylase by tropolones *in vitro* prompted us to study the inhibition *in vivo*. The conversion *in vivo* of dopamine to norepinephrine was not suitable for these

studies, since dopamine is a substrate of dopamine-β-hydroxylase and of catechol methyl transferase and since both of these enzymes are inhibited by tropolone. Tyramine is a substrate of dopamine-β-hydroxylase[2] and not of catechol methyl transferase and, therefore, in these studies the conversion of tyramine to the β-hydroxylated product octopamine was investigated. Rats pretreated with iproniazid were injected with tyramine-1-^{14}C (0·15 mg/rat with sp. act. of 1·8 mc/mmole), and the radioactive precursor and product were isolated from the heart and spleen tissues by previously described procedures.[9] As shown in Table 2, the dopamine-β-hydroxylase activity was not inhibited when the rats were treated with 20 mg 4-isopropyltropolone (thujaplicin)/kg. When dosage of the inhibitor was increased to 100 mg/kg, marked inhibition of tyramine to octopamine conversion was observed. Table 2 shows that the content of octopamine^{14}C is decreased approximately 50 per cent in the heart and spleen of tropolone-treated animals. The decrease in the octopamine-^{14}C content in the heart and spleen is associated with a comparable increase of the tyramine-^{14}C content, which demonstrates that the lower levels of octopamine are due to the inhibition of octopamine synthesis.

TABLE 2. TYRAMINE-^{14}C AND OCTOPAMINE-^{14}C ISOLATED FROM VARIOUS TISSUES OF 4-ISOPROPYLTROPOLONE TREATED AND CONTROL RATS

Treatment*	Heart		Spleen	
	Tyramine	Octopamine	Tyramine	Octopamine
	(cpm/5 g tissue × 10^4)			
Control	0·33 ± 0·03	1·47 ± 0·15	0·25 ± 0·02	1·1 ± 0·1
4-Isopropyltropolone, 20 mg/kg	0·37 ± 0·03	1·30 ± 0·15	0·32 ± 0·03	1·0 ± 0·1
4-Isopropyltropolone, 100 mg/kg	0·75 ± 0·07	0·72 ± 0·07	0·59 ± 0·05	0·52 ± 0·05

Results represent averages of 3 experiments in each series.

* The control animals were injected with 100 mg iproniazid/kg 19 hr prior to the injection of tyramine-^{14}C; 4-isopropyltropoline was injected 18 hr after the treatment with iproniazid and, 1 hr later tyramine-^{14}C was given. One hour after the injection of tyramine-^{14}C the animals were killed.

The inhibition of dopamine-β-hydroxylase activity by a variety of chelating agents suggests that dopamine-β-hydroxylase is a metalloenzyme. From the present results it cannot be concluded which metal is essential for the enzymatic activity. It is noteworthy that tropolone forms the most stable complex with copper.[10] However, the inhibition by tropolone could not be reversed by Cu^{2+}, and Cu^{2+} ions inhibit the enzymatic β-hydroxylation.[4] The finding that the inhibition produced by the chelating agents can be reversed by subsequent dialysis demonstrates that the metal is tightly bound to the protein. The firm bond between metal ions and protein is a characteristic for copper proteins which distinguishes them from many other metalloproteins.[11]

From the present and previous work[5, 12] it is evident that tropolone affects, *in vivo*, the metabolism and biosynthesis of catecholamines. It will be of interest to investigate whether the endogenous levels of dopamine and norepinephrine are selectively altered by tropolone. Such a specific effect should prove to be useful in the evaluation of the physiological role of these two catecholamines separately.

Neurochemistry Laboratories,
Department of Psychiatry and Neurology,
New York University Medical Center,
New York, N.Y., U.S.A.

M. GOLDSTEIN†
E. LAUBER
M. R. MCKEREGHAN

† Research Career Development Awardee of the United States Public Health Service.

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