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An Investigation into the Nature of Action of the Hyperglycemic Factor Present in the Suprapharyngeal Ganglia of Lumbricus Terrestris

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AN INVESTIGATION INTO THE NATURE OF ACTION OF THE HYPERGLYCEMIC FACTOR PRESENT IN THE SUPRAPHARYNGEAL GANGLIA OF *LUMBRICUS TERRESTRIS*

by

Bruce M. Taylor

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Bruce M. Taylor

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INTRODUCTION

The presence of neuroendocrine cells in the central nervous system of the earthworm, Lumbricus terrestris, were first described by Ernst and Berta Scharrer in 1937. The existence of these cells was subsequently confirmed by Schmid (1947). A number of studies have been carried out to elucidate the location, structure, and function of these cells in the earthworm. Several investigators have described two types of secretory cells in a variety of oligochetes, the A-cells and the B-cells (Herlant-Meewis, 1957; Hubl, 1953, 1956; Brandenberg, 1956; Marapo, 1959). Histochemical investigations of the suprapharyngeal ganglia (Goudie, 1968) and the first few ganglia of the ventral nerve cord, including the subpharyngeal ganglia (Orenstein, 1971) of L. terrestris confirmed the presence of these two types of cells on the basis of their size and granulation. The A-cells are larger with large granules and the B-cells are smaller with fine, dark staining granules. The B-cells have been shown to contain monoamines while the A-cells exhibit different enzymic activities (Teichmann and Goslar, 1968; Vigh-Teichmann and Goslar, 1969). Substances present in the suprapharyngeal ganglia of L. terrestris have been implicated as having a regulatory effect on several metabolic processes such as reproduction, regeneration, osmotic regulation, and some other metabolic activities (Golding, 1974). Nelson (1969) and Papoe (1973) have demonstrated the presence of a factor in the suprapharyngeal ganglia of this worm which plays a role in oxidative metabolism. These various factors are

presumably produced by the neurosecretory cells which are present in the central nervous system. In support of this Goudie (1969) and Orenstein (1971) were able to show that the osmoregulatory factor was probably produced by the A-cells because when worms were subjected to osmotic stress these cells became degranulated. Factors having hyperglycemic activity have also been shown to be present in these ganglia (Lawrence, et al, 1972). Their removal resulted in the fall of blood glucose levels to zero by the end of 24 hours and injection of crude brain extracts resulted in a significant elevation of blood glucose. VandenBosch (1970) developed a method for the preparation of a partially purified water extract of the suprapharyngeal ganglia. Four ninhydrin positive fractions were found to be present in this extract and one, possibly two, of these fractions was shown to have hyperglycemic activity.

The purpose of this investigation was to determine the site of action of this hyperglycemic factor(s). In invertebrates various structures have been shown to be analogous in function to that of the mammalian liver. For example, the insect fat body has been shown to carry on many of these functions in the insect (Kilby, 1965) as does the hepatopancreas in the crustaceans. In the earthworm, Lumbricus terrestris, the intestine particularly the anterior end, functions analogously to liver tissue in that glycogen and lipids are stored in this tissue primarily in the chlorogocytes (Roots, 1960). Based upon this information the anterior intestine was selected as the subject of this investigation into the site of action of the earthworm hyperglycemic factor. In addition, an en-

deavor was made to determine if the hyperglycemic factor(s) present in the partially purified extract of VandenBosch is the sole hyperglycemic factor or if it acts in concert with one of the monoamines as is the case with mammalian glucagon and epinephrine. Finally, an investigation was made to check for cross-reactivity of mammalian glucagon and small peptide hormones such as vasopressin and oxytocin in the earthworm. This final study was run in light of evidence that mammalian glucagon had some activity in elevating blood glucose in the crayfish, Orconectes virilis (Leinen and McWhinnie, 1971).

REVIEW OF LITERATURE

In mammals regulation of blood glucose is fairly well understood. Although the methods by which the invertebrates regulate their body carbohydrates are far less well understood than such regulation in the vertebrates, some work has been done to elucidate these methods. Existence of a hyperglycemic factor in the corpus cardiacum has been demonstrated in several species of insects. Bowers and Friedman (1963) demonstrated that in the cockroach, Periplaneta americana, injection of small amounts of corpra cardiaca (CC) extract (equivalent to 0.1 cardiaca) produce an increase in haemolymph glucose and trehalose. They also showed that CC extract caused increased glycogen mobilization from the fat body and that the trehalose is synthesized from the mobilized glucose. Similar findings in the locust, Locusta migratoris, were reported by Goldsworthy (1969) and Mordue and Goldsworthy (1969). Vejbjerg and Normann (1974) investigated the effect of long term flight on haemolymph trehalose levels in the blowfly, Calliphora erythroephala. Their studies indicate that in flies with intact corpus cardiaca haemolymph trehalose levels remained relatively constant at about 2.3% during 45 minutes of continuous flight. However, if a cardiacectomy or cardiaca denervation are carried out, within 15 minutes of flying trehalose decreased by about 33.3 percent and within 45 minutes these cardiacectomized animals experience complete flight exhaustion. With rest recovery occurs at a very slow and apparently a hormone independent rate. Although,

if denervated CC are squeezed *in situ* a steep, temporary rise in haemolymph trehalose occurs.

Several studies indicate that this hyperglycemic factor is neuroendocrine in origin. Normann and Duve (1969) showed that electrical stimulation of the CC causes release of a hyperglycemic factor (neurohormone) in the blowfly, *Calliphora*. Normann (1973) presented electron microscopic evidence of intrinsic neurosecretory activity in blowflies undergoing long term flights. Highman and Goldsworthy (1972) demonstrated the neuroendocrine origin of a hyperglycemic factor in *Locusta migratoris* by producing *de novo* corpra cardiaca, i.e., severing nervous connection with the organ and allowing time for recovery from the surgery. Like the intact organ the contents of *de novo* cardiaca produce a hyperglycemic effect. However, this effect is of a lesser magnitude than that of the neurosecretory lobe of the normal organ. It was concluded that the hyperglycemic factor in the neurosecretory lobe originates in the medial neurosecretory cells of the brain.

Steel (1963), in addition to demonstrating that CC extracts exert their hyperglycemic effect by mobilizing fat body glycogen in *Periplaneta americana*, demonstrated that the increase in haemolymph trehalose is accompanied by a parallel decrease in fat body glycogen which is probably due to increased phosphorylase activity. Wiens and Gilbert (1966) showed that fat body phosphorylase is maximally activated within 10 minutes after being exposed to CC extracts in the locust. Goldsworthy (1970) compared the effects of CC extracts from the different lobes of this organ in *L.*

migratoria and found that all lobes have some phosphorylase activating properties. However, the glandular lobe had a higher potency than the storage lobe. Wyatt (1974) in studying the biochemical events accompanying fat body glycogen depletion in Cercopia silkmoth pupa showed an increase in cAMP levels and enhanced phosphorylase activation in the presence of methylxanthanes. Thus it would appear that the action of the insect neuroendocrine hyperglycemic hormone is similar to that of mammalian glucagon in that it acts by converting glycogen phosphorylase to the active form presumably via the second messenger cyclic-AMP.

In the crustacean, blood sugar regulation appears to be more complex than that of the insect. Hyperglycemic hormones have been shown to be present in the eyestalk, with its contained neurohaemal sinus-gland, in several species of crabs (Kleinholz, 1966; Kleinholz, et al, 1967; Kleinholz and Keller, 1973). Ramamurth, et al, (1968) demonstrated that the hyperglycemic hormone(s), present in the eyestalk, exerts its effect via inhibition of the glycogen synthesizing enzyme, UDPG - glycogen transglycosylase, and converting the glycogenolytic enzyme, phosphorylase, to its active form in the hepatopancreas of two species of crab, Hemigrapsus nudus and Cancer magister.

Recently, evidence, in Scylla serrata, has been presented to demonstrate that there are actually two hormones for blood sugar regulation in the eyestalk of S. serrata: a hypoglycemic and hyperglycemic factor (Deshmukh and Rangneker, 1974). This investigation indicated that injection of eyestalk extract into normal crabs leads

to hyperglycemia. However, bilateral extirpation of the eyestalks does not lead to hypoglycemia, but rather, hyperglycemia, indicating the possibility of an extra-eyestalk hyperglycemic principle produced elsewhere. When eyestalk extract is administered to destalked crabs blood glucose drops to preoperative levels, indicating the possible existence of a hypo- as well as hyperglycemic factor in the eyestalk.

Evidence has been presented by Nagabhushanam and Diwan (1972) indicating that eyestalk removal leads to an immediate hyperglycemia followed by hypoglycemia in 24 hours in Barytelphusa cunicularis. Injection of eyestalk extract into destalked crabs causes hyperglycemia within one hour which drops to normal levels within 24 hours. Additional evidence for the existence of both hyper- and hypoglycemic factors in the eyestalk of S. serrata has been presented by Rangneker and Momin (1975). They demonstrated that bilateral eyestalkectomy leads to increased glycogen concentration in the hepatopancreas in 24 hours suggesting a hypoglycemic factor. Injection of eyestalk extract to normal or destalked crabs causes glycogen depletion from the hepatopancreas suggesting a hyperglycemic factor.

Leinen and McWhinnie (1971) studied the effect of two mammalian pancreatic hormones, insulin and glucagon, on the blood sugar levels of the crayfish, Orconectes virilis. In this study insulin was shown to have no significant effect on blood sugar. However, glucagon (0.5 mg) caused a significant hyperglycemia within two hours in animals in the intermolt stage. This action appears to be an indirect one in which glucagon stimulates the production or release

of an eyestalk related hyperglycemic regulator since glucagon had no effect in destalked animals.

Lawrence, et al (1972) demonstrated the presence of a hyperglycemic factor in the suprapharyngeal ganglia of the earthworm, Lumbricus terrestris. In this study blood glucose levels as measured enzymatically for normal worms were 5.6 mg percent. When the worms were debrained within 24 hours blood glucose concentration dropped to zero. Administration of homogenized suprapharyngeal ganglia resulted in an elevation of blood glucose to above normal levels which were significantly greater than those of control worms injected with saline or protein solutions. In an attempt to isolate the factor(s) which are active hormonally, VandenBosch (1970) developed a method to partially purify crude ganglia homogenates. By this technique lipids and monoamines are extracted into organic solvents, the extract dissolved in water and all insoluble materials discarded leaving a water soluble extract. This water extract was separated into four fractions by chromatographic techniques. Two of the fractions so obtained were active hyperglycemic agents. All fractions were characterized as ninhydrin positive and probably polypeptides.

Investigations into the effects of the monoamines on the body sugars in various invertebrates have implied hormonal effects of these agents. For example, in the liver fluke, Fasciola hepatica, serotonin has a hyperglycemic affect analogous to that of epinephrine in mammals. 5-HT acts via the second messenger cyclic-AMP to convert phosphorylase to its active conformation, thereby, stimulating glycogenolysis

(Mansour, 1959, 1967; Mansour et al, 1960).

Monoamines have been shown to be present both intra-neurally and extra-neurally in a variety of arthropods. Dopamine and to a lesser extent norepinephrine are the predominate monoamines in most species of arthropods, however, dopamine plays a role as a structural precursor in cutical formation as well as a transmitter role in the nervous system. A neurohormonal role for dopamine has been implied by its uptake by neurohaemal organs in several species of arthropods (Murdock, 1971).

The location and function of monoamines in Annelids have been the subject of several investigations. Rude (1969) via extraction and thin layer chromatographic analysis of the ventral nerve cord of L. terrestris demonstrated dopamine to be the predominate catecholamine with norepinephrine present in lesser quantities. No epinephrine was present, however, DOPA was occasionally detected. A study of the morphology of amine secreting neurons of the subpharyngeal ganglia of the earthworm, Octolosium complanatum, demonstrated that amine secretion is associated exclusively with the perikaryon. Further, these amine secretions are suspected to be discharged directly into the blood stream, where they may act as a true hormone (Bianchi, 1967). Electron microscopic examination of the cerebral ganglion and ventral nerve cord of L. terrestris by Myhrberg (1972) demonstrated the presence of norepinephrine, dopamine, and/or serotonin in small granules in the neuropile. However, most neurosecretory neurons which contain large granular vesicles were shown not to contain monoamines.

METHODS AND MATERIALS

Animals

Mature worms, selected on the basis of size, appearance of a mature clitellum, and general physical condition, were obtained from a local bait shop in groups of 100 or more. They were maintained at 4°C in media composed of Buss bedding, soil, walnut meal, coffee grounds, lab rabbit chow, and peanut oil (Cooper, et al, 1970).

Worms maintained in this media remained in very good condition for up to five months. This was indicated by their firm, plump appearance and by glycogen levels of intestinal tissues which reached and maintained a fairly constant level (range: 500 to 700 milligram percent) as compared to highly variable levels when worms arrived from the dealer (range: 300 to 3000 milligram percent).

Tissue Preparation

Animals were removed from their bedding and rinsed in tap water to remove any adhering media. Next, the worms were cut into 3 sections. The middle section, which was reserved extended approximately 2 cm posteriorally from the clitellum. A lateral incision was made through the body wall of this middle section so that the wall could be pinned out to expose the intact intestine. The incision was made laterally in order to avoid damaging the glycogen containing chloragogen cells which are located primarily in the anterior third of the intestine. A similar incision was then made in the intestine and the

intestine washed thoroughly with earthworm saline (Drews and Pax, 1974) to remove any fecal matter and digestive enzymes present in the gut lumen. The section of intestine was then cut into quarters and distributed to four different assay tubes as described in the following section (see Figure 1).

Experimental Design

Preliminary investigations demonstrated an extreme inter-animal variation in intestinal glycogen content making evaluation of glycogenolytic activities of suspect agents difficult, if not impossible. To cope with this problem, a four by four Latin square experimental design was employed (Cochran and Cox, 1957). Before explaining this design, several terms should be defined and/or clarified. An experiment represents all assays run comparing the effect of a prospective agent to those of concurrently run controls. An assay consists of a group of four tubes which receive tissue from the same worms. Two of these tubes were treated as controls and the remaining two as experimentals. Tissue distribution to each of the tubes in an assay followed the Latin square shown in Figure 2. As described previously, the test section of intestine was quartered and the quarters designated A through D as per Figure 1. This process was carried out on four different worms and the quarter sections distributed to the four tubes of the assay as indicated in Figure 2. For example, assay tube one in an experiment would receive subsection A from worm 1, subsection B from worm 2, subsection C from worm 3, and subsection D from worm 4, while assay tube three would receive sub-

section A from worm 4, subsection B from worm 3, etc. The final outcome of this experimental design is a series of 4 assay tubes with each containing tissues from each of four different worms such that each tube contains the equivalent of one whole section of intestine, i.e., each contains a subsection A, B, C, and D. In order to further reduce the intra-assay variation in tissue glycogen content the Latin square procedure was repeated with 4 additional worms to yield four tubes containing the equivalent of two intestinal sections. In this way the variation in glycogen content is buffered because each tube in the assay contains tissue fragments from the same eight worms. Also, since each assay contains its own control and experimental samples small differences in tissue glycogen content can be detected among these samples. In all experiments the 95 percent confidence level was considered the minimum for statistical significance.

Figure 1

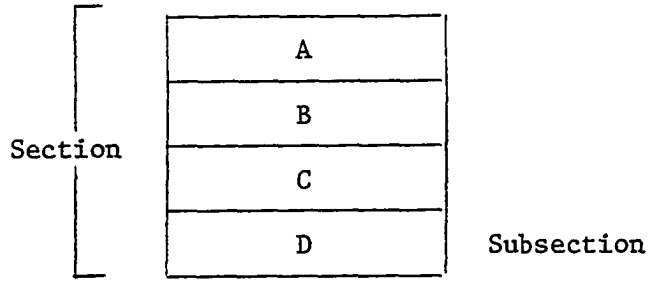


Diagram of intestine showing method of sectioning. Each section was cut into four subsections (A through D).

Figure 2

Assay Tube \ Worm Number	1	2	3	4
1	A	B	C	D
2	B	A	D	C
3	D	C	B	A
4	C	D	A	B

Diagram of Latin square used to guide tissue dispersion. The letters represent the intestinal tissue subsections (See Figure 1).

Glycogen Assay

Intestinal glycogen levels were determined via a modification of the anthrone technique of Roe and Daily (1966). By this method the tissue samples were removed to tared assay tubes containing 1 ml earthworm saline without sucrose (Drews and Pax, 1974) and the tissue wet weight determined. In all procedures sucrose was omitted from Drews' earthworm saline in order to avoid any possible source of sugar contamination. After incubation either in saline as a control or in saline containing a test agent, saline was decanted with a pipette. The tissue was next subjected to hot alkaline digestion in 3 ml of 1N sodium hydroxide in a boiling water bath for fifteen minutes with occasional shaking to insure complete digestion. The digested tissue solutions were then cooled in running water, made acidic with 6 ml of 1N perchloric acid, the volume adjusted to 10 ml with water and mixed. The tissue digests were allowed to

stand for fifteen minutes at room temperature to precipitate protein which was then removed by filtration through perchloric acid washed filter paper (Whatman No. 1). The filtrate was then mixed well with a Vortex Geni mixer (without adequate mixing the heavier molecules such as glycogen tended to become more concentrated at the bottom of the tube). One ml of filtrate was transferred to a glass stoppered 35 ml centrifuge tube containing 3 ml of 95% ethanol with 0.1% lithium chloride and incubated at room temperature overnight to precipitate glycogen. More effective glycogen precipitation is achieved at room temperature than at colder temperatures.

On the following day tubes were placed in an International Model HR-1 refrigerated centrifuge and spun at 10,000 X G for 40 minutes. The supernatant liquid was decanted via a pipette, the tubes inverted, and allowed to drain for an additional 10 minutes. Two ml water and 10 ml anthrone reagent (0.5% anthrone and 3% thiourea in 72% (v/v) sulfuric acid) were added to the precipitate and the tubes thoroughly mixed. Upon completion of this colorimetric reaction, tubes were cooled to 23°C prior to spectrophotometric analysis at 620 nm on a Coleman Jr Spectrophotometer. Using the following formula the glycogen content of the tissue was calculated by comparison of the optical density of the sample with that of a concurrently run standard containing 0.1 mg glucose.

$$\frac{DU}{DS} \times 0.1 \times 10 \times \frac{1}{Wt} \times 0.9 = \frac{\text{mg glycogen}}{\text{gm tissue}}$$

This calculation involves dividing the optical density of the unknown (DU) by the optical density of the standard (DS) and multi-

plying by the following: weight of glucose in the standard in milligrams (0.1), a dilution factor (10), the reciprocal of the tissue weight in grams (1/Wt), and a factor to convert from glucose to glycogen (0.9).

Experimental Procedure

After preparation of the assays (see Experimental Design for details) all four tubes within the assay contained tissue fragments in one milliliter of earthworm saline without sucrose. The sucrose was omitted in order to avoid any possible sugar contamination which might affect the assay results. One milliliter of test or control solution was added to the assay tubes as described below.

Upon addition of the test or control solution, the assay tubes were placed on a rotary shaker and incubated at room temperature with intermittent shaking for 15 or 30 minutes. Incubation times were chosen because preliminary unpublished studies by Donald Ramsey (1973) at Western Michigan University indicated maximal depletion occurred within these periods. After this incubation all solutions were decanted and the tissue fragments washed twice with 1 ml of saline without sucrose to remove any liberated glucose and then digested and assayed for glycogen content.

Experiments with whole "brain" homogenate

Test solutions were prepared by exposing the suprapharyngeal ganglia via a mid-dorsal incision through the body wall of the first five segments, removing it with fine forceps to a ground glass homogenizer. Measured amounts of saline were then added and homog-

enization carried out for fifteen minutes in an ice bath. Since the tissue had previously been placed in 1 ml of saline, solutions of whole "brain" homogenate and all other test agents were prepared such that they would contain two times the desired concentration of said agent. This would, upon addition to the milliliter of saline bathing the tissue, yield the desired concentration, e.g., four ganglia were homogenized per ml and added to yield a final concentration of 2 "brains" per ml. A control experiment was run with 25 µg BSA per ml vs. saline to see if a protein alone would stimulate glycogen depletion. 25 µg is approximately equivalent to the weight of two suprapharyngeal ganglia.

Experiments with partially purified "brain" extract

The procedure for preparation of a partially purified water extract of the earthworm suprapharyngeal ganglia was developed by VandenBosch (1970). By this method "brains" were homogenized in methanol and the methanol removed by flash evaporization. The dry residue was then extracted in a chloroform:methanol:water mixture and the organic phase discarded to remove lipids. Next, via an ether:water extraction monoamines were removed from the extract. This ether extract was then freeze-dried. It was calculated that each milligram contained the equivalent of three "brains". The test solution was prepared by dissolving 2 mg of the extract in 1 ml of earthworm saline and adding this milliliter to the assay preparation to yield a final concentration of 1 milligram (or 3 "brains") per milliliter. Again BSA was used as a control (1 mg/ml).

Experiments with the monoamines

All monoamines were obtained from Sigma Chemical Co., St. Louis, MO. Test solutions were prepared by dissolving epinephrine, serotonin, or dopamine in earthworm saline to yield a final concentration of 33.5 µg per ml, a concentration two times greater than that shown to affect oxidative metabolism by Papoe (1973). One milliliter of earthworm saline was added to control tubes.

Experiments with mammalian peptide hormones

Glucagon was kindly supplied by Dr. Mary Root at The Lilly Research Laboratories in Indianapolis, Indiana. The hypophyseal hormones, arginine vasopressin and oxytocin were obtained through Sigma Chemical Co. Glucagon solutions were prepared in earthworm saline to yield a final concentration of 0.5 mg per ml; a concentration at which Leinen and McWhinnie (1971) showed hyperglycemic activity in the crayfish. The concentration of the hypophyseal hormone test solutions was based upon a concentration five times greater than was necessary to affect water and ion transport in the toad bladder or a final concentration of 250 mIU per ml (Ferguson and Twite, 1974). Control solutions were prepared to contain 0.5 mg BSA per ml.

RESULTS

Several problems were encountered while conducting this study, the largest being the tremendous inter-animal variation in intestinal glycogen levels observed. This problem was greatly reduced via the introduction of a Latin square experimental design. However, with this design the inter-assay group (animal) variation continued to be large. The existence of this problem of variation is indicated by the high probabilities of true differences existing between blocks calculated by the analysis of variance. This statistic indicates a highly significant difference existing between the assay groups in every experiment conducted. A second major problem is the inherent variation seen in the anthrone glycogen assay itself. The coefficient of variation for this assay is between 4 and 5 percent which makes detection of 5 to 11 percent differences rather difficult. A third problem encountered was the tremendous variation in glycogen levels from one lot of worms to the next which was solved, more or less, by the introduction of Cooper's bedding (Cooper, et al, 1970).

The effects of various agents which are both intrinsic or extrinsic to the earthworm, on the stores of intestinal glycogen in this organism are presented in Tables 1 through 4. The experimental results were analyzed by two different methods.

In the first, percent change in glycogen levels between control tissues and experimental tissues was calculated for each of the assay or Latin square groups in an experiment. That is, the mean glycogen level for the tissues in the two control tubes in the assay

group minus the mean glycogen level for the experimental tubes divided by the mean glycogen level for the control tissue for the assay times 100.

$$\text{Percent Change} = \frac{[\text{control glycogen}]-[\text{experimental glycogen}]}{[\text{control glycogen}]} \times 100$$

The percent change was calculated for each assay group in the experiment and the mean change for the experiment is presented in the tables. Since we were looking for factors which would cause a decrease in glycogen levels the percent of assay or Latin square groups in each experiment showing a decrease in glycogen content or negative change is presented in parentheses. It should be noted that due to variations in tissue glycogen levels in the same assay groups an increase in tissue glycogen content was sometimes observed. A true glycogen increase seems improbable due to the lack of a glucose source in the incubation media. When the net change in tissue glycogen levels is in the negative direction the proportion of assay groups showing a decrease will aid in the determination of the validity of statistically nonsignificant decreases. For example, if a decrease is seen in an experiment which is not significant at the 95 percent confidence level ($P>0.05$), observation of a decrease occurring in less than 50 percent of the assay groups would indicate that the observed decrease was probably an experimental artifact due simply to tissue variation rather than hormonal action. However, if a nonsignificant decrease is seen in an experiment but a decrease is detected in the majority of assay groups, say 80 percent, the results would indicate that the decrease was probably

stimulated by hormonal action of the test agent (e.g., "brain", monoamine, etc.).

In the second method of analysis of results, two-way analysis of variance were employed in order to partition the variances between assay groups ("animals"), represented by blocks in the tables, from variances between control and experimental samples. The probability of a true difference is then calculated for each of these factors (Cochran and Cox, 1958).

The effects of whole "brain" homogenate on glycogen levels in the excised intestinal tissues are presented in Table 1. This data indicates an ability of a factor or factors present in the earthworm suprapharyngeal ganglia to stimulate the mobilization of intestinal glycogen. Incubation of tissues in a saline solution containing two "brains" per milliliter (four "brains" total in two ml) for a period of 15 minutes produced a decrease in intestinal glycogen as compared to controls from the same assay group. Although this decrease equalled 10 percent after 15 minutes with 75 percent of the assay groups showing a decrease, it was not statistically significant ($P<0.15$) due to an extremely high intra-assay group variation or variation between blocks ($P<0.001$). The experiment was repeated using a 30 minute incubation period which resulted in a net decrease in intestinal glycogen of 7.5 percent with a negative change occurring in 83 percent of the assay groups. However, the decrease after 30 minutes was significant ($P<0.01$) in spite of a highly significant intra-assay group variation ($P<0.001$). Similarly, when the concentration of homogenized ganglia was in-

Table 1

Effect of Whole Brain Homogenate on Intestinal Glycogen

Agent Concentration	[GLYCOGEN] (±S.E.)* Saline Control	Experimental	Percent Change**	Probability of*** difference between: Samples	Blocks
2 brains/ml (15 min) N=4	6.6 ± 0.5	6.1 ± 0.5	-10.0% (75%)	p<0.15	p<0.001
4 brains/ml (15 min) N=5	6.3 ± 0.2	5.7 ± 0.2	-9.1% (80%)	p<0.02	p<0.001
2 brains/ml (30 min) N=6	5.4 ± 0.2	4.9 ± 0.2	-7.5% (83%)	p<0.004	p<0.001
25 µg BSA/ml (15 min) N=5	6.3 ± 0.2	6.8 ± 0.3	+7.4% (20%)	p<0.06	p<0.001
25 µg BSA/ml (30 min) N=5	5.7 ± 0.2	5.3 ± 0.1	-5.4% (60%)	p<0.08	p<0.004

All solutions prepared in earthworm saline (Drews and Pax, 1974).

*Mean glycogen expressed as milligrams glycogen per gram intestinal tissue (wet weight).

**Percent change in glycogen in experimental as compared to control. Second number indicates the percent of samples within a paired group to show a decrease.

***Probabilities were determined by two-way analysis of variance. Between blocks indicates the probability of a true difference existing between Latin square groups in the same experiment. Between samples indicates the probability of a true difference existing between control and experimental groups in the same experiment.

creased to four "brains" per milliliter and incubated for fifteen minutes, a 9.1 percent decrease in tissue glycogen was observed. A negative change was observed in 80 percent of the assay groups and the glycogen depletion was significant ($P<0.02$), although, inter-animal variation remained highly significant ($P<0.001$). The results of control experiments with bovine serum albumin (BSA) are also shown in Table 1. These experiments were run to determine whether or not the decrease in glycogen was an artifact caused simply by a foreign protein. For these experiments a solution of 25 micrograms BSA per milliliter was employed which was approximately equal to that in three earthworm "brains". After 15 minutes of exposure to this solution no decrease in intestinal glycogen was detected. However, after a 30 minute exposure to this solution a net decrease of 5.4 percent over saline controls was detected which approached significance ($P<0.08$). This decrease appeared in 60 percent of the assay groups. Because of this decrease in glycogen content demonstrated with a half-hour exposure to a foreign protein, a decision was made to run all subsequent experiments for only fifteen minutes.

The results of experiments to determine if the partially purified "brain" extract of VandenBosch (1970) was active in causing glycogen depletion are presented in Table 2. Partially purified extract was administered in a concentration roughly equivalent to three homogenized ganglia per milliliter (1 mg/ml). The results indicate that the hyperglycemic agent(s) shown to be present in this water extract from which lipids and monoamines had been removed were

Table 2

Effect of partially purified brain extract on intestinal glycogen

Agent Concentration	[GLYCOGEN] (±S.E.)* Saline Control	Experimental	Percent Change**	Probability of*** difference between: Samples	Blocks
Partially Purified Extract (1 mg/ml, 15 min) N=5	7.6 ± 0.2	7.2 ± 0.2	-5.0% (60%)	$p<0.20$	$p<0.001$

All solutions prepared in earthworm saline (Drews and Pax, 1974).

*Mean glycogen expressed as milligrams glycogen per gram intestinal tissue (wet weight).

**Percent change in glycogen in experimental as compared to control. Second number indicates the percent of samples within a paired group to show a decrease.

***Probabilities were determined by two-way analysis of variance. Between blocks indicates the probability of a true difference existing between Latin square groups in the same experiment. Between samples indicates the probability of a true difference existing between control and experimental groups in the same experiment.

active in causing glycogen depletion of 5 percent. A decreased glycogen content appeared in 60 percent of the assay groups. However, the observed decrease was not statistically significant by analysis of variance ($P<0.20$).

The experiments testing the effect of monoamines (Table 3) indicate that serotonin and to a lesser extent epinephrine both at a concentration of 33.5 micrograms per milliliter were active in causing intestinal tissue glycogen depletion while dopamine at the same concentration caused no depletion. Serotonin, which is the most abundant monoamine in the earthworm, caused a 6 percent decrease in intestinal glycogen content and epinephrine caused a 3.2 percent decrease. With both of these amines a negative change occurred in 80 percent of the assay groups. From two-way analysis of variance they were not significant ($P<0.10$ and $P<0.26$, respectively). However, a large inter-animal variation in intestinal tissue glycogen content was also observed ($P<0.002$ and $P<0.001$).

The results of experiments designed to determine whether or not any degree of cross-reactivity exists between earthworm intestinal tissue and several mammalian polypeptide hormones are presented in Table 4. These studies were run in light of recent publications which report a hyperglycemic response in the crayfish following injection of glucagon (Leinen and McWhinnie, 1971). Oxytocin and vasopressin were also run to determine whether other small peptide hormones might cause intestinal glycogen depletion and presumably hyperglycemia in L. terrestris. Glucagon (Lilly) at a concentration of 0.5 milligrams per milliliter failed to cause any intes-

Table 3

Effect of the monoamines on intestinal glycogen

Amine Concentration	[GLYCOGEN] (±S.E.)* Saline Control	Experimental	Percent Change**	Probability of*** difference between Samples	Blocks
Epinephrine (Sigma) (33.5 µg/ml)	7.0 ± 0.3	6.7 ± 0.2	-3.2% (80%)	p<0.26	p<0.001
Serotonin (Sigma) (33.5 µg/ml)	9.0 ± 0.5	8.1 ± 0.2	-6.0% (80%)	p<0.10	p<0.002
Dopamine (Sigma) (33.5 µg/ml)	11.9 ± 0.3	12.0 ± 0.3	+1.2% (40%)	p<0.83	p<0.03
N=5 (15 min)					

All solutions prepared in earthworm saline (Drews and Pax, 1974).

*Mean glycogen expressed as milligrams glycogen per gram intestinal tissue (wet weight).

**Percent change in glycogen in experimental as compared to control. Second number indicates the percent of samples within a paired group to show a decrease.

***Probabilities were determined by two-way analysis of variance. Between blocks indicates the probability of a true difference existing between Latin square groups in the same experiment. Between samples indicates the probability of a true difference existing between control and experimental groups in the same experiment.

Table 4

Effect of mammalian peptide hormones on intestinal glycogen

Agent Concentration	[GLYCOGEN] (±S.E.)* Saline Control†	Experimental	Percent Change**	Probability of*** difference between: Samples	Blocks
Glucagon (Lilly) 0.5 mg/ml, 15 min	6.2 ± 0.1	6.8 ± 0.3	+7.7% (20%)	p<0.06	p<0.001
Arginine Vasopressin (Sigma) 250 mIU/ml N=5 15 min	12.3 ± 0.3	13.5 ± 0.8	+9.8% (40%)	p<0.12	p<0.002
Oxytocin (Sigma) 240 mIU/ml, 15 min N=2	12.5 ± 0.4	11.8 ± 0.3	-5.7% (50%)	p<0.11	p<0.04

All solutions prepared in earthworm saline (Drews and Pax, 1974).

*Mean glycogen expressed as milligrams glycogen per gram intestinal tissue (wet weight).

**Percent change in glycogen in experimental as compared to control. Second number indicates the percent of samples within a paired group to show a decrease.

***Probabilities were determined by two-way analysis of variance. Between blocks indicates the probability of a true difference existing between Latin square groups in the same experiment. Between samples indicates the probability of a true difference existing between control and experimental groups in the same experiment.

†Saline controls contained 0.5 mg BSA/ml.

tinal glycogen depletion. Similar results were obtained with 250 milliunits per milliliter arginine vasopressin (Sigma). However, oxytocin (Sigma) at a concentration of 240 milliunits/milliliter appeared to be active in mobilizing intestinal glycogen. Oxytocin caused a 5.7 percent decrease in intestinal glycogen stores; this depletion appeared in 50 percent of the assay groups ($P<0.11$ from ANOV).

DISCUSSION

Although most of the data obtained in this study failed to demonstrate a high level ($P<0.05$) of statistical significance, it does give some insight into the process of blood glucose regulation in the earthworm. The earthworm, like the insect and the crustacean, has a hyperglycemic factor which originates in nervous tissue and is probably a neurosecretion (Lawrence, et al, 1972). In addition, present data indicates that the main site of action is probably the intestine, specifically the anterior portion of the intestine, where a substance, or substances, present in the cerebral ganglia exert a true hormone-like effect. Preliminary studies indicated that the intestine is the primary if not sole site of glycogen mobilization in response to incubation with whole "brain" homogenate. The pharyngeal tissue was found to contain extremely low levels of glycogen (1.3 ± 1.2 mg/gram tissue) while body wall tissue, although rich in glycogen (15.6 ± 6.8 mg/tissue) was relatively inert to stimulation by whole "brain" homogenate. Body wall tissue probably uses its glycogen only for its own needs.

The glycogen depleting agent(s) present in whole "brain" homogenate has been shown here to exert its effect in a dose and time dependent manner. Although a glycogen depleting effect could be seen when two homogenized ganglia per milliliter were incubated with intestinal tissue for 15 minutes this depletion became statistically significant when the incubation time was increased to 30 minutes. A similar level of significance was obtained when the ganglia concen-

tration was increased to 4 "brains" per milliliter. These two properties, time and dose dependence, help to demonstrate the hormone-like nature of the intestinal tissue glycogen depleting factor(s) present in the suprapharyngeal ganglia.

Data presented here also supports the idea that in the earthworm there may be more than one substance capable of mobilizing intestinal tissue glycogen. When whole "brain" homogenate was treated to remove lipids and monoamines, as per VandenBosch (1970), and incubated with intestinal tissue glycogen depletion was detected. However, this depletion was not as great as that seen when intestinal tissue was incubated with whole "brain" homogenate.

This concept that more than one hyperglycemic agent is produced by the suprapharyngeal ganglia was given further support when serotonin, which has been shown to be present in earthworm neural ganglia (Rude, 1969), and to a lesser extent, epinephrine, which is foreign to the earthworm but is an active hyperglycemic agent in the mammal, caused depletion of intestinal tissue glycogen. Also, the monoamines have been implicated as having a role as true hormones, exerting their effect at a distance in the insects (Murdock, 1971) and they may serve that function in the earthworm. Serotonin and/or epinephrine may function in concert with the peptide like (ninhydrin positive) factor or factors isolated by VandenBosch (1970) to regulate blood glucose levels in the earthworm much as epinephrine and glucagon do in the mammal.

The possibility exists that blood glucose regulation in the earthworm may involve additional factors which are produced by sev-

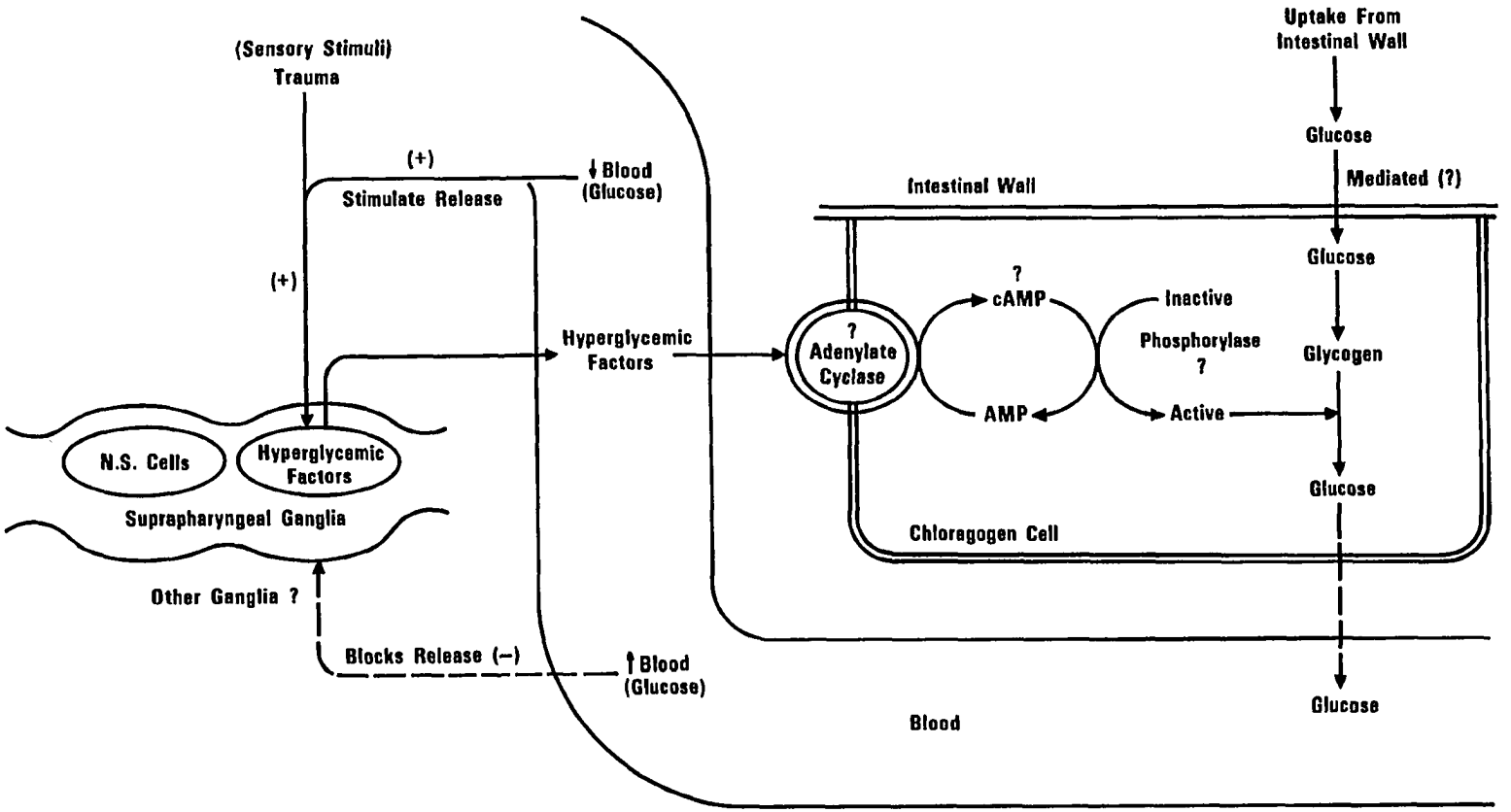
eral areas in the nervous system or in other tissues as has been demonstrated in the crab. In many of the experiments in this study an increase in intestinal glycogen was detected upon incubation with a test agent implying the presence of a hypoglycemic factor in Lumbricus terrestris. However, there was no readily available source of glucose for incorporation into glycogen and preliminary investigations by Fetterer and Lawrence (1976) have been unable to detect the existence of such a factor in the neural ganglia or intestinal tissue of L. terrestris. Here again, it should be emphasized that this study was plagued with extreme interworm variation in intestinal glycogen levels which, although attempts to reduce this variation were employed, could be responsible for these observations of increased glycogen. Also, there may be other areas of the nervous system capable of producing hyperglycemic factors. As Orenstein (1971) demonstrated, the subpharyngeal ganglia and ventral nerve cord apparently also produce osmoregulatory substances as does the suprapharyngeal ganglia as evidenced by the loss of granulation in the A-cells located in these regions when the worms are subjected to osmotic stress following debraining.

Crossreactivity between earthworm intestine and mammalian glucagon was not demonstrated by this investigation. However, before this possibility can be dismissed, an investigation should be conducted in vivo in intact worms since in the crab Leinen and McWhinnie (1971) could not demonstrate hyperglycemia in destalked crabs while they could in intact animals implying an indirect action for this

agent. Perhaps this same indirect activity can be demonstrated where glucagon triggers the release of intrinsic hyperglycemic agents from the suprapharyngeal ganglia of L. terrestris. The other peptide hormones were investigated because they have a neural origin in the vertebrates and they may contain a structure similar to that of the neural factors in the earthworm ganglia. However, no or at best only slight activity could be assigned to these agents either.

Based upon the information available to date a preliminary model of blood glucose regulation in the earthworm can be proposed. As blood glucose levels rise, glucose is deposited as glycogen in the chlorogocytes of the intestine. As the animals' blood glucose levels drop a release of one or more hyperglycemic factors (hormone) from the suprapharyngeal ganglia is triggered. Also, release could be triggered by means of sensory input resulting from trauma, such as surgery. It has been shown that blood glucose levels are sharply elevated immediately following both debraining and sham procedures before dropping to zero levels by 24 hours (Lawrence, et al, 1972). The factor(s) then travels via the blood to the intestine where they cause glycogen to be broken down to glucose. Presumably the hyperglycemic factor(s) exerts its effect through the second messenger, cyclic AMP, which activates phosphorylase. The resulting release of glucose then probably feeds back to the suprapharyngeal ganglia to block the release of the hyperglycemic factor(s) (See Figure 3).

Figure 3. Proposed model for blood glucose regulation in the earthworm L. terrestris. See text for explanation.



SUMMARY

1. The Anthrone glycogen assay of Roe and Daily (1966) was modified for analysis of earthworm intestinal glycogen content. This assay was employed in conjunction with a Latin square experimental design to test various glycogen depleting agents for activity.

2. Homogenized whole "brains" and partially purified "brain" extract were tested and both were shown to be active in depleting intestinal glycogen, although whole "brain" homogenate had a greater effect.

3. The monoamines, dopamine, serotonin, and epinephrine were also tested demonstrating that serotonin, and to a lesser extent epinephrine, were active while dopamine showed no effect.

4. The mammalian hormones glucagon, oxytocin and arginine vasopressin were all shown to be inactive in intestinal glycogen depletion.

5. Finally, a model for blood glucose regulation in L. terrestris is proposed.

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