Neural Pathways Mediating the Increase in Adrenal Medullary Secretion Produced by Hypoglycemia.* (28572)

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An increase in catecholamine secretion after insulin injection has been repeatedly demonstrated in animals(1-4) and humans (5,6,7). This response is caused by hypoglycemia rather than insulin *per se,* since it is abolished when hypoglycemia is prevented by simultaneous administration of glucose(4,8).

The locus at which the hypoglycemia acts to increase catecholamine secretion is unsettled. The increase is blocked by adrenal denervation(1,8-10) indicating that a neural mechanism is involved. Brooks(11) suggested that this mechanism was spinal since he found that cats with cervical cord transections survived following the injection of doses of insulin that were regularly fatal in cats with denervated adrenals. However Duner(3) suggested that the hypothalamus was involved because he found that when he produced local hyperglycemia by injecting glucose solution directly into the hypothalamus, catecholamine secretion fell.

In the present studies it was found that the transection of the midbrain and cervical spinal cord in dogs did not eliminate the marked increase in catecholamine secretion following injection of insulin, but removal of the midthoracic portion of the spinal cord abolished it.

Materials and methods. Thirty-three male mongrel dogs weighing 8.5-18.6 kg were fasted for 24 hours, then anesthetized with pentobarbital and subjected to cannulation of the right lumboadrenal vein by the method of Hume and Nelson(12). Cannulas were also placed in the femoral artery and vein. No further surgery was performed in 5 dogs, which served as controls. In 4 animals, the brainstem was transected at the midcollicular level through a parieto-occipital craniotomy. In three animals, the spinal cord was transected at the level of the second cervical segment, and in one at the level of the seventh cervical segment. In 6 dogs the thoracic and lumbar portions of the spinal cord were removed. This was accomplished by performing laminectomies in the lower cervical and upper lumbar regions, dividing the cord, threading a heavy wire through the spinal canal between the laminectomies, attaching a gauze plug to the wire and extracting the cord segment by pulling the plug through the spinal canal. Bleeding was controlled by packing the canal with gauze. In 14 dogs, smaller segments of the spinal cord were removed.

In all dogs, 1-2 units of crystalline zinc insulin per kg of body weight were injected one to two hours after all surgical manipulations were completed. Samples of adrenal venous and peripheral arterial blood were collected 15 and 5 minutes before, and 15, 30, 60, 90 and 120 minutes after the injection. The blood collected was generally replaced by transfusion of blood from normal dogs. Blood pressure was monitored continuously using a femoral arterial cannula, a Statham Strain Gauge and a Grass model 5 polygraph. Body temperature was monitored by means of a thermistor probe in the rectum, and body temperature was maintained above 35°C by the use of external heat when necessary. At the end of the experiment the dogs were sacrificed and the level and completeness of the transections or extent of the spinal cord removal verified at autopsy.

All adrenal venous specimens were collected in iced heparinized tubes, centrifuged promptly, and the plasma frozen. The epinephrine and norepinephrine content of the plasma was subsequently determined by the method of Goldfien *et al*(6) and the output of these hormones calculated by multiplying their adrenal venous concentration by the adrenal plasma flow per minute. Arterial

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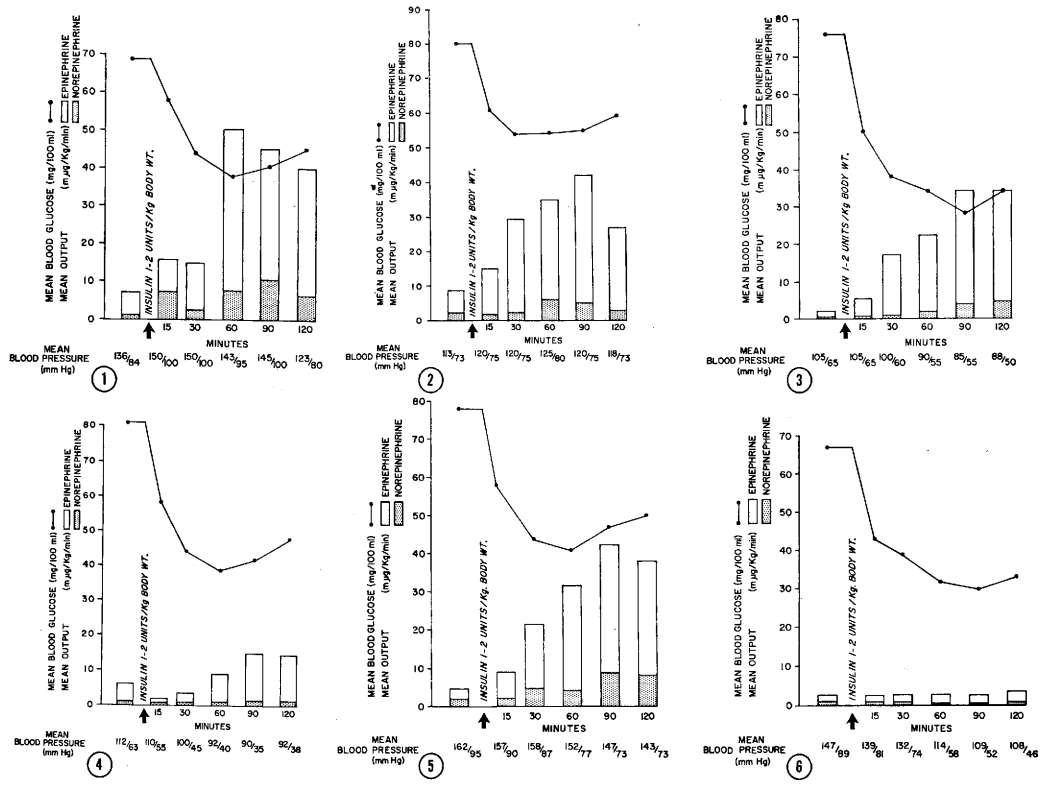
blood was collected in tubes containing sodium fluoride and its glucose concentration determined by the glucose oxidase method(13).

Results. Mean values for epinephrine and norepinephrine output, blood sugar and blood pressure in the 5 animals with intact nervous systems are shown in Fig. 1 Mean output of epinephrine in these animals increased more than 500% after injection of insulin. The increase from the mean output during the 2 control periods was statistically significant ($p < 0.05$). The much smaller increase in norepinephrine secretion was not statistically significant.

The results in the 4 dogs in which the midbrain had been transected are summarized in Fig. 2. A significant ($p < 0.025$) increase in epinephrine secretion also occurred after the injection of insulin in these animals, and the response was similar to that in the controls.

In the 4 dogs in which the spinal cord



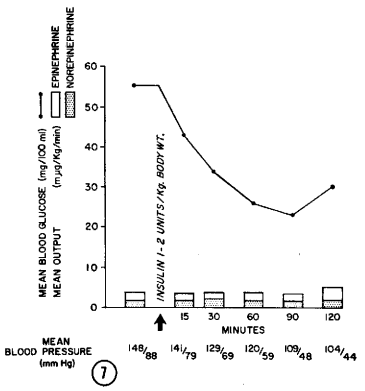


FIG. 1. Effect of hypoglycemia on adrenal medullary secretion, blood glucose, and blood pressure in 5 normal dogs.

FIG. 2. Effect of hypoglycemia in 4 dogs with transection of the midbrain at midcollicular level.

FIG. 3. Effect of hypoglycemia in 4 dogs with spinal cord transection in cervical region.

FIG. 4. Effect of hypoglycemia in 6 dogs with removal of spinal cord from C_{5-8} to L_{1-4}.

FIG. 5. Effect of hypoglycemia in 4 dogs with removal of spinal cord segments between T_{7-10} and T_{11}-L_5.

FIG. 6. Effect of hypoglycemia in 6 dogs with removal of spinal cord segments between T_{4-5} and T_8-L_7.

FIG. 7. Effect of hypoglycemia in 4 dogs with localized removal of fifth through seventh thoracic spinal cord segments.

had been transected at the cervical level, the mean epinephrine output during the control period was slightly but not significantly lower than it was in the group with intact nervous systems (Fig. 3). Following insulin administration, mean epinephrine secretion increased less rapidly than it did in the group with intact nervous systems. However, epinephrine output increased at least 600% in each of the 4 animals and the mean increase in epinephrine output in these dogs was not significantly less than it was in the dogs with intact nervous systems. All these dogs were moderately hypotensive.

The mean epinephrine and norepinephrine outputs, blood sugar levels, and blood pressure of the 6 dogs in which the spinal cord was removed are shown in Fig. 4. There was a slight rise in mean epinephrine output because in one of these dogs epinephrine output increased to 75.1 mμg/kg/min after administration of insulin, and in another it increased to 15.3 mμg/kg/min. However, in the remaining 4 dogs, it remained in the control range. Blood pressure levels in these dogs were similar to those in the group in which the spinal cord had been sectioned. There was no correlation between blood pressure level and catecholamine output.

Ten dogs in which 3 to 15 segments of the spinal cord had been removed were also studied. The results in 4 dogs in which the portion removed was below the sixth thoracic segment are summarized in Fig. 5. Epinephrine output increased 600% or more in each of these dogs, and the mean increase in epinephrine secretion was statistically significant ($p < 0.05$). On the other hand, there was little or no increase in 6 dogs in which the resections included the fourth to seventh thoracic cord segments. The results in these animals are summarized in Fig. 6.

Finally, studies were carried out in 4 dogs in which only the fifth to seventh thoracic cord segments were removed. There was no significant increase in epinephrine secretion in these animals (Fig. 7).

Discussion. The increase in total catecholamine secretion produced by hypoglycemia in this study and in previous studies in the dog (4) was due principally to an increase in secretion of epinephrine. The rise in epinephrine output was unaffected by transection of the midbrain, indicating that neural pathways from the hypothalamus are not essential for this response. Transection of the spinal cord in the cervical region also failed to abolish the response. On the other hand, the response was absent in 4 of 6 dogs from which the thoracic and lumbar portions of the spinal cord had been removed. The small increase in epinephrine secretion in one of these dogs and the large increase in the other are unexplained. However, in the subsequent experiments on the effect of partial spinal cord resection, the response to hypoglycemia was consistently blocked when the fifth to seventh thoracic spinal cord segments were removed. A normal increase in epinephrine secretion occurred in the dogs in which segments below this level were resected. The dogs in which the response was blocked were generally moderately hopotensive. However, epinephrine secretion increased in spite of equally low blood pressures in the dogs in which the spinal cord was transected. This makes it unlikely that the blockade of the response produced by resection of the thoracic spinal cord can be explained by circulatory insufficiency.

Brooks(11) found that cats in which the spinal cord had been transected in the cervical region were more resistant to insulin than cats in which the adrenals had been denervated. His work and the present studies indicate that the spinal cord is the only part of the central nervous system that is essential for the adrenal medullary response to hypoglycemia. Our experiments also indicate that the response is mediated through the midthoracic portion of the spinal cord, although it cannot be stated that this is the only part of the cord involved, since the effects of localized resection above the fourth thoracic segment were not studied. The hypoglycemia could be stimulating the thoracic spinal cord directly, or there may be a receptor outside the nervous system with afferent fibers to this portion of the cord in the dorsal roots of the spinal nerves.

Summary. Insulin-induced hypoglycemia produced a marked increase in epinephrine secretion in fasted dogs. The rise was not abolished by transection of the midbrain or

cervical spinal cord. It was completely abolished by removal of the thoracic and upper lumbar spinal cord in 4 of 6 dogs studied. It was also blocked by localized resection of the fifth to seventh thoracic segments of the spinal cord, but not by removal of more caudal segments. These data indicate that the portion of the spinal cord above the seventh thoracic segment is the only part of the central nervous system which is essential in the dog for the increase in epinephrine secretion produced by insulin-induced hypoglycemia.

1. Cannon, W. B., McIver, M. A., Bliss, S. W., *Am. J. Physiol.*, 1924, v69, 46.

2. Houssay, B. A., Molinelli, E. A., Lewis, J. T., *Rev. Assn. Med. Argent.*, 1924, v37, 486.

3. Duner, H., *Acta Physiol. Scand.*, 1954, v32, 63.

4. Goldfien, A., Zileli, M. S., Despointes, R. H., Bethune, J. E., *Endocrinology*, 1958, v62, 749.

5. Von Euler, U. S., Luft, R., *Metabolism*, 1952, v1, 528.

6. Goldfien, A., Zileli, M. S., Goodman, D., Thorn, G. W., *J. Clin. Endocrinol. and Metab.*, 1961, v21, 281.

7. Goldfien, A., Moore, R., Zileli, M. S., Havens, L. L., Boling, L., Thorn, G. W., *ibid.*, 1961, v21, 296.

8. Duner, H., *Acta Physiol. Scand.*, 1953, v28, suppl. 102, 7.

9. Britten, S. W., Geiling, E. M. K., Calvery, H. O., *Am. J. Physiol.*, 1928, v84, 141.

10. Schlossberg, T., Sawyer, M. E. M., Bixby, E. M., *ibid.*, 1933, v104, 190.

11. Brooks, C. M., *ibid.*, 1933, v106, 251.

12. Hume, D. M., Nelson, D. H., *Surg. Forum*, 1954, v5, 568.

13. Teller, J. D., *Abst. of Papers*, 130th Meeting, Am. Chem. Soc., Sept. 1956, 69c.

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Preparation of Mammalian Cell Cultures with Enzyme from *Aspergillus oryzae*. (28573)

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For quantitative studies with a subline of Earle's L strain cells adapted to grow in a chemically defined medium(1), the need arose for a suitable enzyme that would release cells from glass without injurious effects. Preliminary studies suggested that this need was satisfied by a proteolytic enzyme isolated from an *Aspergillus oryzae* fermentation and designated Aspergillin-O(2). We therefore undertook to determine whether Aspergillin-O was equally effective in preparing primary cultures and cultures from established cell lines. Our findings are reported here.

Materials and methods. Enzyme stock solutions of 1% trypsin (Difco, 1:250) and 0.1% Aspergillin-O were prepared in glucose-potassium-sodium (GKN) solution(3) and sterilized by passage through EO Ertel and UF sintered glass filters, respectively. Stocks stored at −20°C were appropriately diluted in solution GKN and adjusted to pH 7.4 with 7% sodium bicarbonate prior to use.

The Aspergillin-O was from lot #P-73. It was prepared in these laboratories by submerged fermentation of a strain of *Aspergillus oryzae* and was highly purified. The preparation used assayed 24,000 units per mg when tested by a fibrinolytic plate assay(4) which was modified for use in these laboratories. This preparation is many times more potent on a weight basis than the preparation described by Stefanini *et al.*(5).

Preparation of primary cultures. Trypsin (0.25%) or Aspergillin-O (0.01%) was used to prepare cell cultures from 10-day-old chick embryos and from rhesus and African green monkey kidneys according to the technique described by Younger(6). For cultivation, chick cell suspensions were diluted with chemically defined medium CMRL-1066*

* CMRL-1066 is identical with medium 858(7) except that the fat soluble vitamins (A, D, E, and K) and ferric nitrate included in medium 858 have been omitted from CMRL-1066; also, 5 B vitamins that were present in earlier media, but omitted from medium 858, have again been added. (G. M. Healy and R. C. Parker, unpublished experiments.)