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Received June 2, 1967. P.S.E.B.M., 1967, v126.

Incorporation of C^{14}-Orotic Acid and C^{14}-Amino Acid into Pigeon Pancreas Slices Following Cholinergic Stimulation.* (32400)

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Hokin and Hokin(1) have reported that stimulation of enzyme secretion by carbamylcholine and acetylcholine is not accompanied by any increase in rate of incorporation of P^{32} into RNA in slices of pigeon pancreas incubated *in vitro*. Schucher and Hokin(2) also reported that cholinergic stimulation of enzyme secretion is not accompanied by increased enzyme synthesis. These views on the lack of an obligatory link between protein synthesis and RNA turn-over, and the lack of an effect of secretion on the rate of synthesis have been re-presented in a more recent symposium(3). In view of current theories of regulation of protein synthesis it seemed improbable that the synthesis of pancreatic enzymes proceeds at a constant rate which is independent of secretory activity. Therefore, experiments were performed on the effect of altered secretory rate on incorporation of labeled orotic acid into RNA and of labeled protein hydrolysate into protein.

Materials and methods. Preparation and incubation of tissues. Male White King pigeons (*c* 600 g) were fasted for 24 hours before sacrifice. The pancreas was removed under ether anesthesia, trimmed free of connective tissue and fat, chilled in ice and sliced with a Stadie-Riggs slicer. The thickness of the slices was approximately 0.5 mm. The entire pancreas was sliced and alternate slices were used for the experimental and control conditions. The slices were placed in 5 ml of bicarbonate-saline buffer(4) containing glucose (200 mg/100 ml), amino acid mixture (20 mg/100 ml), sodium pyruvate (0.02 M final concentration) and 20 μc orotic acid-6-C^{14} (Nuclear-Chicago, specific activity 44.5 mc/mM). These conditions are essentially similar to those of Hokin and Hokin(1,5). Acetylcholine (to a final concentration of 1 μg/ml) was added to one vessel. The incubation vessels were gassed with 95% O_2, 5% CO_2 for 90 minutes at 40°. The slices were then washed with buffer for an hour with 6 changes in a Dubnoff shaker. Some experiments were carried out with carbamylcholine given intramuscularly (0.15 mg/kg) an hour before killing of the birds. In this case a bird given an injection of 1 ml of saline served as the control. In experiments to study the incorporation of labeled amino acids, 20 μc of labeled protein hydrolysate

* Supported by Grant GB-5736 from National Science Foundation.
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(Nuclear-Chicago, specific activity 640 μc/mg) was added to the incubation media instead of the orotic acid and the unlabeled amino acid pool was omitted. In these experiments the washing in the Dubnoff shaker was omitted.

Extraction of RNA. After washing, the tissue was denatured with 0.8 M $HClO_4$ and ground in a tissue homogenizer. The tissue was then centrifuged and the supernatant discarded. This was followed by two washes with 0.4 M $HClO_4$, two washes with 95% ethanol, two with ether:ethanol(3:1, by volume) and one in anhydrous ether. The sample was then dried and extracted for an hour at 95-100° using 8 ml of 10% NaCl, 0.5 ml of saturated $NaHCO_3$ and 1-2 drops of 1 N NaOH. The sample was filtered through glass wool into excess 95% ethanol. The precipitate was collected, dried and dissolved in 2 ml of 0.2 N NaOH. Half of the sample was added to 10 ml of Bray's solution for liquid scintillation counting. The RNA content of the other half of the sample was measured on a Beckman DU spectrophotometer at 260 mμ(6).

Extraction of protein. To measure the total amount of labeled protein produced during the incubation both the incubation liquid and the tissue were extracted. The tissue was homogenized in the incubation media, centrifuged and the precipitate discarded. The protein was then precipitated with cold 10% trichloroacetic acid (TCA) and washed 4 times with 5% TCA containing 0.1 M unlabeled amino acids. The protein was then dissolved in 2 ml of 0.2 N NaOH. Half of the sample was added to a filter paper disc and treated by the method of Mans and Novelli(7) before being counted in a liquid scintillation counter. A blank for the scintillation counter was obtained by subjecting the incubation media without the tissue to the same procedure. The nitrogen content was measured colorimetrically by means of the Nessler reagent reaction(8).

Autoradiographs. After the completion of the *in vitro* incubation in the media containing labeled orotic acid slices of pancreas were washed and then fixed in formaldehyde. After embedding in paraffin and sectioning at 6 μ the sections were dipped in Ilford L4 emulsion and stored for 7-21 days before developing. Some sections were pretreated with RNase (0.2 mg/ml, 4 hours at 45°) before being set up for autoradiographs.

Results. The data in Table one show clearly that there was an increase in the amount of incorporation of orotic acid into the pigeon pancreas slices when the cholinergic stimulation was given *in vitro* at the start of incubation. Although there is considerable variation from experiment to experiment and some overlap in the values for experimental and control slices, the ratio of the paired slices always shows an increase in the case of cholinergic stimulation. The 'T' test shows that this increase is significant at the 1% level.

In another series of experiments carbamylcholine (0.15 mg/kg) was injected *i.m.* one hour before the pigeon was killed. The pancreas slices were incubated for 90 minutes in media containing labeled orotic acid. The mean value of the specific activity of the extracted RNA (counts/min/mgRNA) from 8 birds was 142 with a standard deviation of 38. Control value from 8 birds injected with 1 ml of saline was 132 with a standard deviation of 32. Thus no significant difference was obtained between experimental and control animals. In this experiment it was not possible to use paired slices. The effect of prior cholinergic stimulation *in vitro* was also studied by incubating the slices in 1 μg/ml acetylcholine before addition of the labeled orotic acid. The mean value of the specific activity of the extracted RNA (counts/min/mgRNA) for the slices from 9 birds was 119 with a standard deviation of 33. The mean value for the paired slices used as control was 106 with a standard deviation of 44. Again the difference is not significant. The values for the specific activity obtained by *in vivo* and *in vitro* cholinergic stimulation are similar.

The increase in specific activity of the protein following cholinergic stimulation is shown in Table II. The variation found was similar to that for orotic acid incorporation and the 'T' test shows that the increase is significant at the 1% level.

Autoradiographic studies showed consider-

able incorporation of labeled orotic acid into the pancreas. Penetration to the center of the slice of the labeled material was found although the concentration was higher near the edge of the slices. No definite localization was observed; both the nucleus and cytoplasm were labeled to a similar extent. The counts were reduced to background levels by pre-incubation with RNase showing the specific nature of the labeling.

Discussion. In the completely *in vitro* system where the cholinergic agent is added directly to the incubation media at start of the experiment there is a significant increase in the incorporation of orotic acid into RNA (Table I). If, however, the cholinergic agent

TABLE I. Effects of Acetylcholine on *in vitro* Incorporation of Orotic Acid into Pancreas Slices. Acetylcholine (1 μg/ml) and 20 μc C^{14}-orotic acid added to incubation media at start of experiment. Alternate slices used for experimental (acetylcholine) and control. Incubation 90 min. at 40°. Incubation media and RNA extraction procedures are given in Material and Methods section.

	Specific activity of RNA, counts/min/mg RNA		
	Control	Experimental	Ratio
Exp 1	198	629	3.2
2	129	203	1.6
3	104	192	1.8
4	126	327	2.6
5	157	358	2.2
6	235	346	1.5
7	74	182	2.4
8	105	161	1.5
9	120	500	4.2
10	296	492	1.7
11	134	261	1.9
12	135	217	1.6
13	64	118	1.8
Avg	144	307	2.1

'T' test of sample variance from zero gives value of 4.5 with 12 degrees of freedom. Significant at 1% level.

is injected into the pigeon an hour before the *in vitro* incubation this increase is not seen. The latter result is in agreement with previous work(1). These findings suggest that the changes in rate of synthesis of RNA occur early in the cycle of events that follow cholinergic stimulation. Therefore if the stimulation is given an hour before the start of incubation the changes in the rate of RNA synthesis are not seen. That the difference is due to the timing of the experiments rather than to some difference in the means of giving the cholinergic stimulation was shown by pre-incubation with acetylcholine for an hour before addition of the labeled orotic acid. In this case no increase in the amount of label incorporated was found.

TABLE II. Effect of Acetylcholine on *in vitro* Incorporation of Amino Acids into Pancreas Slices. Acetylcholine (1 μg/ml) and 20 μc C^{14} protein hydrolysate added to incubation media at start of experiment. Alternate slices used for experimental and control conditions. Incubation 90 min at 40°. Incubation media and protein extraction procedures are given in Material and Methods section.

	Specific activity of protein, counts/min/mg nitrogen		
	Control	Experimental	Ratio
Exp 1	1918	2471	1.3
2	821	1638	2.0
3	1622	2580	1.6
4	1132	1860	1.6
5	1647	1799	1.1
6	892	1704	1.9
7	2696	3880	1.4
8	2222	5516	2.5
9	1547	2906	1.9
10	1728	3384	2.0
Avg	1622	2774	1.7

'T' test of sample variance from zero gives value of 4.1 with 9 degrees of freedom. Significant at 1% level.

A significant increase in the amount of incorporation of labeled amino acids was found following cholinergic stimulation (Table II). This is in disagreement with the conclusion of Schucher and Hokin(3) that there is no increase in rate of synthesis following cholinergic stimulation. The difference between the two results appears to lie in the experimental procedure. Schucher and Hokin determined the levels of amylase, ribonuclease and lipase by specific actions of the enzymes. In our experiment the amount of synthesis of new protein was determined by the amount of incorporation of labeled amino acids during the period of incubation. It appears that the amount of new protein synthesized *in vitro* is too small, compared to the amount of enzyme present in the tissue, to be detected unless it is specifically labeled.

The mechanism by which cholinergic stimulation causes increased RNA and protein synthesis is not known. Acetylcholine could

conceivably function as a component of a primary signal for new RNA and protein biosynthesis. More likely the primary effect of the cholinergic stimulus is to increase the secretory rate of the gland. RNA and protein synthesis may then be stimulated by a signal that is generated by the release of stored protein. Experiments with the ampullate silk gland of the spider have shown that acetylcholine causes the secretion of preformed silk protein followed by increased protein synthesis(9). In this case puromycin inhibits the increased synthesis of new protein but does not affect the initial secretion.

Summary. It is found that the rate of synthesis of RNA is increased in the pigeon pancreas following cholinergic stimulation. This increase occurs in the first hour following stimulation. There is also an increase in the rate of protein synthesis following cholinergic stimulation. Both of these results, while in agreement with current theories of protein synthesis, are in disagreement with previously published work on the cholinergic stimulation of the pigeon pancreas.

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Received June 6, 1967. P.S.E.B.M., 1967, v126.

Studies of the Diabetogenic Action of Streptozotocin.* (32401)

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Streptozotocin is an antibiotic extracted from *Streptomyces acromogenes* and prepared in highly purified form. Its molecular weight is 265 with the empirical formula $C_8H_{15}N_3O_7$ containing a N-nitrosomethylamide function(1). The substance has also been shown to exert antitumoral activity in leukemia L 5178 Y, Ehrlich carcinoma and Walker 256 carcinosarcoma(2). In 1963, Rakieten *et al* (3) further reported that streptozotocin is diabetogenic, since its intravenous administration led to frank diabetes in dogs and rats. On the basis of their histologic studies, they attributed this diabetes to damage to the pancreatic B-cells. However, Evans *et al*(2), while confirming the diabetogenic action, suggested that it might not result from permanent damage or necrosis of the B-cells, but rather from an inhibition of production and/or secretion of insulin. Similarly, Arison *et al* (4) have concluded from their studies, including electron microscopy, that streptozotocin produces degranulation of B-cells without necrosis.

In all of the studies reported so far, some doubt remained as to the purity of the streptozotocin preparation, since Evans *et al*(2) stated that many preparations made available prior to 1965 were contaminated with as much as 15% of another compound, Zedalan (3 - [oximinoacetamido] acrylamide, U-15,774). Indeed, these authors suggested

* Supported by grants-in-aid of the Fonds National Suisse de la Recherche Scientifique (No. 3618 and 4237). A.E.L. is Aspirant of the Fonds National Belge de la Recherche Scientifique.