A new technique for conditioned hypoglycemia¹

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A simple technique for obtaining repeated blood samples was developed to test its applicability to psychological experimentation. With this technique, rats which received insulin injections for 15 days were conditioned to lower their blood glucose levels to a saline injection on the 16th day. Control animals, which got saline throughout, showed no drop in blood glucose.

Investigators from two laboratories have reported that the effects of injecting insulin into a S can be conditioned. These reports are interesting in themselves, quite aside from any such practical implications as might relate to diabetes, for example, because the existence of such conditioning would challenge our understanding either of the mechanisms that control blood glucose, or of the necessary conditions for learning. Yet, these reports have escaped the attention they deserve. This may be at least partly because they are difficult to interpret. Reiss (1958), using insulin as a US, reported a conditioned comatose state in rats. His conclusions, however, were based upon the visual observations of a single observer and raise a number of issues, including questions about the physiological mechanism. Alvarez-Buylla and co-workers (Alvarez-Buylla & Carrasco-Zanini, 1960; Alvarez-Buylla, Segura, & Alvarez-Buylla, 1961; Alvarez-Buylla & Alvarez-Buylla, 1964) have reported a conditioned hypoglycemia in dogs, but a different design, including control Ss, for example, would have made these more physiological findings easier to interpret. Zakharov's failure to condition hypoglycemia in dogs (1960) may have contributed to the inattention given the positive results, but his report lacked sufficient information to interpret his negative findings.

On the basis of positive results in pilot work, we planned this experiment to test a technique developed for investigating mechanisms affecting blood glucose. We report the present study because it demonstrates the usefulness of the technique used and because it supports the earlier reports of conditioned hypoglycemia. A more extensive series of parametric studies on the conditioned hypoglycemia is now in progress.

Method

The Ss were 7 male Wistar rats about 90 days old at the beginning of the experiment. The Ss were housed individually and maintained on ad lib food (Purina rat chow) and water.

On Day 1, the distal 1 mm of each S's tail was cut off with scissors. For this procedure, and for the drawing of each blood sample, Ss were placed in a

small animal restrainer which firmly holds the body, but allows free access to the tail. After the cut was made, a blood sample was taken from the wound. In this procedure, the tail was "milked" while the drops of blood obtained were collected in a heparanized microcapillary tube (1.5 x 75 mm). ("Milking" involves gently applying pressure with the thumb and forefinger as the hand is slowly moved from the base to the tip of the tail.) Immediately, 0.05 ml of blood was removed from the tube with a volumetric micropipette and added to 1.55 ml of distilled water. Blood glucose determinations were made using the glucose oxidase method. An important feature of this technique is that the cut is made distal of the tail bone. This technique yields capillary blood, a factor which is not important here, but could be in some experiments.

After the blood samples were taken, Ss were returned to the home cage. On Days 2-5, one blood sample/day was taken from each S. These, and all subsequent samples, were obtained by removing the scab from the tip of the tail and then milking the tail as described above. Days 1-5 allowed the Ss to become accustomed to the blood drawing apparatus and procedure. After this period, the Ss were divided randomly into two groups. The four experimental Ss got insulin injections on training days, and the three control Ss got saline.

The following procedure was used throughout the training session. A rat was removed from its home cage and taken to another room where the first blood sample for the day was drawn. The S then got a subcutaneous injection of 1 unit of insulin/20 gm body weight, or an equivalent volume of physiological saline. The S was then placed into an individual, enclosed chamber (6 x 10 x 10 in.) in a room with 85 dB of white noise. Exactly 20 min after the first blood sample was taken, a second sample was taken in the blood drawing room, after which S was returned to his home cage. Thus, over a period of 40 min, two blood samples were taken from each of seven Ss, and each was given 20 min of training.

The 15 conditioning days were distributed over a period of 33 days in order to permit the physiological state of the Ss to return to the pretrial conditions after each trial. On the test day (two days after the last conditioning day), the same procedure was followed except that the experimental Ss got saline instead of insulin. Blind procedures insured that no E had information on which kind of injection was associated with any particular S or blood sample.

Results

There were no differences in blood glucose levels

Fig. 1. Mean changes in blood glucose levels in control and experimental Ss. The bars represent standard errors of the means.

between the two groups on the five preconditioning days or on the first blood samples taken on the conditioning and test days. Figure 1 gives the blood glucose changes for the two groups during the conditioning and test days. On the 15 conditioning days, injection of insulin was followed by a decrease in blood glucose of 14 to 50 mg%, but injection of saline was followed by an increase in blood glucose on 41 out of 45 determinations, the magnitude of the changes ranging from -3 to +37 mg%. On the test day, the decrease in blood glucose level in the experimental group was significantly different from the increase in the control group ($t=4.88$, $p<0.01$), and from no change in blood glucose at all ($t=3.90$, $p<0.05$). The change in blood glucose on the test day was significantly different ($t=8.34$, $p<0.01$) from the change on the last conditioning day for the experimental group, but not for the control group.

Discussion and Conclusions

These results demonstrate that this technique for collecting blood samples from rats, although not new, has greater potential for behavioral research than has been realized in the past. During pilot work, no change in the ease of obtaining blood samples occurred over three months. Furthermore, neither special skill nor manual dexterity is required to take a blood sample within 1 min.

The results of the experiment are clear in spite of the small number of Ss. The blood glucose levels of Ss that had been getting insulin decreased on a day when they received only saline. These changes were significantly different from those of the controls ($p<0.01$) and from no change at all ($p<0.05$).

These results are typical for a classical conditioning procedure: the US, insulin, elicits the UR, a decrease

in blood glucose level; the CS, a complex stimulus including white noise and many if not all of the components of the entire procedure beginning with the removal of S from his cage, leads to the CR, a change in blood glucose level in the same direction as the UR, but smaller. However, the conclusion that the phenomenon is classical conditioning is complicated by the fact that the US, insulin, elicits the UR without initiating any known reflex arcs. According to the classical view of the mechanisms controlling blood glucose level, this result is analogous to conditioning the contraction of a denervated muscle by direct, electrical stimulation of the muscle.

We believe that recent contributions to the understanding of these mechanisms (Frohman, Ezdinli, & Javid, 1967; Porte, 1967; Creutzfeldt, 1967) provide a reasonable basis for the present findings, that is consistent with classical learning theory. This explanation will appear in a longer paper (in preparation).

The 20 min interval between insulin injection and measurement of its effect on blood glucose level may not be optimum because the response to insulin is not maximum until at least 60 min after injection. It was chosen here for the sake of expedience and because pilot work showed it to be satisfactory. Work to measure the time course of the conditioned response is under way. The 20 min interval between CS and CR is in line with that of previous conditioned drug effects (Loucks, 1937).

References

- ALVAREZ-BUYLLA, R., & CARRASCO-ZANINI, J. A conditioned reflex which reproduces the hypoglycemic effect of insulin. *Acta physiol. Latinoamer.*, 1960, 10, 153-158.
- ALVAREZ-BUYLLA, R., SEGURA, E., & ALVAREZ-BUYLLA, E. Participation of the hypophysis in the conditioned reflex which reproduces the hypoglycemic effect of insulin. *Acta physiol. Latinoamer.*, 1961, 11, 113-119.
- ALVAREZ-BUYLLA, R., & ALVAREZ-BUYLLA, E. The reflex mechanism which controls the release of ACTH stored in the hypophysis. *Acta physiol. Latinoamer.*, 1964, 14, 251-254.
- CREUTZFELDT, D. Factors influencing insulin secretion. A lecture at the University of Washington, November 22, 1967.
- FROHMAN, L., EZDINLI, E., & JAVID, R. Effect of vagotomy and vagal stimulation on insulin secretion. *Diabetes*, 1967, 16, 443-448.
- LOUCKS, R. Humoral conditioning in mammals. *J. Psychol.*, 1937, 4, 295-307.
- PORTE, D., JR. Personal Communication, 1967.
- REISS, W. Conditioning of a hyperinsulin type of behavior in the white rat. *J. comp. physiol. Psychol.*, 1958, 51, 301-303.
- ZAKHAROV, S. On conditioned-reflex insulin hypoglycemia. *Zh. vyssh. nervn. Deiatel.*, 1960, 10, 280-284.

Notes

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