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Glucagon-Like and Insulin-Like Hormones of the Insect Neurosecretory System

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Aqueous extracts of corpus cardiacum–corpus allatum complexes of the adult tobacco hornworm *Manduca sexta* produced both glycogenolysis and hypoglycaemia when injected into the larval form of the same species. Application of specific radioimmunoassays to similar extracts showed also that these gland complexes contain both glucagon-like and insulin-like peptides. Further, the partially purified immunoreactive peptides had the expected biological activities. The former decreased the glycogen content of the fat-body and the latter the circulating trehalose levels in recipient animals. These results suggest the existence of hormones in these invertebrates having both biological and structural similarities to vertebrate insulin and glucagon.

Insect haemolymph contains as the major form of circulating carbohydrate the non-reducing disaccharide trehalose. Although smaller amounts of glucose and other monosaccharides also occur, fluctuations in the levels of the disaccharide appear to reflect the state of glycaemia under conditions of, for example, starvation or exercise (Wyatt, 1967). The regulation of haemolymph trehalose levels in insects by means of a hyperglycaemic factor has been recognized since 1961 (Steele, 1961). Experimental approaches implicating the corpus cardiacum and other components of the neurosecretory system in the storage and secretion of this hormone include ablation of the endocrine tissue by surgery (Normann & Duve, 1969; Vejbjerg & Normann, 1974), stimulation of the tissue electrically or mechanically (Normann & Duve, 1969; Vejbjerg & Normann, 1974) and injection of tissue extracts into test animals (Steele, 1963; Brown, 1965; Natalizi & Frontali, 1966; Keeley & Friedman, 1967; Mordue & Goldsworthy, 1969; Goldsworthy & Mordue, 1974). Typically, injections of gland extracts result not only in hyperglycaemia, but also in depression of the glycogen content of the fat-body (Goldsworthy, 1970; Wiens & Gilbert, 1967), indicating the profound similarity of the action of this factor in insects to that of glucagon in vertebrates.

Although studies on the existence of an opposing, insulin-like hormone in insects have received considerably less attention, Dixit & Patel (1964) showed that honeybee royal jelly contains a substance promoting glucose oxidation in mammalian adipose tissue. The insulin-like hypoglycaemic activity of extracts of whole *Drosophila* in mice (Meneses & Ortiz, 1975) and the morphogenic effects of mammalian insulin on *Drosophila* cells in culture (Seecof & Dewhurst, 1974) have since been documented. Normann (1975) has also suggested that the hyperglycaemia associated with decapitation of the blowfly *Calliphora* results from the lack of hypoglycaemic hormone of cephalic origin. In order to delineate the identities of these hyperglycaemic and hypoglycaemic factors and their roles in insect physiology, we began an examination of neuroendocrine tissues in insects for peptide hormones capable of influencing carbohydrate metabolism. Earlier, we reported the presence of a peptide with glucagon-like immunoreactivity in extracts of corpus cardiacum–corpus allatum complexes from the lepidopteran *Manduca sexta* (L.) and proposed that the peptide was the insect hyperglycaemic hormone (Tager *et al.*, 1975). We extend our observation here and report additional results strongly suggesting that peptides of *Manduca* immunologically similar to insulin and glucagon have intraspecific biological activities corresponding to those of the respective vertebrate hormones.

Materials and Methods

Insects

Eggs of *Manduca sexta* were a gift from Dr. R. A. Bell (U.S. Department of Agriculture, Fargo, ND, U.S.A.), and were hatched and reared at 28°C and 60% relative humidity during a 16h light–8h dark photoperiod on a modification of the standard diet (Yamamoto, 1969). Adults of *Plodia interpunctella* (Hübner) and *Periplaneta americana* (L.) were from the U.S. Grain Marketing Research Center Laboratories and from Dr. C. Pitts (Department of Entomology, Kansas State University, Manhattan, KS, U.S.A.) respectively.

Dissections and tissue extractions

Adult and larval insects were anaesthetized by cooling to 4°C before dissection of tissues by standard techniques (Schneiderman, 1967). Tissue specimens (corpora cardiaca–corpora allata complexes or the separated glands) were rinsed in 0.15M-NaCl containing 1mg of bovine serum albumin/ml and either 1mM-di-isopropyl phosphorofluoridate or 5000 units of the protease inhibitor Trasylol*/ml (FBA Pharmaceuticals, NY, U.S.A.) and were then freeze-dried and stored at −20°C. The desiccated tissues were homogenized by using small glass tissue grinders in either the above solution lacking proteinase inhibitors for biological assays, or in that solution containing 6M-guanidine hydrochloride for gel filtration. The homogenates were clarified by centrifugation at 10000*g* for 10min at 4°C.

Biological assays

Larval *M. sexta* on day 1 of the fifth instar (2.0± 0.3g) were anaesthetized by cooling. The appropriate gland extract or other test solution (usually 0.01ml) was then injected into the haemocoel through a proleg of the sixth abdominal segment. After the animals had been incubated at 22°C for 60min without feeding, haemolymph was collected as previously described (Kramer *et al.*, 1974) and the fat-body was removed and freeze-dried. The desiccated tissue was dissolved in hot aq. KOH (30g/100ml) and glycogen was precipitated by the addition of 4vol. of ethanol at 4°C (Carroll *et al.*, 1956). The glycogen was purified by twice redissolving the precipitate in 1ml of KOH (5g/100ml) followed by the addition of ethanol. The purified material was dissolved in 1ml of aq. 72% H_2SO_4 and immediately subjected to the anthrone reaction (Roe, 1955). Colour values were compared with those obtained with glucose as a standard.

Trehalose, the major circulating saccharide of *M. sexta*, was purified in each case from other anthrone-positive saccharides present in blood by gel filtration of 0.1ml of haemolymph on a column (0.9cm× 120cm) of Bio-Gel P-2 (Bio-Rad Laboratories, La Jolla, CA, U.S.A.) equilibrated with 0.02M-Tris-(hydroxymethyl)aminomethane (2-amino-2-hydroxymethylpropane-1,3-diol)/0.1M-NaCl, adjusted to pH 7.3 by the addition of HCl. The identity and purity of the gel-filtered disaccharide was confirmed by t.l.c. (Bassal, 1973) and the sugar was quantified by the anthrone reaction.

Column chromatography and radioimmunoassays

Tissue extracts prepared as described above were gel-filtered at 22°C on a column (0.9 or 1.5cm×60cm) of Bio-Gel P-10 by using a solution containing 3M-

* Mention of a proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

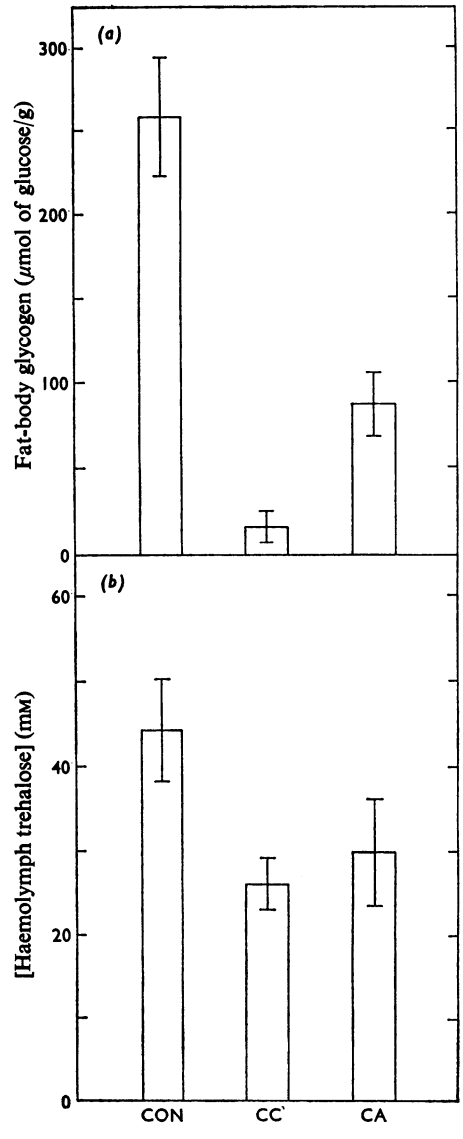

Fig. 1. *Effects of injection of extracts of corpora cardiaca and corpora allata from adult M. sexta on fat-body glycogen content (a) and haemolymph trehalose concentration (b) in larval insects*

Dissected glands were extracted and amounts of extract equivalent to four corpora cardiaca (CC) or four corpora allata (CA) were injected as described in the Materials and Methods section. Fat-bodies and haemolymph samples were removed 60min after injection for determination of glycogen content and trehalose concentration respectively. Control animals (CON) were injected with the solvent used for tissue homogenization. The height of the bars represents the mean value±S.E.M. from ten experiments.

acetic acid, 0.5mg of bovine serum albumin/ml, and either 0.1M-ammonium acetate or 0.05M-NaCl. Samples (0.05–0.5ml) of each 1–2ml fraction obtained after gel filtration were dried in a vacuum desiccator to remove solvent and the residue was dissolved in 0.5ml of 0.1M-Tris(hydroxymethyl)-aminomethane/0.05M-NaCl containing 1mg of bovine serum albumin/ml, adjusted to pH7.7 by the addition of HCl. Radio-labelled hormone (either ^{125}I-insulin or ^{125}I-glucagon; 0.1ml in the above buffer) was added to the test samples and to standard solutions of bovine glucagon or insulin. The appropriate antiserum (0.05ml of a dilution shown to bind 50% of the labelled peptide in the absence of added hormone) was then added; the solutions were mixed and incubated at 4°C for 24h. Bound hormone was separated from free by centrifugation 30min after precipitation of the former by the addition of 0.05ml of normal pooled rabbit serum and 0.5ml of aq. 24% (w/v) polyethylene glycol 6000 (J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A.). Anti-glucagon serum was a gift from Dr. L. Heding (Novo Laboratories, Copenhagen, Denmark); anti-insulin serum, ^{125}I-glucagon and ^{125}I-insulin were gifts from Dr. A. H. Rubenstein and Dr. S. Kuku (The University of Chicago). All values reported here were obtained by evaluation of experimental data against standard curves prepared with either bovine glucagon or bovine insulin.

Results and Discussion

An early experiment designed to detect the presence of carbohydrate-regulating hormones in corpus cardiacum–corpus allatum complexes of adult *Manduca* initially gave conflicting results. As expected, injection of an extract equivalent to three gland complexes into a larval insect decreased the fat-

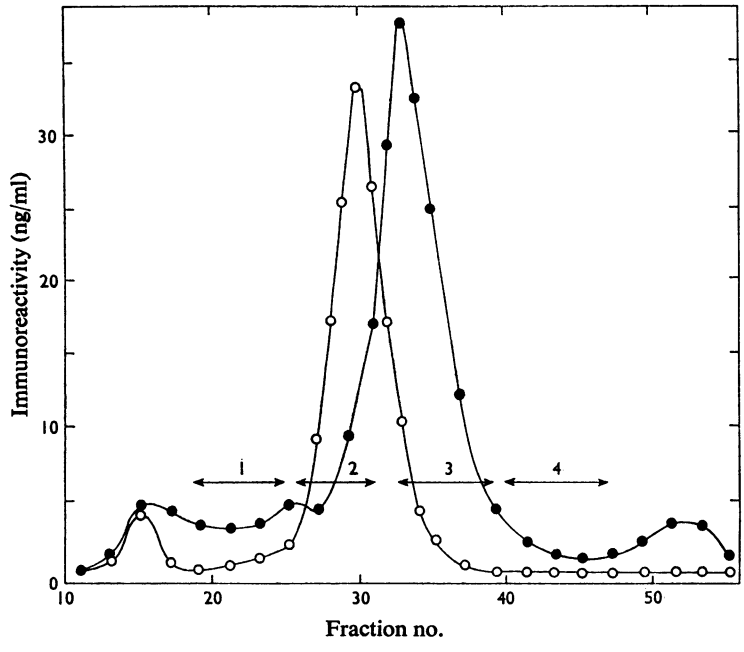

Fig. 2. *Profiles of insulin-like* (○) *and glucagon-like* (●) *immunoreactivity obtained after gel filtration of an extract of corpus cardiacum–corpus allatum complexes from adult M. sexta*

Tissue (100 gland complexes) was extracted with aq. guanidine hydrochloride and gel-filtered over a column (1.5cm×60cm) of Bio-Gel P-10 by using 3M-acetic acid containing albumin and ammonium acetate as described in the Materials and Methods section. The flow rate was 7ml/h and the fraction size 1.6ml. Samples (0.05ml) of each fraction were dried under vacuum in small glass tubes and the resulting residues dissolved in Tris buffer for immunoassay. Data points were compared with standard curves prepared with either bovine insulin or bovine glucagon for determination of the values reported. The remaining portions of the column fractions were combined into four separate pools as indicated by the numbered horizontal arrows. These pools were concentrated on the rotary evaporator and the residual material was repeatedly freeze-dried from water to obtain a product free from both solvent and ammonium acetate for use in the biological assays reported in Fig. 3.

body glycogen content of the test animal from a control value of 230 μmol of glucose equivalents/g desiccated tissue to 51 μmol of glucose/g of desiccated tissue. The concentration of haemolymph trehalose, however, fell from 45 mM, a concentration very similar to that reported by Dahlman (1975), to 25 mM, producing hypoglycaemia. When an extract containing the equivalent of four corpora cardiaca from adult *Manduca* was injected into larval animals, the glycogen content of the fat body fell dramatically to, on average, only 9% of its control value (Fig. 1*a*). A parallel injection of an extract containing four corpora allata was also effective at stimulating glycogenolysis, although its activity was somewhat lower than that of the extract of corpora cardiaca. Fig. 1(*b*) shows that extracts of both glands also had hypoglycaemic activity and decreased haemolymph trehalose concentrations by 30–40% of that found in injected control animals. Extracts of adult *Manduca* brains were only marginally effective in promoting either glycogenolysis or hypoglycaemia, producing decreases in fat body glycogen content or trehalose concentration of only about 10–20% (not shown here). Thus, although hyperglycaemic and glycogenolytic activity

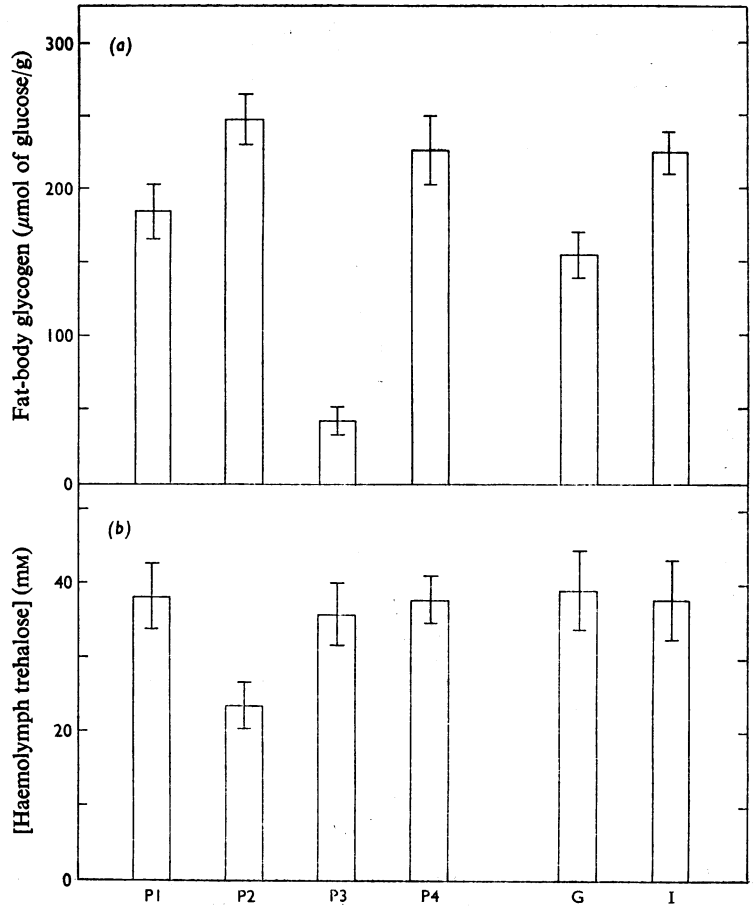

Fig. 3. *Effects of injection of partially purified insulin-like and glucagon-like immunoreactive peptides from corpus cardiacum–corpus allatum complexes of adult M. sexta on fat body glycogen content (a) and haemolymph trehalose concentration (b) in larval insects*

Material obtained from each of the four pools shown in Fig. 2 (P1–P4) was dissolved in 0.25 ml of water and samples equivalent to three gland complexes were injected into larval animals for determination of their glycogenolytic and glycaemic activities as described in the Materials and Methods section. The Figure also shows the effects of injecting bovine glucagon (G) or bovine insulin (I) at doses of 50 nmol/kg body wt. Mean values ± S.E.M. from ten experimental animals are shown.

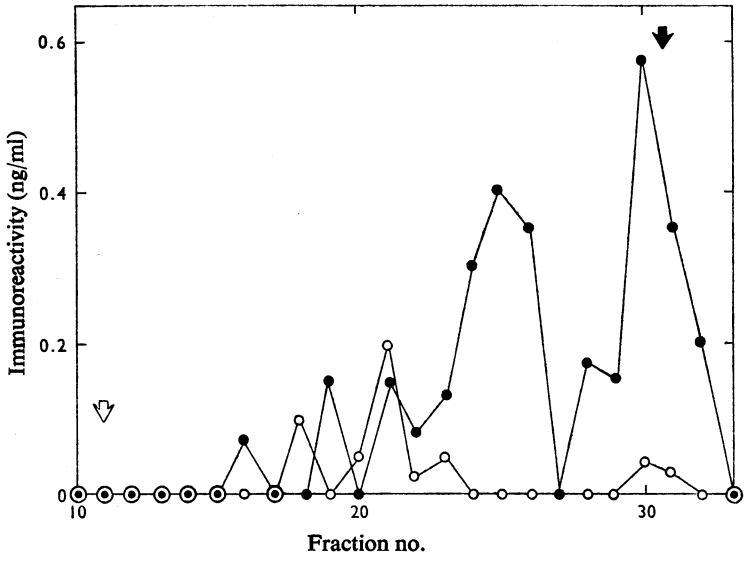

Fig. 4. *Profiles of insulin-like* (○) *and glucagon-like* (●) *immunoreactivity obtained after gel filtration of an extract of whole heads of Plodia interpunctella*

Tissue (200 whole desiccated *Plodia* heads) were extracted and gel-filtered as described in the legend to Fig. 2 and in the Materials and Methods section. The column (0.9cm×60cm) of Bio-Gel P-10 was eluted with 3M-acetic acid containing albumin and NaCl at a flow rate of 4ml/h, and fractions (0.9ml) were collected. Samples (0.4ml) of each fraction were dried under vacuum for assays of insulin-like and glucagon-like immunoreactivity. The open arrow at the left of the Figure indicates the void volume of the column, and the solid arrow at the right, the elution position of ^{125}I-glucagon used as an internal column marker.

has often been ascribed only to the corpus cardiacum (Steele, 1961; Friedman, 1967; Wiens & Gilbert, 1967; Mordue & Goldsworthy, 1969), our results with *Manduca*, as well as those of others using different insects (Goldsworthy, 1970; Highnam & Goldsworthy, 1972), indicate that the hormone is stored in both the corpus cardiacum and the corpus allatum.

Profiles of insulin- and glucagon-like immunoreactivity obtained after gel filtration of an extract of *Manduca* corpus cardiacum–corpus allatum complexes are shown in Fig. 2. Immunoreactive insulin-like material was detected in two peaks, the major one at fraction 30 representing 90% of the total activity. The glucagon-like material was eluted in a single major peak and three minor peaks as shown. The elution volumes of the major components are essentially those of bovine insulin and glucagon, respectively, the vertebrate hormones also being poorly resolved by gel filtration. The hormone content of each gland, based on measurement of immunoreactivity with the bovine hormones as standards, was about 1ng for both insulin and glucagon. Since we do not know the extent to which each of the insect peptides reacts with the respective antiserum, however, these absolute values may be underestimates and this apparent equivalency might be misleading.

The remainder of the fractions obtained after gel filtration of the corpus cardiacum–corpus allatum extract were combined into four pools, as illustrated in Fig. 2, for subsequent biological assay. The glycogenolytic and glycaemic activities of these pools are shown in Figs. 3(*a*) and 3(*b*) respectively. Injection of the equivalent of four gland complexes from the immunoreactive glucagon-enriched pool (3) decreased the fat body glycogen content of larval *Manduca* by 80%. Pool (1) was considerably less potent, and pools (2) and (4) were essentially without activity. Fig. 3(*a*) also shows that bovine glucagon at an immunoreactive dose 100 times greater than that of pool (3) produced only a 40% fall in fat-body glycogen content, whereas bovine insulin was totally ineffective.

As shown in Fig. 3(*b*), only the immunoreactive insulin-enriched pool (2) obtained from the gel-filtered extract of corpus cardiacum–corpus allatum complexes produced a hypoglycaemic effect when injected into *Manduca* larva. Neither bovine insulin, at a dose 50 times that of the immunoreactive component in pool (2) injected, nor bovine glucagon altered the concentration of haemolymph trehalose. Although the failure of the immunoreactive glucagon-containing pool (3) to induce hyperglycaemia was

unexpected, we calculated that conversion of the entire glycogen content of a typical fat-body into trehalose would have increased the trehalose concentration in haemolymph by only about 10% of the control value. This projected change is within the biological variation among test animals.

The results presented here document the existence of biologically active and immunologically reactive glucagon- and insulin-like peptides in extracts of corpus cardiacum–corpus allatum complexes of *M. sexta* and indicate functional and structural similarities between the insect hypoglycaemic and glycogenolytic factors and their vertebrate counterparts. Our observations present the first evidence for the presence of the insect hyperglycaemic hormone in a lepidopteran and show that a proposed insect hypoglycaemic factor (Normann, 1975; Meneses & Ortiz, 1975) is present in extracts of neuroendocrine tissue and is functional in animals of the class Insecta.

An examination of other insects for immunoreactive peptides similar to those found in *Manduca* resulted in our detection of immunoreactive glucagon in the Indian meal moth *Plodia interpunctella* and the cockroach *Periplaneta americana*. In contrast with the relatively simple gel-filtration profile shown for *Manduca* (Fig. 2), extracts of 200 adult *Plodia* heads (Fig. 4) and of 35 corpus cardiacum–corpus allatum complexes of *Periplaneta* (not shown) contained several components having glucagon-like immunoreactivity. In each case one was the molecular weight of bovine glucagon and the others larger. The source of this heterogeneity is not clear, but the complex profiles are consistent with the multiplicity of hyperglycaemic peptides present in neuroendocrine tissues of both the cockroach (Natalizi *et al.*, 1970) and the locust (Mordue & Goldsworthy, 1969). Insulin-like immunoreactivity was low in the gel-filtered extracts of tissues from both *Plodia* and *Periplaneta*. Depending on the nutritional requirements of an insect species or on the stage of development of a group of animals, one or the other of these hormones may play the more important physiological role in regulating carbohydrate metabolism. Although the chemical structures of these hormones and the mechanisms for their secretion and action remain to be determined, the immunological and biological similarities of the insect insulin- and glucagon-like peptides to their vertebrate counterparts suggests that these hormones arose remarkably early during animal evolution.

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