THE CONDITIONED HYPOGLYCEMIC RESPONSE IN RATS

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ABSTRACT

This study was conducted to test the hypothesis that stimuli preceding the injection of insulin would evoke an anticipatory conditioned hypoglycemic response.

The subjects were 11 male rats 120-150 days old, with five subjects in the experimental group and six subjects in the control group. The experimental session consisted of two phases. In Phase I a blood sample was collected from the tip of the subject's tail. It was then given an injection of .085 saline solution and put in the conditioning chamber for 15 minutes. At the end of this period, the subject was taken out, and another blood sample taken. Phase II began when the subject received 80 units of insulin per kilogram of body weight if it belonged to the experimental group, or an equivalent volume of saline if it belonged to the control group. It was subsequently put back into the chamber for an additional 20 minutes. At the end of this period a third blood sample was taken and the subject was returned to its home cage. Tests were carried out after six and 12 conditioning trials (on the seventh and 14th trials) with the same procedure as above adhered to, except that for the experimental group saline was substituted for insulin on these test trials. The Somogyi-Nelson method was used to determine blood glucose level.

The results indicated that a slight anticipatory CR appeared on the first test trial but not on the second. The typically measured conditioned hypoglycemic response, elicited when saline was substituted for insulin, was evident in the experimental group on both test trials. The Pavlovian

mechanism of inhibition of delay was used to explain the disappearance of the anticipatory CR after 12 conditioning trials.

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CHAPTER I

INTRODUCTION

HISTORICAL BACKGROUND

Since 1922, when insulin was isolated in the beta cells of the pancreas (Banting and Best, 1922), physiologists have found it difficult to agree on its basic mode of action. Since the overall effects of insulin in terms of the lowering of blood sugar, glycogen synthesis, and glucose utilization was well established, scientists have sought for many years to understand completely its mechanism of action. Alvarez-Buylla and Carrasco-Zanini (1960) cite a study by Savchenko (1939) who reported, in the Soviet literature, the existence of a neurohumoral system which is responsible for insulin induced hypoglycemia. Savchenko also reported that the system could be classically conditioned. By repeatedly pairing a conditioned stimulus (CS) with an injection of insulin, the undonditioned stimulus (UCS), this previously neutral stimulus (CS) in time comes to evoke a hypoglycemic response which closely 'resembles' that produced by insulin.

Several years following Savchenko's report, some physiologists have used the classical conditioning procedure as a means of investigating the physiology of hypoglycemia as well as to identify the reflex pathway which participates in conditioned hypoglycemia. Although physiologists have repeatedly demonstrated the conditioning of the insulin hormonal system, psychologists have practically ignored this area of investigation. As a

result, most of the literature available for review has been derived from the physiologist's laboratory. As physiologists, they were not primarily concerned with the associative aspects of learning involved in this conditioned response but rather with the physiological processes underlying this phenomenon.

As far as the available literature shows, the phenomenon of conditioned hypoglycemia was most extensively investigated by Alvarez-Buylla and his associates (Alvarez-Buylla & Carrasco-Zanini, 1960; Alvarez-Buylla, Segura & Alvarez-Buylla, 1961a; Alvarez-Buylla, Segura & Alvarez-Buylla, 1961b) in an attempt to fathom the psychological processes responsible for conditioned hypoglycemia.

Alvarez-Buylla and Carrasco-Zanini (1960) postulated that one possible mechanism which might be involved in the reduction of blood glucose could be the increased excretion of glucose via the urine. If this was the case, then hypoglycemia should always be accompanied by glycosuria. They tested this hypothesis by using the classical conditioning procedure. In a controlled experiment dogs were presented with the sound of a metronome (CS) paired with .05 U insulin per kilogram of body weight. One trial a day was administered for eight consecutive days. On the ninth day (the test day) instead of insulin an equivalent volume of saline solution was administered. When blood samples taken immediately before the injection and 15 minutes after the injection and onset of the CS were compared, a hypoglycemic response equal in magnitude to the unconditioned response was revealed, without any accompanying glycosuria.

In the same experiment, using the above conditioning procedure,

Alvarez-Buylla and Carrasco-Zanini (1960) investigated the importance of the beta cells of the pancreas in hypoglycemia. Dogs were made diabetic (sustained hyperglycemia between 220 and 330 Mg.% glucose for 2½ months) by a single injection of alloxan. After eight CS-UCS pairings, the CS plus an injection of saline was given on the ninth trial. A comparison of blood samples, taken immediately before and 15 minutes after injection, showed an average reduction of blood glucose of 233 Mg.% in these experimental animals.

Alvarez-Buylla and Carrasco-Zanini (1960) extended their investigation in a further attempt to discover if there was any mechanism in the pancreas which was active in conditioned hypoglycemia. In this experiment they surgically removed the entire pancreas from three dogs and subjected them to the conditioning procedures described previously. Once again the results revealed, as in the above studies, a conditioned hypoglycemic response which was practically equal in magnitude to the hypoglycemic response produced by insulin in the conditioning trials.

The pronounced hypoglycemic response to the conditioned stimulus in the alloxan diabetic dogs and in the animals chronically deprived of their pancreas led these investigators to postulate the existence of a potent hypoglycemic factor other than pancreatic insulin but practically as effective as the hormone in lowering blood glucose concentration. They concluded that since the reduction of blood glucose in conditioned hypoglycemia occurs without any exogenous or known endogenous supply of insulin, it appears necessary to propose a potent hypoglycemic mechanism mediated by the central nervous system (CNS) which is active in this conditioned response.

The above thesis was indirectly supported in a study by V.S. Kulilova

(1956) in which she reported that the threshold dose required to induce a perceptible reduction in blood sugar was four to five times higher for decorticated dogs than for intact animals. On the basis of her results, she proposed that insulin plays the role of a stimulator of interoceptive reflexes active in hypoglycemia.

The importance of the connections of the spinal medulla with the cerebral nervous system in the development of conditioned hypoglycemia was evaluated in an experiment by Elena R.de Alvarez-Buylla (1961). Two weeks after spinal section at the level of the sixth cervical vertebra, a group of dogs received one conditioning trial per day for eight consecutive days. The sound of a bell was the CS, and .025 U insulin per kilogram of body weight was the UCS. On the ninth day an equivalent volume of saline solution was substituted for insulin and was found to reduce blood sugar level in these dogs by an average of 24.6 Mg.%, a response not significantly different from the unconditioned response (UCR). She suggested from this observation that the afferent center and efferent mechanism of conditioned hypoglycemia must be located in the nerve paths above the level of this section.

For a long time it was suggested (Housay & Biosotti, 1931) that the hypophysis-hypothalamus complex played an integral role in the homeostasis of the glucose mechanism. Alvarez-Buylla, Segura, and Alvarez-Buylla (1961b) investigated the role of this complex in conditioned hypoglycemia by means of the classical conditioning procedure. They were seeking answers to three basic questions. First, whether .05 U insulin per kilogram of body weight, the dose used to establish a recognisable UCR and CR in normal dogs, will be

effective in establishing a comparable CR in hypophysectomized animals. Second, if this dose was ineffective, would increasing the dose enable these animals to acquire the CR. Third, whether hypophysectomy would eliminate a previously well-established conditioned hypoglycemic response.

They found that it was not possible to condition a hypoglycemic response in hypophysectomized dogs with .05 U insulin per kilogram of body weight, even after 20 conditioning trials, and in spite of an average UCR of 25 MG.% reduction in blood glucose during conditioning, and although it was possible to condition defensive and alimentary reflexes. They found, however, that only by increasing the above dose by 20 times were they able to obtain a significant CR in these animals after 10 conditioning trials. Their results also showed that only animals conditioned with very large doses of insulin (3 to 5 U per kilogram of body weight) before hypophysectomy were able to reacquire a CR similar to that of pre-hypophysectomy. Even this was only possible after the use of a minimum dose of 1 U insulin per kilogram of body weight in postoperative trials. The authors postulated on the basis of their findings that a hypoglycemic factor, probably of hypothalamic origin, is stored in the pituitary and plays an important role in hypoglycemia. They reasoned that the appearance in the blood of 'aggressive' dosages of insulin capable of producing shock, as that administered to the hypophysectomized animals, would mobilize directly the producers of the hypoglycemic factor without the necessity for the intervention of the intermediary deposit in the hypophysis.

Alvarez-Buylla, Segura and Alvarez-Buylla, (1961a) through a series of surgical operations, which involved vagotomy on dogs, cats and rabbits, and

the use of the classical conditioning procedure, have advocated that the vagus nerve forms an integral segment in the conditioned hypoglycemic reflex.

If the amount of research reported in this area by psychologists is any indication of their interest in studying the problem of neurohumoral conditioning, then it may be assumed that their interest in this problem is meagre. It is surprising that so much evidence is presented supporting the notion that such phylogenetically ancient systems, such as humoral systems, are subject to the laws of learning. Yet, psychologists have not attempted to investigate whether the general principles of Pavlovian conditioning apply in the same way to these interoceptive reflexes as they do to the familiar exteroceptive ones. If these principles do not apply, then what unique properties characterize the former.

In the available Western literature, the first psychologist to report a study using insulin as the UCS was Reiss (1958). He attempted to show that the overt behavioral manifastation of hyperinsulinism could be classically conditioned. In his procedure a series of 10 daily injections of 1 unit of insulin per 12 grams of body weight was administered to rats in the presence of a CS, an intense light which was on for 20 minutes after the injection. He defined the CS as composed of the sensory stimulation incident to passing the hypodermic needle through the skin and peritoneum, plus the intense light. The UCR was described as extreme muscular flaccidity and retardation of normal movements. On the test trials the same procedure as above was carried out as in conditioning, except that an equivalent volume of saline was substituted for insulin. It was observed on the first test

trial that the latency and length of the UCR was not significantly different from that of the CR. There was, however, a progressive extinction over five trials. Although his results were positive as far as his hypothesis was concerned, the fact that they were based on the visual observation of a single person plagued his conclusions.

Segura (1962) studied the effects of size and frequency of reinforcement on the acquisition of the conditioned hypoglycemic response. He factorially combined frequency of reinforcement and dosage for different groups of experimental animals, where low and high frequency were one trial per day and three or more trials per day, respectively, and low and high dosage were .05 U per kilogram and 1 U per kilogram of body weight, respectively. An intertrial interval of three to six hours was used for the high frequency groups in order to insure that their basal blood glucose level was similar to the pre-insulin level before administering the next insulin injection. His results indicated that there was greater acquisition of the CR in the high frequency-high dosage, and high frequency low dosage groups. However, the former group was difficult to work with as their hyperinsulin syndrome could not always be brought under control. His results were also in agreement with the general findings of classical conditioning experiments on the size and frequency of reinforcement on the acquisition of the CR. In this study also Segura (1962) investigated the possibility that the conditioned hypoglycemic response might have been attributed to pseudoconditioning, with negative results.

Balagura (1968) reported a study in which he demonstrated that another pancreatic hormone, glucagon which has the opposite effect of insulin on

blood glucose, can be used to establish a conditioned hyperglycemic response. In this study the CS was defined as the injection procedure itself, and 18 rats were assigned to four groups. The group to be conditioned, (N=5) was deprived of food for two hours at the same time each day and received .25 milligram of glucagon intraperitoneally following each deprivation period for five consecutive days. Following treatment, a series of three saline injections (one per day) was given instead of glucagon, and blood samples were taken 20 minutes after injection. To test for pseudoconditioning, another group (N=5) was submitted to the same schedule as the conditioned group but was given .25 milliliter of saline solution instead of the hormone. A third group (N=4) was run to test for possible cumulative effects of the glucagon injections. This group was deprived of food for two hours at the same time each day but was given five daily injections at random times. On the sixth day blood samples were taken without submitting these subjects to an injection treatment. Group 4 (N=4) was used to provide a measure of the size of the UCR. In one session this group was deprived of food for two hours, injected with .25 milligram of glucagon, and blood samples taken 20 minutes later.

The results of this experiment showed no cumulative effect of glucagon. On the first extinction trial hyperglycemia in the conditioned group was equal in magnitude to the hyperglycemia of the group given a single injection of glucagon, and both of these groups showed a hyperglycemia which was significantly different from that of the cumulative and pseudoconditioning groups. These two latter groups in turn were not significantly different from each other. Balagura (1968) concluded that his results provide a clear

example of classical conditioning of a neurohumoral system.

In the same report Balagura (1968) suggested that insulin induced hyperphagia could be classically conditioned. In this study six female Sherman rats were kept under a 12 hour light, 12 hour dark cycle, with continuous reinforcement for an adaptation period of two weeks. Bar-press rate was recorded for each subject during a control, an experimental, and an extinction period, arranged to coincide with the two hours in which the least amount of food was eaten. During the control sessions, the subjects were handled and sham injected with an empty syringe daily for four consecutive days. The number of bar-presses for the two hours following injection was recorded. Immediately following the control session the experimental or insulin treatment was begun. An intraperitoneal injection of .025 U of regular insulin was administered daily for 12 consecutive days at the same time of the day as the control injection had been given, and a record of bar-presses for the two hours after injection was taken. Following this treatment, four subjects were given normal saline injection for six consecutive days on the same schedule. The remaining two subjects were switched to .025 U of glucagon on the same schedule. A relatively stable pattern of eating was maintained during the control, experimental, and extinction conditions.

The results of this study showed that operant responding to obtain food increased significantly from a mean of 14 bar-presses in the control sessions to a mean of 39 bar-presses following insulin injections. During extinction the frequency of responses following the first three saline injections was not significantly different from those obtained after insulin injections. During the last three injections the rate of response rapidly returned to

the control baseline level. The rate of response of the two subjects given glucagon following insulin treatment immediately returned to the baseline level.

On the basis of his results, Balagura (1968) inferred that the link between the injection procedure and the observed significant increase in operant foodgetting response during the extinction trials was a conditioned hypoglycemia, and he further postulated that neurohumoral conditioning modulates foodgetting responses. His above inference gains support not only from all the studies reviewed so far, but also from Mayer's (1953) revised glucostatic theory of hunger.

All the studies reviewed so far, with the exception of those of Reiss (1958) and Balagura (1968), have used dogs as experimental subjects, possibly because of the greater convenience, and control achieved when these animals are used in this area of investigation. For example, the Pavlovian harness and sound proof conditioning chambers, the method of remote injection of drugs and collecting blood samples deployed when dogs are used, all contribute to the realization of better control in the experiment.

Woods, Makous, and Hutton (1968) suggested a technique which, although not clearly as efficient as the above used with dogs, should be of interest to psychologists whose budgets generally do not afford them to work with dogs. The technique suggested consists mainly of a method for collecting blood samples from rats with relatively little stress to the animal, a factor which is particularly important in studying the conditioned hypoglycemic response. This technique demands very little skill on the part of the experimenter and takes less than one minute to execute. It involves

slicing off the distal 1 millimeter of the rat's tail and collecting .05 milliliter of blood by gently 'milking' the tail. The blood sample is collected by means of a micropipette and analysed by the glucose oxidase method.

In another report, Woods, Makous, and Hutton (1969) using the above mentioned blood drawing technique, described a series of experiments in which they studied the temporal parameters of conditioned hypoglycemia in rats. In these experiments they used a white noise of 82 decibels plus the injection procedure as the CS. Their general procedure was as follows: two days before conditioning, about 1 millimeter of the tip of the tail of each subject was cut off. On all other trials, each subject was transferred directly from his home cage to a restrainer, where a blood sample was taken by removal of the scab of the wound. After this, the subject was given a subcutaneous injection of either insulin (1 unit per 20 grams of body weight), or an equivalent volume of saline, then put in individual enclosed chambers for 20 minutes (except in the time course study). At the end of this period, the subject was returned to the restraining apparatus, where a second blood sample was taken, and then finally returned to its home cage. Subjects were treated on alternate days.

The first experiment was designed to study the effects of varying the number of trials on the magnitude of the conditioned hypoglycemic response, with an aim of determining the optimum number of trials for conditioning. This experiment consisted of two studies with 13 subjects each. Different groups receiving insulin, received either two, four, or six trials; or five, seven, or nine trials. Control animals for each study were given

equivalent volume of saline. All subjects in the insulin groups were given three daily extinction trials in which saline was given instead of insulin. The results of the study showed that blood glucose changes in subjects receiving two, four, five, or nine conditioning trials were not significantly different from their controls, on the days they received saline instead of insulin. The blood glucose changes observed in groups receiving six or seven conditioning trials however, were significantly different from their controls on the first extinction trial at least. Woods et. al. (1969) concluded from these results that the optimum number of conditioning trials necessary for obtaining a significant conditioned hypoglycemic response in rats is six.

The second experiment was designed to test the effect of an olfactory cue on the acquisition of the conditioned hypoglycemic response. The odor was introduced within the chambers where the subjects were kept between blood drawings by applying Mentholatum to a gauze pack taped to the inside of the chambers out of the subject's reach. Groups of suspects receiving insulin were given one, two, four or six conditioning trials, and in another group, insulin subjects were given five, six, seven, or nine trials. All groups that were significantly different from their respective control groups on the first extinction trial were given at least two more such trials. The results show that all differences on the first day of extinction were statistically significant except for those of the subjects receiving only one conditioning trial. If the results of this experiment were compared with those of the preceding experiment, it will be revealed that the process of conditioning is in some fundamental way more rapid with the

menthol cue than without it. Another comparison between this experiment and the former indicates that, whereas the results of the former experiment suggest that the magnitude of the conditioned hypoglycemic response deteriorates after six or seven conditioning trials, the results of the latter experiment showed that even after nine conditioning trials, the magnitude of the CR was still significantly different from that of the appropriate control group when an olfactory cue was used during conditioning.

The third experiment was designed to investigate the magnitude of the CR and UCR following different intervals of time after injection of insulin and was aimed at determining the optimal interval between injection and the drawing of the second blood sample. The interval of time between the injection and the second blood drawing was varied in geometric progression from five to 160 minutes. Half of the subjects were given a menthol cue and half were not. Half of each of these groups received insulin and half received saline on the conditioning trials. Subjects with the menthol cue were given five conditioning trials, and those without were given nine conditioning trials. All subjects received saline on the extinction trials. The results of this experiment showed that subjects in the saline control groups experienced a slight increase in blood glucose that returned to baseline within 160 minutes. The insulin injection caused a decrease in blood glucose that peaked between 80 and 160 minutes after injection. Menthol had no statistically significant effect on the UCR either to the saline or to insulin. The CRs for both insulin groups followed the UCR very closely for the first 10 minutes. However, the CR of the subjects with the menthol cue followed the UCR for 10 minutes longer than that

of the subjects without the menthol cue, although the difference lacked statistical significance. The CRs peaked at about 20 minutes after injection and returned to the level of the control groups within 80 minutes.

Statement of the Problem

A review of the available literature, strongly suggests that conditioned hypoglycemia is a demonstration of Pavlovian conditioning of a neurohumoral system. This phenomenon has been repeatedly demonstrated. Unlike the investigation of most other classically conditioned response (eg., GSR, conditioned eyelid response and conditioned salivary response etc.). The investigation of classical conditioning of hypoglycemia has not gone beyond the mere demonstration of it.

The absence of such research may be attributable to reasons implied earlier: the apparent lack of enthusiasm of psychologists in this area of investigation, combined with the physiologist's interest in this conditioned response merely as a tool to provide information of physiological processes underlying the phenomenon.

In view of the present state of psychological research in this area, the present study was conducted not only to demonstrate conditioned hypoglycemia but also to investigate the possibility of establishing a conditioned anticipatory response in the insulin hormonal system similar to that established in the familiar exteroceptive conditioning situation.

If the general characteristics of the conditioned hypoglycemic response apart from its long latency (an important consideration in the present study) are similar to those of exteroceptive conditioned responses, then it

would be predicted that remote stimuli, repeatedly paired with the UCS would elicit an anticipatory CR (Pavlov, p.40, 1927). Specifically in the present study the anticipatory CR in the experimental animals was expected to be manifested on the second blood sample and to be elicited by the whole complex of stimuli preceding this blood sample starting with the removal of the subject from its cage.

The present study is comparable with an interoceptive conditioning experiment by Krylov, reported by Pavlov (p. 35, 1927). Krylov demonstrated that a series of previously neutral stimuli eg., the sight of the experimenter and the sight of a syringe, when repeatedly paired with an injection of apomorphine, in time, come to evoke interoceptive reflexes equal in strength to those evoked by the morphine itself.

CHAPTER II

THE INVESTIGATION

Subjects

The subjects were 11 male Holtzman Albino rats, 120-150 days old at the beginning of the experiment. They were individually housed, and kept on an ad lib diet of Purina Chow and water.

Apparatus

The conditioning chambers consisted of two wooden chambers, 10 inches long by 10 inches wide by 14 inches deep, painted black on the inside. They were fitted with milk glass tops and grid floors two inches from the base. The two chambers were connected by a strip of plywood to insure the same distance and position from each other throughout the experiment. A 100 watt, 120 volt incandescent lamp, mounted on the strip of plywood midway between the chambers and 10 inches from their tops, provided the only illumination in the room during the experimental session. The noise level in the room with the air conditioning fan on was 74 decibels, as measured with a Brüel and Kjaer sound level meter (type 4131/32). A 1-c.c. tuberculin syringe, with a $\frac{1}{2}$ inch #25 gauge needle, was used for injecting the subjects. Disposable blood collecting and diluting pippetes and vials (#2742 Becton, Dickinson & Co.) containing a 1.3 milliliter heparinized diluent were used to collect and store (under refrigeration) blood samples.

Procedure

On Days 1 and 2 each animal was handled for five minutes and then re-

turned to its home cage. The subjects were then randomly assigned to one of two groups, with five animals in the experimental group and three in the control group. Three additional control animals were run two weeks later. Each animal was assigned to a particular chamber for the duration of the experiment.

On Day 3 conditioning began and the following procedure was carried out throughout the experiment. Each animal was weighed, then taken to the experimental room where it was firmly wrapped in a piece of black cloth which was secured with safety pins allowing free access to the tail. Then the distal 1 millimeter of the tail was cut off with a sharp razor blade. After the cut was made, a kind of sham blood sample was taken which consisted of 'milking' four drops of blood unto a clean piece of tissue paper. '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.

After the above procedure the animal was given an intraperitoneal injection of 1 c.c. of .085 saline solution per kilogram of body weight. Control tests (Woods et. al., 1969) have shown that this concentration of saline has no detectible difference in effect as compared to the insulin vehicle. After this injection, the animal was put into the conditioning chamber for 15 minutes. At the end of this period, it was taken out of the chamber and a second sham blood sample was taken. The subjects in the experimental group were then injected with 80 units of regular insulin (Connaught Medical Research Laboratories) per kilogram of body weight, while those in the control group, with an equivalent volume of saline. This dose was used as it was found through pilot studies to reduce blood glucose

level by 25Mg.% - 35Mg.% 20 minutes after insulin injection. The subjects were then returned to the chamber for a further 20 minutes. At the end of this period a third sham blood sample was taken. These and all subsequent samples were obtained by removal of the blood clot or scab from the wound. In order to permit the physiological state of the animal to return to its pre-trial, the animals were run every other day. Thus, the CS consisted of all the stimuli associated with the events occurring prior to the hypothesised triggering of hypoglycemia by insulin (Alvarez-Buylla and Carrasco-Zanini, 1960). In the present study, these events, considered in a backward temporal order were: insulin injection, drawing of the second blood sample, exposure to the chamber cues, saline injection, and drawing of the first blood sample.

On testing days 9 and 16 (Trials 7 and 14) the same procedure as above was followed, except that the experimental animals received saline on their second injection instead of insulin and actual blood samples were collected instead of sham samples. The blood was drawn into a 20ul microcapillary tube by the capillary action of the tube. Immediately after removal of a blood sample, it was added to the premixed heparinized diluent. It took less than one minute to obtain a fully prepared blood sample. Blood glucose determination was made by the Somogyi-Nelson method (Grant and Moorhouse, 1966).

CHAPTER III

RESULTS

The results of the experiment are shown in Figure 1. The median change in blood glucose level (Mg.%) is plotted for Trial 7 and Trial 14 in the left panel and right panel, respectively. At the zero point on the abscissa an arbitrary zero change in glucose level is indicated. The data points at 15 minutes are medians of the first sample (taken at the beginning of the session) subtracted from the second sample (taken at 15 minutes), while plotted at 35 minutes are medians of the second sample subtracted from the third sample (taken at 35 minutes). These changes in glucose level were analysed by the Mann-Whitney U test for very small samples (Siegel, 1956). Although the UCR to insulin on conditioning days is not shown in the present study, pilot studies have indicated quite definitively that the daily dosage used here produced a reliable decrease in blood glucose over several days of conditioning of 25Mg.% - 35Mg.%.

Since a comparison of the blood glucose level in the control and the experimental groups on the first blood sample taken on the two test days yielded no significant difference between groups ($U=14$, $P=.465$ on both occasions), the possibility that the hypoglycemic response might have been caused by a cumulative effect of insulin should be dismissed.

Table 1 presents the change in blood glucose from the first blood sample to the second blood sample which was taken 15 minutes later. Inspection of this table reveals that the median change (zero) in blood glucose level in the experimental group was significantly different from

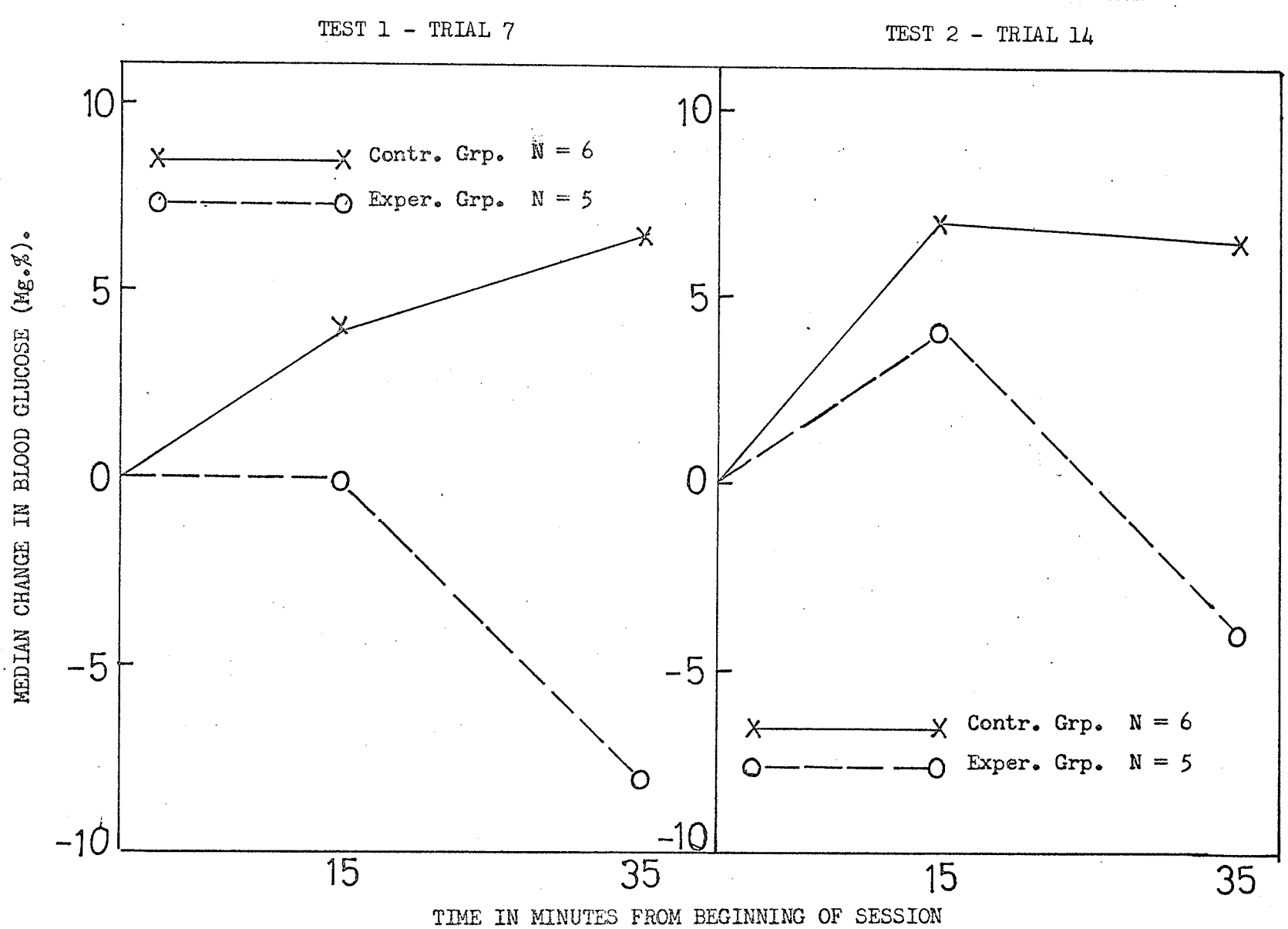

Fig. 1. Change in blood glucose level from the first sample (Time = 0) to the second sample, (15 minutes) and from the second sample to the third sample (35 minutes).

TABLE 1

Values of Mg.% glucose in the Mann-Whitney U Test for Test 1 and Test 2: difference scores between first and second samples, median difference scores, values of U, and the associated P-values.

TEST 1

EXPERIMENTAL GRP.

5 OBSERVATIONS

8.00 0.0 0.0 0.0 -5.00 Median = 0

CONTROL GRP.

6 OBSERVATIONS

5.00 3.00 8.00 2.00 0.00 5.00 Median = 4.0

*U = 5

*P = .041

TEST 2

EXPERIMENTAL GRP.

5 OBSERVATIONS

3.00 14.00 0.0 30.00 4.00 Median = 4.0

CONTROL GRP.

6 OBSERVATIONS

8.00 -14.00 8.00 6.00 8.00 4.00 Median = 7.0

U = 15

P = .535

the median change (an increase) in blood glucose concentration in the control group after six conditioning trials (U=5, P=.041). After 12 conditioning trials, however, as the lower portion of Table 1 shows, there was no significant difference between the experimental and control groups in their blood glucose changes from Sample 1 to Sample 2 (U=15, P=.535). These results suggest that the apparently weak, but statistically significant anticipatory hypoglycemic response, became inhibited on the second test trial. As depicted in Figure 1, the glycemic response of the experimental animals to saline injection closely followed that of the control group.

Table 2 shows the change in blood glucose level from the second blood sample to the third sample, taken 20 minutes later, for both groups. After six conditioning trials, the decrease in blood glucose level from Sample 2 to Sample 3 in the experimental group was significantly different from the increase in blood glucose level for the same period in the control group (U=0, P=.002). Making the same comparison after a further six conditioning trials reveals a significant difference in blood glucose levels between the experimental and control group (U=1, P=.004). These results, together with those mentioned in the preceding paragraph, indicate that although there was a 'slight' anticipatory hypoglycemia after the first six conditioning trials, the conditioned hypoglycemic response came to be associated almost exclusively with the second injection procedure as is clearly shown in Figure 1.

The overall trend in blood glucose change for each test session, is depicted in Table 3. These difference scores were derived from the change in blood glucose from Sample 1 to Sample 3. Actually, they are the algebraic

TABLE 2

Values of Mg.% glucose used in the Mann-Whitney U Test for Test 1 and Test 2: difference scores between second and third samples, values of U, and the associated P-values.

TEST 1

EXPERIMENTAL GRP.

5 OBSERVATIONS

-8.00 -19.00 0.0 -10.00 0.0 Median = -8.0

CONTROL GRP.

6 OBSERVATIONS

3.00 8.00 5.00 17.00 11.00 0.0 Median = 6.5

**U = 0

**P = .002

TEST 2

EXPERIMENTAL GRP.

5 OBSERVATIONS

-15.00 -4.00 -3.00 -24.00 -4.00 Median = -4.0

CONTROL GRP.

6 OBSERVATIONS

10.00 10.00 8.00 5.00 -4.00 0.0 Median = 6.5

**U = 1

**P = .004

TABLE 3

Values of Mg.% glucose used in the Mann-Whitney U Test for Test 1 and Test 2: difference scores between first and third samples, median difference scores, values of U, and the associated P-values.

TEST 1

EXPERIMENTAL GRP.

5 OBSERVATIONS

0.0 -19.00 0.0 -10.00 -5.00 Median = -5.0

CONTROL GRP.

6 OBSERVATIONS

8.00 11.00 13.00 19.00 11.00 5.00 Median = 11.0

**U = 0.0

**P = .002

TEST 2

EXPERIMENTAL GRP.

5 OBSERVATIONS

-12.00 10.00 -3.00 6.00 0.0 Median = 0.0

CONTROL GRP.

6 OBSERVATIONS

18.00 -2.00 16.00 11.00 4.00 4.00 Median = 8.5

U = 7

P = .089

sum of the difference scores between Sample 1 and Sample 2, and between Samples 2 and 3. After six conditioning trials, the decrease in blood glucose from Sample 1 to Sample 3 in the experimental group, was significantly different from the overall increase in blood glucose from Sample 1 to Sample 3 in the control group, ($U=0$, $P=.002$). After 12 conditioning trials, however, blood glucose levels in the experimental and control groups were not significantly different from each other over the same period ($U=7$, $P=.089$). An examination of Figure 1 will help to explain this situation. As shown in Figure 1, the median blood glucose change in the experimental group from Sample 1 to Sample 2 was zero after six conditioning trials. As a result there was no hyperglycemia after the first injection to subtract from the distinct hypoglycemia after the second injection, as was manifested on the second test trial.

CHAPTER IV

DISCUSSION

All investigators who have demonstrated conditioned hypoglycemia have interpreted their findings as a demonstration of Pavlovian conditioning of a neurohumoral sustem. Although the investigation of a number of Pavlovian conditioning phenomena has not as yet been attempted in this area, some of the fundamental requirements of Pavlovian conditioning have been satisfied by the present study.

In accordance with the above considerations it was hypothesised at the outset of the study that by repeatedly presenting the subjects during the conditioning trials with cues, which were expected to become associated with the UCR, for an interval of time preceding the UCS, the subjects would develop an 'anticipation' of the UCS and display an anticipatory CR on the test trials. This reasoning was based on the principles of the development of conditioned reflexes as discussed by Pavlov (Lecture 111, 1927). In this lecture Pavlov described an experiment by Dr. Krylov of the Tashkent Biological Laboratory in which he used apomorphine as a UCS in a classical conditioning study. He described the experiment in the following manner:

> It is well known that the first effect of a hypodermic injection of morphine is to produce nausea with profuse secretion of saliva followed by vomiting ... Dr. Krylov however, observed when the injection of morphine was repeated regularly, that after five or six days the preliminaries of the injection were themselves, sufficient to produce all

> of these symptoms... Under these circumstances the symptoms are now the effect, not the morphine acting through the blood stream directly on the vomiting centre, but all the external stimuli which previously had preceded the injection of morphine. The connection between the morphine itself and the various signals may in this instance be very remote, and in the most striking cases all the symptoms could be produced by the dogs simply seeing the experimenter. Where such a stimulus was insufficient it was necessary to open the box containing the syringe, to crop the fur over a small area of skin and wipe with alcohol, and perhaps even to inject some harmless fluid before the symptoms could be obtained ... (Pavlov, 1927, p. 35).

Inspection of Figure 1 shows that the results of this investigation partially support the hypothesis in question. On the first test trial the glycemic response of the experimental group (no change) to the first injection plus the 15-minute pre-exposure to the cues of the conditioning chamber, was significantly different from the glycemic response of the control group to the same stimuli. This indicates that the stimuli preceding the injection procedure of insulin evoked a slight, though statistically significant anticipatory CR in the experimental animals. On the second test trial, however, the results show that there was no appreciable difference in blood glucose level between the experimental and control groups under the same circumstances as above. It appears that the concept of the development of inhibition of delay as advanced by Pavlov (1927) may explain the absence of an anticipatory CR on the second test trial. According to Pavlov (1927), in the early stages of conditioning an unreinforced stimulus preceding a

reinforced conditioned stimulus, comes to elicit a fraction of the CR. However, as this situation is repeated this 'anticipatory' response decreases until its value becomes zero. Thus, in addition to the apparent demonstration of an anticipatory response, it seems that the classical conditioning phenomenon of inhibition of delay was an important finding of the present study. This inhibition is not an independent feature, however, but the product of the dynamic interaction of the above 'sub features', i.e., anticipation of the UCS and the development of inhibition to an unreinforced stimulus. It is possible, of course, that random variation in responses which may occur in a relatively small group of subjects may also have affected these results. The fact that a definitive decrease in blood sugar level became manifested exclusively during the second interval on both test trials, indicates that the experimental animals were perhaps capable of discriminating between the unreinforced stimuli, presented before the second injection, and those they received after this point. Whether this discrimination is based on temporal stimuli or upon external stimuli is unclear. Perhaps this development of differentiation may be attributed to the very different physiological responses to saline and to insulin. This suggestion gains support from the fact that previous studies (Woods, et. al., 1968; 1969) showed that the response by rats to injection of saline is a slight hyperglycemia. Also in the present study, Pavlov's (1927) proposition, that the duration of the time interval can acquire properties of a conditioned stimulus, cannot be discounted. This situation is not difficult to envision, as it may be recalled that each experimental session was composed of two distinct intervals.

A very noticeable feature of the results of the present study, and others using rats as subjects (Woods, et. al., 1968 & 1969), is the small size of the CR as compared with those studies which used dogs as experimental subjects. As implied earlier, this difference in the magnitude of the conditioned hypoglycemic response between rats and dogs may be attributable to the much greater control achieved when dogs are used as experimental subjects in neurohumoral conditioning. Perhaps the hypothesis of the present study could have been more reliably tested if dogs were used instead of rats. In view of the difference in the degree of control achieved, and the difference in the magnitude of the conditioned hypoglycemic response between these species, this is in fact one of those cases in which one can legitimately argue that, the use of a larger and more convenient species to work with e.g., the dog, is a much more reliable way of obtaining accurate information on a number of conditioning phenomena in this area of investigation. This demand may be interpreted as being analagous to a geneticist's use of Drosophilla in genetic research or a neurologist's use of a neuron of the Giant Squid in neurological research.

It is conceivable that, studies like the present investigation, may be extended to reveal that many types of operant behavior may have foundation on a classical conditioned response. For example Balagura (1968) has suggested that his demonstration of conditioned instrumental food getting response might have been elicited by a conditioned hypoglycemia. Bykov (1956) mentioned an experiment in which he demonstrated a conditioned increased metabolic rate by repeatedly pairing the injection of the hormone thyroxine with a light as the CS, and on the test trial substituting saline

for thyroxine. Experiments of this nature could be also used to demonstrate that in many instances of operant conditioning there is an underlying classically conditioned response, as increased metabolic rate, usually is accompanied by increased activity. As more and more of these 'internal' physiological responses become amenable to precise measurement as in the case of conditioned hypoglycemia, the result may produce a less dichotomous state between operant conditioning and classical conditioning. And the operant conditioner may come to accept the fact that the organism is not that 'empty' after all.

On the basis of the results, this investigation has demonstrated that, an 'anticipatory' conditioned hypoglycemic response can occur in the initial stage of conditioning, but that this response changes into an inhibitory response after further conditioning. The results also show the establishment of differentiation in this type of neurohumoral conditioning.

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