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The Effect of Neurosecretions from the Cerebral Ganglia on Blood Glucose Levels in Lumbricus Terrestris

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THE EFFECT OF NEUROSECRETIONS FROM THE CEREBRAL GANGLIA ON BLOOD GLUCOSE LEVELS IN *LUMBRICUS TERRESTRIS*

by

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Joan VanderPol Craig

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INTRODUCTION

The presence of neurosecretory cells in the suprapharyngeal ganglia of Lumbricus terrestris has been well established (B. and E. Scharrer, 1937). Since these cells may well be the only source of hormones in this organism (B. Scharrer, 1959), the question arises as to their role in the animal's regulation of metabolism The particular aspect of this problem investigated here concerns the effect of these neurosecretory substances on the blood glucose levels in L. terrestris.

Preliminary studies by this investigator on glucose levels in earthworms revealed that blood samples of approximately 15 microliters could be obtained from each animal and the glucose levels were very low. This made it necessary to develop an accurate method for determining micro amounts of glucose in ultra-micro samples of blood. It was decided to use a modification of the orthodianisidine enzymatic method (Glucostat, Worthington Biochemical Corp., Freehold, N. J.) due to its specificity in measuring only beta-D-glucose and not other reducing substances. This method could prove useful to other investigators in studying glucose levels in animals, especially where blood samples of several animals could be pooled and then analyzed.

The second aspect of this study was establishing glucose levels in earthworms after specific treatment of varying environmental conditions, worms that had been slit and decerebrated worms over a

period of days.

Thirdly, the effect of the neurosecretory substances from the suprapharyngeal ganglia on glucose levels in earthworms was further studied by decerebrating worms, injecting homogenates of cerebral ganglia and then measuring the glucose levels.

Literature Review

An interesting and important development in physiology during the last thirty years has been that certain nerve cells have been found to produce secretions which act as hormones. According to Prosser and Brown (1961), tissue of the central nervous system originally possessing only a conductile function has gone through an intermediate stage in which cells display their secretory function and still maintain the conductile ability of nerve cells, to a condition where the cells have become specialized to act only in the endocrine capacity. Carlisle and Knowles (1959) suggested that neurosecretory neurons and ordinary neurons differ principally in that the neurosecretory neurons do not form synapses with other neurons nor do they innervate effector organs.

Based on ultrastructural evidence, it is believed that the neurosecretory material may be synthetized in the endoplasmic reticulum, as is the case in other protein secreting cells. It is then passed to the Golgi apparatus, where it is accumulated in vessicles and surrounded by a membrane furnished by the Golgi apparatus, and is then pinched off as a vessicle (E. Scharrer and S. Brown, 1961;

Bullock and Horridge, 1965).

Welsh (1959) has distinguished neurosecretory substances from neurohumors by observing that the neurosecretory substances serve mostly as long-range, long-acting, coordinating agents. They are released into the circulation from storage centers and are more stable than neurohumors, which are short-range, brief-acting agents. Neurosecretions can either act directly upon other endocrine structures, stimulating the latter to secrete their own hormones, or they can be released directly into the blood stream and transported to act upon some distant organ (Prosser and Brown, 1961).

The presence of neurosecretory cells is characteristic of almost all metazoan invertebrates (Gorbman and Bern, 1962). All invertebrate phyla possessing a centralized nervous system, except coelenterates and ctenophorates, have been examined for the presence of neurosecretory cells and in almost all cases, such cells are present (Bullock and Horridge, 1965). In annelids, neurosecretory cells terminate in a neurohemal region in the brain (ibid.). In these less specialized invertebrates, these cells may well be the only sources of hormones (B. Scharrer, 1959).

In general, neurosecretory systems seem to be fundamental to endocrine control throughout the animal kingdom, exerting control over major physiological processes such as growth and reproduction (Bullock and Horridge, 1965). Many neurohormone systems are now known. Examples are the X-organ--sinus gland complex in the eyestalk and brain-secretory cell-postcommissural organ and pericardial

organ-anterior ramifications system of crustaceans, the pars intercerebralis-corpora cardiaca complex of insects and the hypothalamic-hypophyseal complex of vertebrates (ibid.).

Pioneer work was begun in the field of invertebrate neurosecretion in 1912 by Kopeć (1912, 1917, 1922), who studied pupation in Lymantria. Neurosecretory cells were first described by Speidel (1919), based on studies of the caudal spinal cord of elasmobranchs. B. and E. Scharrer wrote what was known of the field of neurosecretion in 1937 and since then have carried out an extensive study of the field. The analogy of neuro-endocrine systems was recognized in invertebrates and comparison of these systems with vertebrate systems was made by E. Scharrer (1959) and Hanström (1953, 1957).

Although neurosecretory cells have been well described in polychaetes and leeches (Bullock and Horridge, 1965), the following description will be confined to that of the neurosecretory cells in oligochaetes. Schmid (1947) confirmed the existence of neurosecretory cells in L. terrestris and demonstrated cyclic variation in the activity of these cells. The principal work on neurosecretory cells in various species of earthworms was done by Herlant-Meewis (1955, 1956, 1956/57, 1959), who studied Eisenia foetida and Hubl (1953, 1956), who studied L. terrestris and Allolobophora. Harms (1948), Brandenberg (1956), Aros and Vígh (1959) and Aros and Bodnár (1960) further defined the histological nature of these cells. Marapao (1959) studied the histological changes in the number and percentage of the different types of neurosecretory cells of L.

terrestris when subjected to pricking and compared these percentages at different seasons of the year.

Both Herlant-Meewis and Hubl described four basic neurosecretory cell types. Three secretory cell types were found in the suprapharyngeal ganglion. These have been designated as: a-cells, b-cells and "large and medium-sized neurons." The fourth type was found in the subpharyngeal ganglion (Herlant-Meewis, 1955, 1956). Hubl noticed still another type, the c-cell, present in the suprapharyngeal ganglion during regeneration. He also found u-cells in the subpharyngeal ganglion during regeneration. These cells described by each investigator may not be completely equivalent, since neurosecretory cell types that have been given the same name by different investigators sometimes seem to be related to different physiological conditions (Bullock and Horridge, 1965). Brandenberg (1956) and Marapao (1959) concluded that some of the cell types described in L. terrestris were actually the same cell type in different phases of the secretory cycle.

At the present time, very little is known about the chemistry of these neurosecretory substances and their mechanism of action at either the molecular or cellular level. The chemical nature of the neurosecretory substances in some, but not all, invertebrates appears to be protein with disulfide groups (Brousse et al., 1958). Extracts from L. terrestris have been found to contain substances that display juvenile hormone activity (Schneiderman and Gilbert, 1958), but the chemical nature of this active substance is unknown.

In oligochaetes, neurosecretory products possibly control four processes: 1) maturation of gametes; 2) development of somatic sex characteristics; 3) regeneration; and 4) physiologic color change. Most work has been done to support their influence on reproduction and regenerative activity (Bullock and Horridge, 1965). Kamemoto (1964) and Dennany (1964) demonstrated that neurosecretion affected osmotic and ionic regulation in earthworms. It was shown that an increase in body weight and decreased sodium concentrations in the blood and coelomic fluid occurred in decerebrated worms. These conditions were reversed by injections of brain homogenates into the coelom.

To date, little information is available as to the normal blood glucose levels in invertebrates in general, and annelids in particular. In studying Helix pomatia, Schwartz (1935) found the glucose level to remain fairly constant from 10 to 30 mg % throughout the year, although there were seasonal fluctuations. Normally fed and starved animals showed no noteworthy differences and injections of insulin or adrenalin did not alter the blood sugar content. The only mention concerning the analytical method used in obtaining these values was that micro methods were used. Bahl (1946), in studying the structure, development and physiology of the nephridia of oligochaetes, made biochemical estimations of nutritive and excretory substances in the blood and coelomic fluid. He used from three to seven determinations and obtained blood glucose levels from 92 to 108 mg %. In 1947 the same investigator, reporting on

excretion in Pheretima posthuma, obtained blood glucose levels of 100 mg %. In his 1946 analyses, Bahl used Hagedorn and Jensen's titration method and Folin and Wu's colorimetric method for analyzing glucose. Both of these methods are described in Hawk et al. (1947). No mention is made as to what method of analysis was used in his 1947 studies.

De Jorge et al. (1965b) made glucose analyses on the snail, Strophocheilus oblongus musculus, and found the level of glucose to be from 18.5 to 25.4 mg % in five specimens. When the same investigator analyzed five specimens of the giant earthworm, Glossoscolex gigantus, the glucose level obtained for this animal was from 19.4 to 21.8 mg % (De Jorge et al., 1965a). The analytical method employed was that of Nelson (1944). It should be noted that the giant earthworms were anaesthetized with prophylene phenoxetol before drawing the blood. It is not known what effect this anaesthesia might have had on the glucose level.

If little is known concerning the glucose levels in invertebrates, still less is known about its mechanism of control. de Ley and Vercruysse (1955) found both glucose-6-phosphate and gluconate-6-phosphate dehydrogenases present in L. terrestris, showing these animals have the enzyme system for the hexose-monophosphate oxidative route or a closely related one. Abramowitz et al. (1944), Renaud (1949), Scheer (1959) and Dean and Vernberg (1965) studied carbohydrate metabolism and blood glucose levels in crustaceans and found that the levels vary considerably with the time of day and

the reproductive stage involved.

Abramowitz and coworkers found a diabetogenic factor in the eyestalks of crustaceans, which may be the agent which regulates the normal sugar metabolism in this group of animals. In these studies the analytical method of Miller and Van Slyke (1936) was used in obtaining their values.

It is not known what method Renaud used in his determinations. Scheer used the anthrone method of Roe (1955) and Dean and Vernberg used the orthodianisidine enzymatic method of Huggett and Nixon (1957).

Goddard et al. (1964) found the albumin gland of Helix aspersa to affect the blood sugar levels in these animals. When albumin gland homogenates were injected into the hemocoel, there was a definite rise in the blood sugar level, significantly larger than in those animals with control injections. In this particular study, King's modification of the Folin-Wu method (King, 1946) was employed. All of the analytical methods used in determining blood glucose levels, except the one used by Dean and Vernberg, measured other reducing substances as well as glucose.

METHODS

The animals analyzed in this study were earthworms of the species L. terrestris, which were purchased from a local bait shop. These worms were collected by the bait shop during early summer and used within two weeks after purchase. Only those displaying a mature clitellum were analyzed. All animals were maintained at 4^{o} C in damp soil in large plastic pans covered with perforated aluminum foil, unless otherwise indicated. All analyses were made in early summer to minimize seasonal changes in the glucose levels.

Preliminary studies revealed only very small samples of blood could be obtained from each animal and the glucose levels were very low. This necessitated developing a satisfactory method for measuring micro amounts of glucose in ultra-micro samples of blood.

The orthodianisidine method (Glucostat, Worthington Biochemican Corp., Freehold, N. J.) for the determination of glucose was chosen because of its specificity in measuring only glucose and not other reducing substances and the ease of running the test in the laboratory with a minimum amount of equipment. This method is a coupled enzyme system which utilizes glucose-oxidase-peroxidase and a chromogen, orthodianisidine. Beta-D-glucose is oxidized to gluconic acid and hydrogen peroxide. The oxygen produced by the peroxidase reacting on the hydrogen peroxide reacts with the chromogenic acceptor, orthodianisidine, forming a stable color complex, which can then be measured on a colorimeter. The color intensity is proportional to the concentration of glucose.

A control serum was run in conjunction with thirteen groups of samples to check the accuracy of the orthodianisidine method of glucose determination. The results are recorded in Table I. The control serum used was Lab-trol (Dade Reagents, Inc., Miami, Fla.), Lot LT-28 XE. The glucose level of this particular lot of Lab-trol was calculated to be 105 mg %, according the the pamphlet enclosed with the serum. In this study, 1:10 and 1:20 dilutions of the Lab-trol were made to represent controls of calculated glucose levels of 10.5 and 5.25 mg % respectively. This is the approximate range of the unknowns' glucose levels. These dilutions of control serum were run along with the standards and unknowns, treating all in exactly the same manner. The same bottle of Lab-trol was used throughout the survey.

Procedure for Glucose Determination

1:5 filtrates (Somogyi, 1930) were made in duplicate by placing 40 microliters of blood into each of two micro centrifuge tubes. 80 microliters of 5% zinc sulfate were added to each tube and the contents of the tubes mixed well. 80 microliters of 0.3N barium hydroxide were added to each tube and again, the contents were mixed well. It is important that the zinc sulfate and barium hydroxide neutralize each other for maximum precipitation of protein. A reagent blank and standards were run in conjunction with each set of unknowns by substituting water for the blood in the reagent blank and substituting known standards in place of the blood. Each stand-

Table I -- Glucose Levels on Control Serum

Lab-trol	
1:20 dilution (calc. 5.25 mg %)	1:10 dilution (calc. 10.5 mg %)
4.2	8.3
4.2	6.7
4.6	10.0
4.5	7.3
3.9	9.4
3.9	9.4
4.7	10.0
4.0	6.0
2.6	7.8
7.8	12.1
5.0	8.3
3.3	8.3
3.9	8.6
Totals 56.6	112.2
Mean 4.4	8.6
Standard Deviation 1.21	1.59

ard was also run in duplicate. It was necessary to use standards that contained glucose in the approximate range of the unknowns. Standards of 10 mg % and 25 mg % were used. The filtrates were allowed to stand at least five minutes and then centrifuged.

100 microliters of the protein-precipitated filtrate were pipetted to the bottom of micro test tubes. With accurate timing between each specimen, 250 microliters of Glucostat color reagent were added to each tube and the contents mixed well. At exactly ten minutes after the addition of the color reagent to each tube, 10 microliters of 4N hydrochloric acid were added to each tube to stop the reaction.

Five minutes after the addition of the 4N hydrochloric acid, the samples were readied for reading on a Bausch and Lomb Spectronic 20 unit with microcell assembly set at 400 mu wave length. The reagent blank was set at 0 optical density and optical densities were read on all standards and unknowns. All samples were read within thirty minutes after the addition of the 4N hydrochloric acid.

The concentration of glucose in the unknowns was calculated using the following formula:

$$\frac{\text{optical density of unknown}}{\text{optical density of standard}} \times \text{concentration of standard} = \text{mg/100 ml (mg \%) glucose}$$

Procedure for Decerebration

The worms were anaesthetized in a 10% ethanol solution for two

minutes, followed by rinsing in tap water to rid the outer surface of the animal of alcohol. A longitudinal slit was made on the dorsal midline between the 2nd and 5th segments and the cerebral ganglia lifted up and clipped off. If the animals were to be placed in the refrigerator, they were kept at room temperature until they recovered from the anaesthesia.

Procedure for Drawing Blood Specimens

Each worm was pinned down after being slit on the dorsal surface in the region of the hearts. The coelomic fluid in the area was drawn off and discarded, and then the hearts were punctured and the blood was drawn into a capillary tube. Since it was not known what effect the trauma of the blood drawing would have on the glucose level, all specimens were drawn within a period of five minutes from the initial pinning down through the drawing of the blood. The temperature of the room during the drawing of the blood specimens was between 25 and 30^{o} C. Approximately 15 microliters of blood per worm was drawn. Each blood specimen was placed in a vial and the vial kept on ice until all samples for that pool were included. The proteins in the blood were precipitated according to the method of Somogyi (1930) immediately upon completion of a pool. The precipitated sample was then refrigerated until the precipitated samples from all the pools and standards to be run at that time were included.

Table II summarizes the second aspect of the study, which involved establishing glucose levels in earthworms after specific treatment of varying environmental conditions, worms that had been slit and decerebrated worms over a period of days. The first group of worms, kept at 4^{o} C in damp soil (Group Ia), was considered the control group. Analyses for this group were made on five different dates. Animals analyzed on May 25 consisted of 2 pools of 10 worms each and on June 2, 2 pools of 8 worms each. On June 7, 2 pools of 8 worms each were analyzed. The group analyzed August 15 consisted of 5 pools of 8 worms each. This latter group was analyzed at the completion of the study to determine whether any seasonal change in the glucose level might have occurred since the beginning of the study. Worms kept at 20^{o} C for 24 hours (Group Ib) included 2 pools of 10 worms each. This group was analyzed to determine what effect the warm temperature to which the animals were exposed at the time of blood sampling would have on the blood glucose level. Animals kept in damp cloth toweling for 48 hours (Group Ic) included 5 pools of 8 worms each. This group represented the fasting glucose level.

The effect of trauma of the slitting prior to decerebration (Group II) was analyzed in 5 pools of 8 worms each.

The response of the glucose level 24 hours after decerebration (Group IIIa) consisted of 5 pools of 8 worms each; 48 hours after decerebration (Group IIIb) consisted of 5 pools of 8 worms each; 5 days after decerebration (Group IIIc) consisted of 4 pools of 8

Table II -- Glucose Levels in Earthworms After Specific Treatment

Group	Sample Size	Date Assayed	Treatment	Mean Glucose (mg %)
I a	20	May 25	Control (early summer)	5.7
	16	June 2	"	6.55
	16	June 7	"	5.45
	40	August 15	Control (late summer)	2.1
b	20	June 25	20° C, 24 hr.	4.6
c	40	June 9	cloth toweling 48 hr.	5.9
II	40	June 28	slit June 27, cloth toweling 24 hr.	9.5
III a	40	June 14	decerebrated June 13, cloth toweling 24 hr.	0
b	40	June 15	decerebrated June 13, cloth toweling 48 hr.	2.4
c	32	June 16	decerebrated June 11, cloth toweling 5 days	0.9
d	40	June 22	decerebrated June 10, cloth toweling 5 days, soil 7 days	1.3
e	20	June 24	decerebrated June 23, 20°C, cloth toweling 24 hr.	0

worms each; 5 days after decerebration and then 7 days in soil (Group IIId) consisted of 5 pools of 8 worms each. Decerebrated worms that had been kept at 20° C for 24 hours (Group IIIe) included 2 pools of 10 worms each.

Blood samples were drawn between 8 a.m. and 9 a.m. on the days analyzed. All these analyses were run in duplicate and means taken for each group. All samples in this aspect of the study were analyzed within a period of one month except those of August 15.

The third aspect of this study is summarized in Tables III, IV and V. Since the blood glucose level goes down to 0 mg % within 24 hours after decerebration, it was decided to analyze worms at various times within this 24-hour period. All of the worms were anaesthetized between 8 a.m. and 10 a.m. with 10% ethanol for two minutes and then rinsed in tap water. Blood was drawn at the exact time interval indicated after anaesthesia (2 hr., 3 hr., 6 hr., 14 hr. and 24 hr.) and at the exact same time each day of analyses. All the worms were kept at room temperature after alcohol anaesthesia except the 14 hr. and 24 hr. groups. These were placed in a 20° C controlled temperature room until time of analyses. Each group consisted of 2 pools of 10 worms each except Group Ie, Table V, which was composed of 5 pools of 8 worms each. This entire aspect of the study was done within one month's time.

The three basic control groups were: non-decerebrated worms that received alcohol anaesthesia only (Group I, Table III); alcohol-anaesthetized slit worms (Group I, Table IV); and alcohol-anaesthe-

Table III -- Treatment Groups of Non-decerebrated Earthworms Under Experimental Conditions

Group	Sample Time (in hours) Post-anaes.	Post-inj.	Date Assayed	Treatment	Mean Glucose (mg %)
I a	2		July 15	Control	11.3
b	3		July 16	"	1.1
c	6		July 15	"	4.0
d	14		July 16	"	3.2
e	24		July 16	"	5.0
II a	3	1	August 2, August 4	Saline inj. 2 hr. after anaes.	5.9
b	6	4	August 2	"	0
c	14	12	August 4	"	4.4
d	24	22	August 11	"	1.1
III a	3	1	August 6, August 9	Cerebral gang. inj 2 hr. after anaes.	11.8
b	6	4	August 6	"	3.7
c	14	12	August 9	"	2.5
d	24	22	August 12	"	2.8

N = 20

All animals anaesthetized with 10% ethanol, then rinsed in tap water.

Table IV -- Treatment Groups of Slit Earthworms Under 10% Ethanol Anaesthesia

Group	Sample Time (in hours) Post-anaes.	Date Assayed	Mean Glucose (mg %)
I a	2	July 28	14.6
b	3	July 27	20.2
c	6	July 27	6.9
d	14	July 27	10.4
e	24	July 28	6.9

N = 20

Table V -- Treatment Groups of Decerebrated Earthworms Under Experimental Conditions

Group	Sample Time (in hours) Post-anaes.	Post-inj.	Date Assayed	Treatment	Mean Glucose (mg %)
I a	2		July 11	Control	4.1
b	3		July 12	"	11.3
c	6		July 12	"	7.3
d	14		July 13	"	5.9
e*	24		June 14	"	0
II a	3	1	July 29, August 11	Saline inj. 2 hr. after decereb.	9.8
b	6	4	July 29	"	4.8
c	14	12	August 3	"	0.8
d	24	22	August 2	"	4.6
III a	3	1	July 19, August 12	Cereb. gang. inj. 2 hr. after dec.	8.2
b	6	4	July 19	"	15.5
c	14	12	August 8	"	1.1
d	24	22	August 6	"	4.1

N = 20

*N = 40

All animals anaesthetized with 10% ethanol, then rinsed in tap water.

tized decerebrated worms (Group I, Table V).

The cerebral ganglia homogenates were prepared in a 0.7 % sodium chloride solution. This made it necessary to also measure the response of both non-decerebrated and decerebrated worms to injections of 0.7% sodium chloride without the cerebral ganglia. The suspension of the cerebral ganglia was prepared so that 0.2 ml. contained approximately one pair of cerebral ganglia, the amount normally present in one animal. 0.2 ml. of cerebral ganglia homogenates was injected into each earthworm in the cerebral ganglia injection group. The saline injection group received 0.2 ml. of the 0.7% sodium chloride solution. The injections were made anterior to the clitellum and in an anterior direction. All injections were made exactly two hours following alcohol anaesthesia.

The statistical analysis is recorded in Tables VI - X. Various groups were compared, the t values for the means were calculated and compared with the allowable t value obtained from the "Student's" t distribution (two-tailed) at the .05 level of confidence.

Table VI -- Two-tailed *t* Test on Means

(see results in Table II)

Group	Mean	D.F.	S.D.	Calculated *t*	Allowable *t* at .05 level	Significance at .05 level
(Comparing all groups with Ia - early summer)						
I a (early summer)	5.7	11	2.30			
I a (late summer)	2.1	7	1.54	3.80	2.1	Significant
I b	4.6	3	2.0	.85	2.14	not sign.
I c	5.9	9	2.91	.17	2.09	not sign.
II	9.5	9	7.70	1.53	2.26	not sign.

Table VII -- Two-tailed *t* Test on Means

(see results of Controls in Tables III, IV and V)

Group	Mean	D.F.	S.D.	Calculated *t*	Allowable *t* at .05 level	Significance at .05 level
(Comparing all groups with Ia, Table III)						
I a (III)	11.3	3	1.96			
I a (IV)	14.6	3	2.26	2.22	2.45	not sign.
I a (V)	4.1	3	.77	6.84	2.45	Significant
(Comparing all groups with Ib, Table III)						
I b (III)	1.1	3	.82			
I b (IV)	20.2	3	.77	34.17	2.45	Significant
I b (V)	11.3	3	4.58	4.41	3.18	Significant
(Comparing all groups with Ic, Table III)						
I c (III)	4.0	3	0			
I c (IV)	6.9	3	.4	14.5	3.18	Significant
I c (V)	7.3	3	1.59	4.13	3.18	Significant
(Comparing all groups with Id, Table III)						
I d (III)	3.2	3	.4			
I d (IV)	10.4	3	2.49	5.72	3.18	Significant
I d (V)	5.9	3	4.12	1.29	3.18	not sign.
(Comparing all groups with Ie, Table III)						
I e (III)	5.0	3	1.18			
I e (IV)	6.9	3	3.15	1.11	2.45	not sign.
I e (V)	0	3	0	0	3.18	not sign.

Table VIII -- Two-tailed *t* Test on Means

(see results in Table III)

Group	Mean	D.F.	S.D.	Calculated *t*	Allowable *t* at .05 level	Significance at .05 level
			(Comparing all groups with I b)			
I b	1.1	3	.82			
II a	5.9	3	2.47	3.69	2.45	Significant
III a	11.8	3	4.17	5.06	3.18	Significant
			(Comparing all groups with I c)			
I c	4.0	3	0			
II b	0	3	0	0	3.18	not sign.
III b	3.7	3	4.28	.14	3.18	not sign.
			(Comparing all groups with I d)			
I d	3.2	3	.4			
II c	4.4	3	1.84	1.3	3.18	not sign.
III c	2.5	3	1.87	.78	3.18	not sign.
			(Comparing all groups with I e)			
I e	5.0	3	1.18			
II d	1.1	2	.98	4.62	2.57	Significant
III d	2.8	3	1.48	2.41	2.44	not sign.

Table IX -- Two-tailed *t* Test on Means

(see results in Table V)

Group	Mean	D.F.	S.D.	Calculated *t*	Allowable *t* at .05 level	Significance at .05 level
			(Comparing all groups with I b)			
I b	11.3	3	4.58			
II a	9.8	3	6.47	.38	2.45	not sign.
III a	8.2	3	6.81	.76	2.45	not sign.
			(Comparing all groups with I c)			
I c	7.3	3	1.59			
II b	4.8	3	2.01	1.89	2.45	not sign.
III b	15.5	3	3.88	3.91	2.45	Significant
			(Comparing all groups with I d)			
I d	5.9	3	4.12			
II c	0.8	3	1.08	2.39	2.45	not sign.
III c	1.1	2	.98	2.22	3.26	not sign.
			(Comparing all groups with I e)			
I e	0	9	0			
II d	4.6	1	.56	17.14	12.71	Significant
III d	4.1	3	1.2	6.67	2.45	Significant

Table X -- Two-tailed *t* Test on Means

(see results in Tables III and V)

Group	Mean	D.F.	S.D.	Calculated *t*	Allowable *t* at .05 level	Significance at .05 level
Ia(III)	11.3	3	1.96			
IIIb(V)	15.5	3	3.88	1.91	2.45	not sign.
Ib(III)	1.1	3	.82			
IIIc(V)	1.1	2	.98	.12	2.57	not sign.
Ic(III)	4.0	3	0			
IIId(V)	4.1	3	1.2	.17	2.45	not sign.

RESULTS AND DISCUSSION

The results of the first aspect of the study are shown in Table I. There is extreme variability, as is often the case in ultra-micro chemical analyses, where very small amounts of sample and reagents are used and the smallest error is multiplied many times.

In Table II are shown the results of the second aspect of the study. The control group, run in early summer, had an average glucose level of 5.7 mg % as compared to a glucose level of 2.1 mg % for the control group run in late summer. These two levels were significantly different at the .05 level of confidence (Table VI). Marapao (1959) found significant differences in the percentage of cell types of the neurosecretory cells in L. terrestris during different times of the year and Goddard et al. (1964), in studying Helix aspersa, found significantly lower results in July than in September. Since our results in this group represent a span of three months, this could be a periodic change in the glucose level of L. terrestris. Bahl obtained glucose levels from 92 to 108 mg % in from three to seven determinations in the earthworm Pheretima posthuma and in later analyses found the level of glucose in the same species of worms to be 100 mg %. These results are considerably different from those obtained in our study and also by other investigators and may be due in part to the fact that so few determinations were carried out. Bahl used Hagedorn and Jensen's

method and Folin and Wu's colorimetric method, both described in Hawk (1947). These methods measure other reducing substances as well as glucose and are not specific for beta-D-glucose, as are the enzymatic methods. This fact should also be kept in mind when comparing the glucose levels obtained by other investigators: levels from 10 to 30 mg %, reported by Schwartz (1935) and levels from 19.4 to 21.8 mg %, reported by De Jorge et al. (1965a) for Glossoscolex gigantus. Schwartz does not mention what method was used in obtaining his levels, but De Jorge et al. used the method of Nelson (1944), which measures other reducing substances as well as glucose.

The worms kept in cloth toweling for 48 hours (Group Ic, Table II) had a glucose level of 5.9 mg %. This could represent the fasting level, but may be introducing a stressful condition to the animal. Not only is the glucose source in the soil taken away, but the animal may be utilizing glycogen in response to the stress. This value was not significantly different from the control animals (Table VI).

Both non-decerebrated and decerebrated worms were maintained in a constant temperature room of 20° C for 24 hours to compare glucose levels with worms maintained at 4° C. The non-decerebrated group (Group Ib) showed a glucose level of 4.6 mg %, not significantly different from the control group (Table VI). The warmer temperature to which the decerebrated worms (Group IIIe) were exposed seemed to have no effect on the glucose levels when compared to

decerebrated worms maintained at 4^{o} C.

Group II was run to demonstrate the effect on the glucose level of the slitting prior to decerebration. 24 hours after slitting, the worms had a glucose level of 9.5 mg %. This group did not differ significantly from the control group (Table VI).

Group IIIa through IIIc were decerebrated worms kept in damp cloth toweling and analyzed at various intervals after decerebration. After 24 hours, the glucose level decreased to 0 mg %, increased to 2.4 mg % 48 hours after decerebration and decreased to 0.9 mg % 5 days after decerebration. When placed in soil for 7 days after 5 days in cloth toweling, Group IIId showed an increase in the glucose level to 1.3 mg %, which was still lower than the pre-treatment level. These levels are graphically represented in Figure 1.

It appears that the decerebration or the response to the alcohol anaesthesia caused the glucose level to drop to 0 mg % in 24 hours after decerebration. Since the glucose level did not rise to the pre-treatment level within 5 days after decerebration, there is evidence that the cerebral ganglia are responsible since it seems by that time the animal should have recovered from the trauma of the operation and, as a result, tended to return to the pre-treatment level. However, if the period were extended beyond 5 days, the glucose level might have returned to its pre-treatment level. The fact that the glucose level does rise above the 24-hour level could represent some recovery from the trauma of decerebration, which

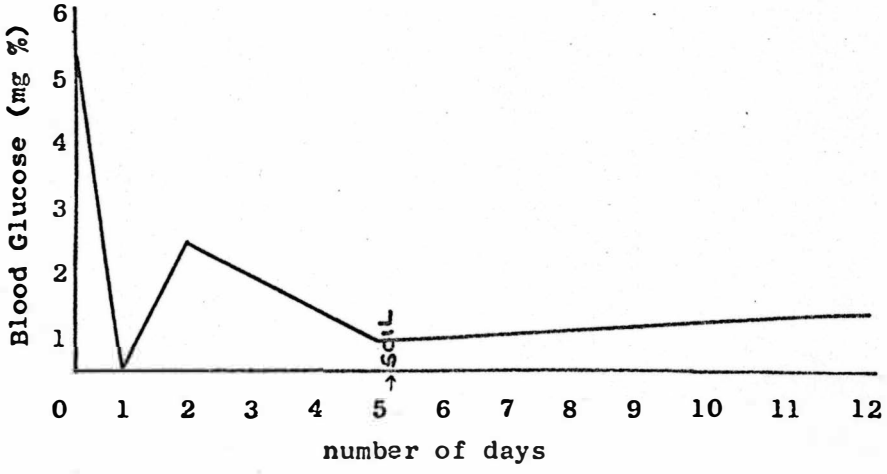

Figure 1 -- Changes in Glucose Levels in Decerebrated Earthworms Over Period of Days

showed its effect most strikingly at 24 hours. This rise could also be due to another mechanism taking over the role of glucose metabolism formerly controlled by the neurosecretory substance of the cerebral ganglia. Another possibility is that this rise could indicate incomplete recovery and no ganglionic control. More extensive studies in this direction could perhaps reveal more definite information along this line of thought.

The third phase of the study was demonstrating the effect of neurosecretory substances from the suprapharyngeal ganglia on glucose levels in earthworms by injecting cerebral ganglia homogenates into both non-decerebrated and decerebrated worms. Tables III, IV and V contain the results of this phase of the study and are represented graphically in Figures 2 through 5. This part of the study was all done within a 24-hour period after anaesthesia to observe the glucose levels at specific time intervals after anaesthesia.

Table III shows the response of the glucose levels in non-decerebrated worms to alcohol anaesthesia, saline injections and cerebral ganglia homogenate injections. The response of the glucose levels in alcohol-anaesthetized worms to the slitting is shown in Table IV. Table V shows the response of the glucose levels of decerebrated worms to alcohol anaesthesia, saline injections and cerebral ganglia homogenate injections.

A comparison was made in Table VII at the .05 level of confidence of the control groups in Tables III, IV and V. The groups which showed significant differences at the .05 level of confidence

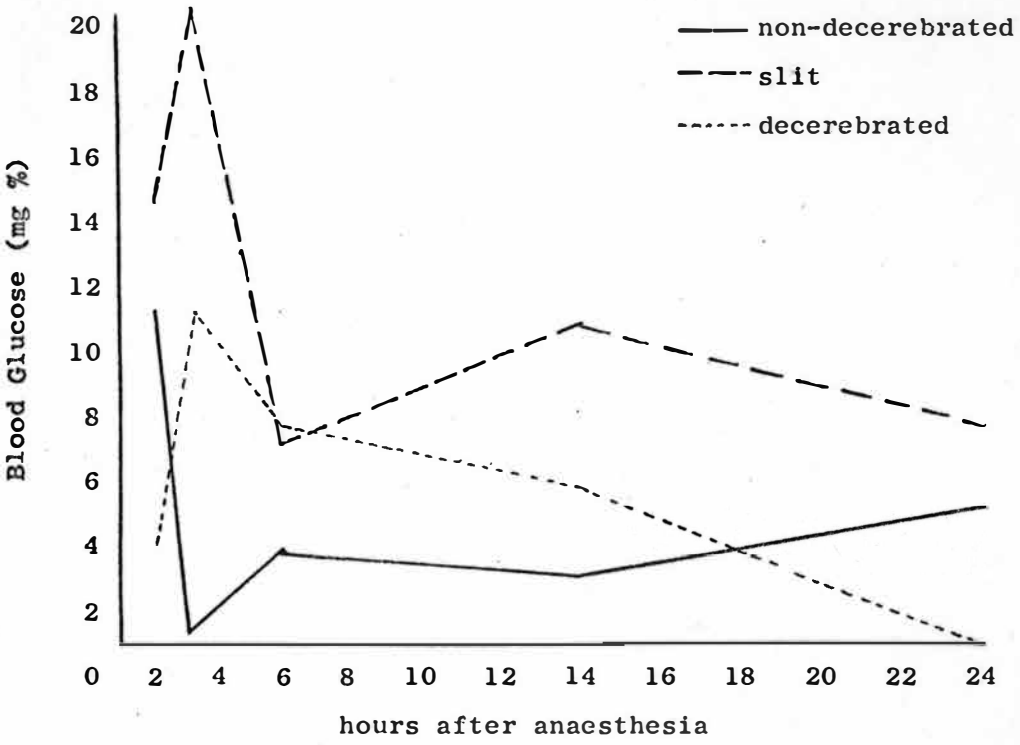

Figure 2 -- Changes in Glucose Levels in Earthworms in Response to Alcohol Anaesthesia, Slitting* and Decerebration*

* All earthworms received alcohol anaesthesia at 0 hr.

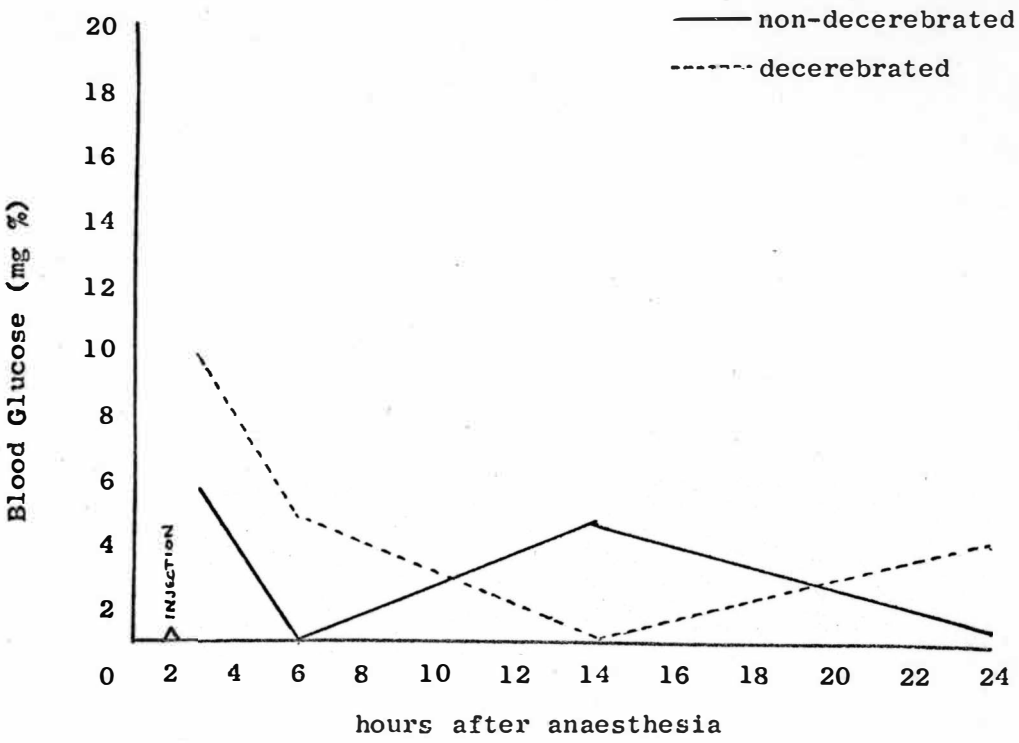

Figure 3 -- Changes in Glucose Levels in Earthworms in Response to Saline Injections*

* All earthworms received alcohol anaesthesia at 0 hr.

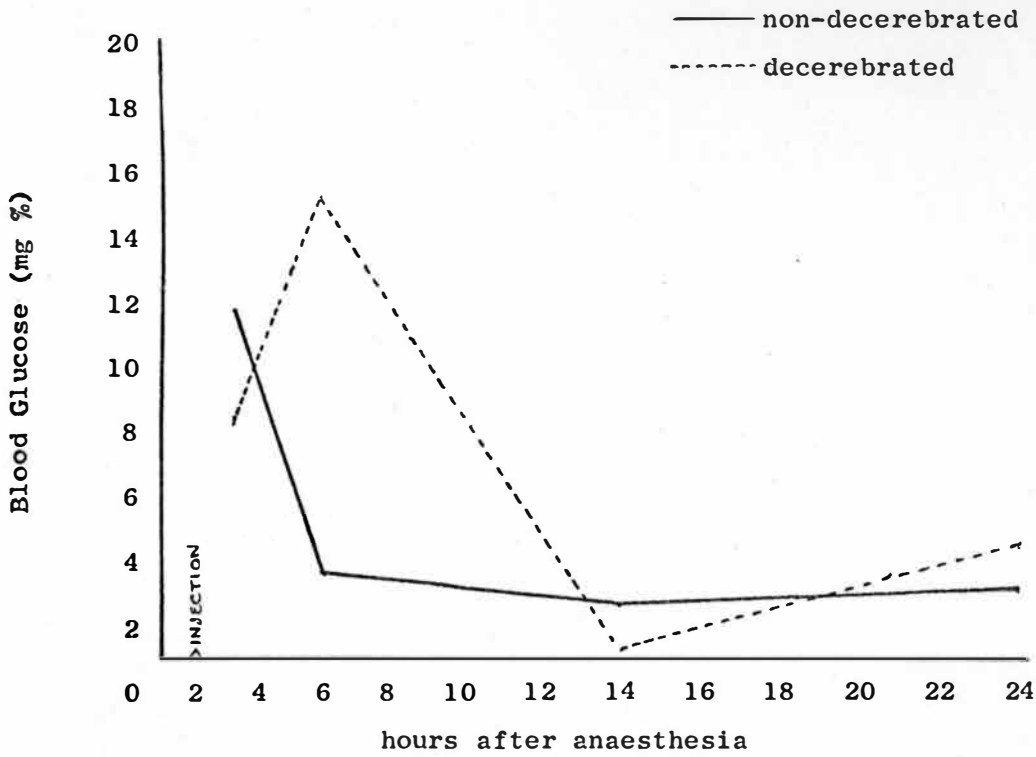

Figure 4 -- Changes in Glucose Levels in Earthworms in Response to Injections of Cerebral Ganglia Homogenates*

*All earthworms received alcohol anaesthesia at 0 hr.

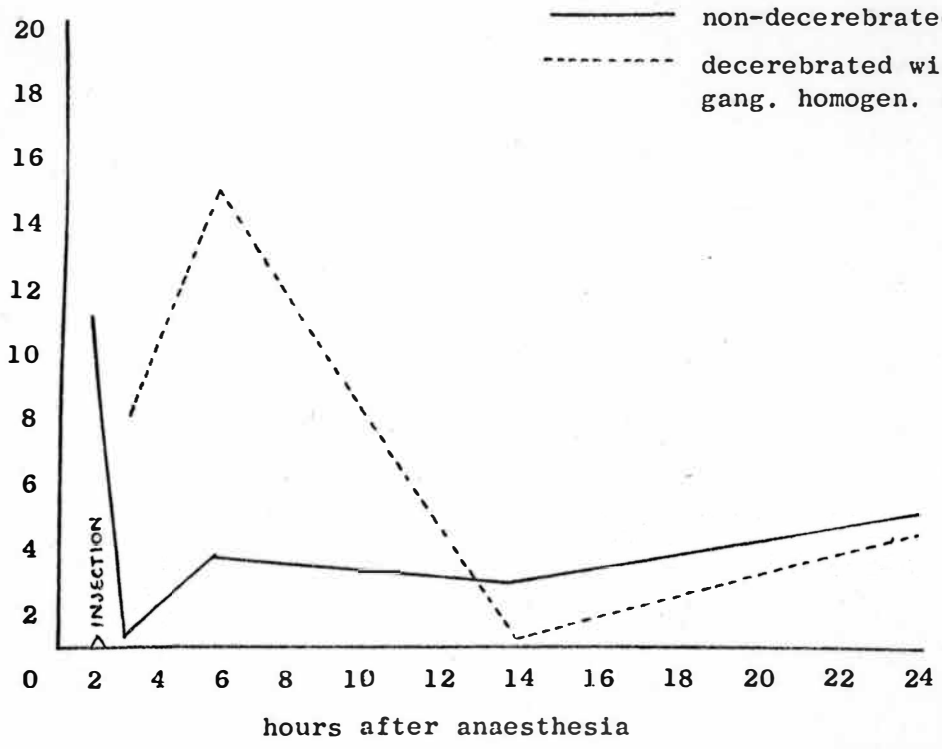

Figure 5 -- Comparison of Glucose Levels in Non-decerebrated Earthworms and Decerebrated Earthworms Which Received Cerebral Ganglia Homogenate Injections*

*All earthworms received alcohol anaesthesia at 0 hr.

were: Group Ia, Table III, and Group Ia, Table V; Group Ib, Table III, and Group Ib, Table IV; Group Ib, Table III, and Group Ib, Table V; Group Ic, Table III, and Group Ic, Table IV; Group Ic, Table III, and Group Ic, Table V; Group Id, Table III, and Group Id, Table IV. These significant differences are more evident when comparing the curves graphically (Fig. 2).

The alcohol appeared to suppress the glucose level in the non-decerebrated and slit worms, but in the decerebrated worms, the suppression was delayed and the glucose level more or less stabilized in 24 hours. The effect of the alcohol anaesthesia may be manifested through the ganglia.

The significant difference that appeared at the 2-hour level seemed to be due to the delayed response of the decerebrated worms. At the 3-hour level, the significant differences appeared to be due again to the delayed response of the decerebrated worms and the slightly delayed response of the slit worms.

In comparing the various groups in Table III, the following showed significant differences at the .05 level of confidence: (Table VIII): Group Ib and IIc; Group Ib and IIIa; Group Ie and IId. These significant differences may be due to the suppression of the glucose level in the alcohol-anaesthetized non-decerebrated worms (Group Ib) at 3 hours post-anaesthesia and the saline injection group (Group IIa) did not have a suppression until 6 hours post-anaesthesia. The cerebral ganglia homogenate injection group (Group IIIa) was at its highest peak at 3 hours post-anaesthesia and then suppressed gradually, leveling off.

The results from Table V show the following significant differences at the .05 level of confidence (Table IX): Group Ic and IIIb; Group Ie and IId; Group Ie and IIId. These significant differences may be due to the fact that the highest level of the decerebrated control worms (Group Ic) was reached at 3 hours post-anaesthesia, then the level was depressed, and the highest level of the cerebral ganglia homogenate injection worms (Group IIIb) was reached at 6 hours post-anaesthesia and then depressed. The differences in Groups Ie, IId and IIId appear to be due to the control group (Group Ie) tapering off to 0 mg % at 24 hours post-anaesthesia and Groups IId and IIId reached their lowest level at 14 hours post-anaesthesia and then their levels increased at 24 hours post-anaesthesia.

When comparing the alcohol-anaesthetized non-decerebrated earthworms (Group I, Table III) with alcohol-anaesthetized decerebrated worms that received injections of cerebral ganglia homogenates (Group III, Table V), the glucose levels were compared on a delayed basis, i.e., comparing the 2 hours post-anaesthesia level of Group I, Table III, with the 6 hours post-anaesthesia level of Group III, Table V; the 3 hours post-anaesthesia level of Group I, Table III, with the 14 hours post-anaesthesia level of Group III, Table V; and the 6 hours post-anaesthesia level of Group I, Table III, with the 24 hours post-anaesthesia level of Group III, Table V. There were no significant differences at the .05 level of confidence (Table X).

Comparisons of glucose levels of worms that have not been de-

cerebrated and decerebrated worms that received cerebral ganglia homogenate injections (Fig. 5) show the two curves approaching each other. Theoretically, the group of decerebrated worms that received the cerebral ganglia homogenate injections should be similar in neurosecretory content to the group of worms that received only alcohol anaesthesia. However, it should be noted that the peak reached in the cerebral ganglia homogenate injection group was higher than the peak reached by the group that received only alcohol anaesthesia and that the lowest levels of both groups were the same. After the lowest level was reached, both groups increased to approximately the same level. The biggest difference was in the somewhat slower response of the decerebrated worms.

The final proof of the regulation of the glucose levels by the neurosecretory material in the cerebral ganglia will be accomplished when the neurosecretory substance is separated from the cerebral ganglia and purified. Then injections of the neurosecretory material alone and the cerebral ganglia minus the neurosecretory material can be made into the animal and the effect on the glucose level analyzed.

SUMMARY AND CONCLUSIONS

The study presented in this paper deals with three different aspects in studying the blood glucose levels in L. terrestris.

The first aspect involved developing a method for the determination of micro amounts of glucose in ultra-micro samples of blood.

The second aspect dealt with establishing glucose levels for non-decerebrated earthworms at two different temperatures when kept in damp soil or cloth toweling, establishing what effect slitting had on glucose levels and the effect of decerebration on the glucose levels over a period of days. The normal glucose level for early summer was established as 5.7 mg %. Decerebrated worms responded to decerebration by a drop in the glucose level to 0 mg % 24 hours after decerebration and rising slightly within 5 days after decerebration to 0.9 mg %. The fact that the level does not remain at 0 mg % may indicate some other mechanism could be taking over the role of the neurosecretory substance of the cerebral ganglia when the cerebral ganglia are removed.

The third aspect of the study was observing the response in glucose levels of both alcohol-anaesthetized non-decerebrated worms and alcohol-anaesthetized decerebrated worms to injections of cerebral ganglia homogenates and saline injections as a control group. Two significant conclusions could be drawn from this aspect of the study. First, the suppression of the glucose level by alcohol an-

aesthesia in non-decerebrated and slit worms and in decerebrated worms the suppression is delayed and more or less stabilizes in 24 hours. The effect of the alcohol anaesthesia may be manifested in the ganglia. Secondly, another significant conclusion that could be drawn from this aspect of the study was that when decerebrated worms (minus their neurosecretory substances from the cerebral ganglia) are injected with homogenates of cerebral ganglia to restore the neurosecretory substances, these animals show a response, although somewhat delayed, somewhat similar to earthworms that have not been decerebrated (those that have a full complement of their neurosecretory substances). The response in the glucose levels to the saline injections in both non-decerebrated and decerebrated worms revealed a somewhat similar response in both groups. From these findings, it seems the neurosecretory substances from the cerebral ganglia may exert an effect in maintaining the blood glucose levels in L. terrestris, but the alcohol anaesthesia has just as drastic an effect on glucose levels as decerebration.

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