of norepinephrine occurs because it is released and destroyed at a faster rate than it is synthesized and stored. Dopamine, on the other hand, is not affected by these particular stimuli. This differential depletion of catecholamines in response to various stimuli can perhaps be explained by the fact that they are distributed differently within the brain. Norepinephrine is primarily located in the hypothalamus and other brain stem areas that are believed to represent the central component of the sympathetic nervous system; dopamine is primarily located in the basal ganglia—areas associated with the extrapyrimidal system. Drugs that deplete both catecholamines probably do so by interfering with the storage mechanisms or binding of the amines (e.g. reserpine). However, in the present study perhaps only neurons of the central sympathetic nervous system were stimulated and thus were partially depleted of their neurotransmitter—norepinephrine. Of course neither norepinephrine nor dopamine is a proven central transmitter substance so that this explanation of the depletion is only speculative.

Aggregation enhanced the toxicity of D-amphetamine in mice, This increased toxicity was accompanied by an increased depletion of brain and heart norepinepnrine stores.[3] Although a thorough examination of the toxicity of D-amphetamine was not made in the present study, it was apparent that grid-shocked animals were especially sensitive to the actions of D-amphetamine. Like the effects of aggregation in mice, grid shock in rats increased the toxicity and norepinephrine-releasing action of D-amphetamine, suggesting that under these conditions the excessive release of norepinephrine may be playing a role in the increased toxicity of D-amphetamine.

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Stimulation by acetylcholine and norepinephrine of glucose oxidation in rat submaxillary gland slices, as influenced by calcium

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THIS report directs attention to the importance of calcium in obtaining a stimulatory effect with neurohormones in the oxidation of ^{14}C-labeled glucose by rat submaxillary slices. Although Deutsch and Raper[1] had observed that acetylcholine increased the uptake of oxygen by cat submaxillary gland slices when glucose was available as a substrate, Sandhu[2] found that neither epinephrine nor acetylcholine increased the production of $^{14}CO_2$ in rat submaxillary slices incubated in Krebs-Ringer phosphate buffer (pH 7·4) from which the calcium was omitted. Since Douglas and Poisner[3] recently reported that calcium is required for the stimulation of secretion by acetylcholine and noradrenaline in perfused submaxillary glands of the cat, the study with rat submaxillary slices *in vitro* has been repeated, with particular attention to the presence of calcium in the medium.

EXPERIMENTAL PROCEDURE

Mature male Sprague-Dawley rats were killed by a blow at the base of the skull. The submaxillary glands were quickly removed, chilled on ice, and sliced by freehand sectioning with a razor blade. The slices were mixed, then weighed and divided into aliquots of 80–100 mg each, and placed in the main chambers of 25-ml Erlenmeyer flasks. The incubation medium in each flask contained one mg D-glucose, with approximately 0·25 μc of radioactivity in the form of either glucose-1-^{14}C or glucose-6-^{14}C, and Krebs-Ringer bicarbonate buffer[4] at pH 7·4 to give a total volume of 2·0 ml. The closed flasks were immediately incubated at 37° in a Dubnoff shaker. After 45 min the reaction was stopped by injection of sulfuric acid, the $^{14}CO_2$ was trapped in Hyamine solution in the center well, and the radioactivity was measured, all essentially as described by Field *et al.*[5] Glucose disappearance was measured by the glucose oxidase method* after tissue slices were incubated for 45 min in 2 ml Krebs–Ringer bicarbonate buffer, pH 7·4, containing 3 mg glucose.

RESULTS

In general, the effect of acetylcholine (146 μM) was measured with eserine present (360 μM), but a few experiments were done with each of these agents separately. Without calcium included in the buffer (Table 1), neither the combination of acetylcholine and eserine (3 experiments), nor either agent alone (1 experiment) had any measurable effect on the oxidation of glucose to $^{14}CO_2$.

TABLE 1. EFFECTS OF ACETYLCHOLINE, ESERINE AND NOREPINEPHRINE ON THE OXIDATION OF RADIOACTIVE GLUCOSE TO $^{14}CO_2$ BY RAT SUBMAXILLARY GLAND SLICES *in vitro*, WITH CALCIUM OMITTED FROM THE INCUBATION MEDIUM*

Expt. no.	Control	ACh + eserine		Norepinephrine		Other treatment		
	(cpm)	(cpm)	(% stim)	(cpm)	(% stim)	(cpm)	(% stim)	(agents)
1	5,110	5,035	(−1·5)			4,970	(−2·7)	Eserine
	5,110					4,880	(−4·5)	ACh
2	2,984	2,973	(−0·4)					
3	3,733	3,822	(2·4)					
4	2,686			2,625	(−2·3)			
5	3,932			4,295	(9·2)			
6	4,190			4,450	(6·2)			
7	3,733			4,453	(19·3)			

* The findings, unless otherwise specified, are reported for slices of male rat submaxillary glands incubated in Krebs-Ringer bicarbonate buffer at pH 7·4, with 1 mg D-glucose and 0·25 μc glucose-1-^{14}C as substrate. The agents tested, wherever present, were included in the medium in the following concentrations: acetylcholine (ACh), 146 μM; eserine, 360 μM; norepinephrine, 295 μM.

Results are presented as counts per minute (cpm) in $^{14}CO_2$ (obtained by tissue oxidation of glucose) per 100 mg fresh tissue, after 45-min incubation at 37°. In each experiment the results with the variable being measured are compared with the results obtained for controls. Each value is the mean of two or more trials. The increases obtained in the counts per minute in the presence of various agents are also reported as the per cent stimulation above the control value, and depressions below the control are presented as negatives.

When the calcium was added in the normal amount for the Krebs-Ringer bicarbonate buffer to give a final concentration of 2·54 mM in the incubation medium, the control values for $^{14}CO_2$ produced (two experiments) did not differ essentially from those obtained when calcium was omitted. With calcium added, eserine alone had a rather variable effect: the mean stimulation over the control values was 13·4%, with a range from no effect to 30·2% (Table 2, Expts. 3–7). One experiment with acetylcholine but no eserine showed a small stimulatory effect (11·8%). The combination of acetylcholine and eserine in the presence of added calcium produced a mean stimulation of 35·0%, with a range from 17·2% to 45·0% (Table 2, 7 experiments).

Norepinephrine was tested at a concentration of 295 μM. Without calcium added, norepinephrine produced a mean stimulation of 8·1%, with a range from no effect to 19·3% (Table 1, Expts. 4–7). With calcium present, norepinephrine consistently gave a greater stimulation in oxidation of glucose

* Glucostat of Worthington Biochemical Corp., Freehold, N.J.

TABLE 2. EFFECTS OF VARIOUS AGENTS ON THE OXIDATION OF RADIOACTIVE GLUCOSE TO $^{14}CO_2$ BY RAT SUBMAXILLARY GLAND SLICES *in vitro*, WITH CALCIUM INCLUDED IN THE INCUBATION MEDIUM*

Expt. no.	Control	ACh + eserine		Norepinephrine		Other treatment		
	(cpm)	(cpm)	(% stim.)	(cpm)	(% stim.)	(cpm)	(% stim.)	(agents)
1	2,057	2,870	(39·5)					
2	2,868	3,362	(17·2)					
3	3,388	4,284	(26·4)			3,909	(15·4)	Eserine
4	5,920	8,000	(35·1)			6,620	(11·8)	ACh alone
	5,920					5,830	(−1·5)	Eserine
5	6,868					8,945	(30·2)	Eserine
6	2,989					3,365	(12·6)	Eserine
7	3,597	5,149	(43·1)	4,781	(32·9)	3,968	(10·3)	Eserine
8	4,826	6,700	(38·8)			4,340	(−10·1)	Atropine
	4,826					4,184	(−13·3)	Atr, ACh, Es
9	2,309			3,432	(48·6)			
10	3,515			4,820	(37·1)			
11	3,020			5,100	(68·9)			
12	4,299			8,972	(108·7)			
13	5,656			9,383	(65·9)			
14	5,630			7,595	(34·9)			
15†	5,345			7,551	(41·3)			
16	4,096			5,350	(30·6)	3,467	(−15·4)	Ergotamine
	4,096					4,208	(2·7)	Erg, NE
17	4,160	6,030	(45·0)	6,165	(48·2)	6,055	(45·6)	ACh, Es, NE
18	2,621					2,638	(0·6)	Insulin
19	4,611					4,994	(8·3)	Insulin

* See footnotes for Table 1. Calcium was added at a concentration of 2·54 mM. The additional agents, wherever tested as indicated, were included in the incubation medium in the following concentrations: atropine, 170 μM; ergotamine, 190 μM; insulin, one unit per 2 ml.

† Tissues from female rats.

to $^{14}CO_2$: the mean effect was 51·7%, with a range from 30·6% to 108·7% (Table 2, 10 experiments). In one experiment in which tissue from female rats was used, the results were similar to those obtained with male rat tissue (Table 2, Expt. 15).

Atropine not only completely blocked the stimulatory effect of acetylcholine and eserine, but also caused a small depression in the $^{14}CO_2$ production below that of the control (Table 2, Expt. 8). Similarly, ergotamine alone depressed the production of $^{14}CO_2$ and prevented the usual stimulation by norepinephrine (Table 2, Expt. 16).

Although the addition of insulin had a greater stimulatory effect on glucose 'uptake' (disappearance) than did acetylcholine plus eserine (68·5% vs. 15·5%), the insulin had only negligible or no stimulation on $^{14}CO_2$ production (Table 2, Expts. 18, 19).

When potassium was omitted from the incubation medium, but calcium was included, the stimulatory effect of acetylcholine and eserine in combination was 36·2%, whereas that of norepinephrine was 50·1%, both within the respective ranges with potassium present.

The $^{14}CO_2$ activity obtained in the controls from oxidation of glucose-6-^{14}C (3355 cpm/100 mg) did not differ substantially from that obtained with oxidation of glucose-1-^{14}C (3267 cpm/100 mg) in slices from the same tissue. Likewise, the stimulation of $^{14}CO_2$ production from glucose-6-^{14}C oxidation by acetylcholine and eserine in combination (45·1%), or by norepinephrine (39·1%), was within the same range as that obtained when glucose-1-^{14}C was used.

The control conversion *in vitro* of radioactive glucose to radioactive carbon dioxide with calcium present was much greater in slices of submaxillary gland (3345 cpm/100 mg) than in a homogenate of the same tissue (645 cpm/100 mg).

DISCUSSION

The findings have established several facts concerning the effects of acetylcholine and norepinephrine on one aspect of the metabolism of glucose by the rat submaxillary gland *in vitro*. With only endogenous tissue calcium present, acetylcholine was essentially without effect on the production of $^{14}CO_2$ from radioactive glucose, and stimulation of the glucose oxidation by norepinephrine was also absent or minimal. When calcium was included in the incubation medium in the concentration

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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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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