

INSULIN-LIKE HYPOGLYCEMIC AND IMMUNOLOGICAL ACTIVITIES IN HONEYBEE ROYAL JELLY

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Abstract—An aqueous extract of royal jelly from *Apis mellifera* produced hypoglycemia when injected into larvae of *Manduca sexta*. Application of specific radioimmunoassay to the partially-purified extract showed that royal jelly contains several insulin-like peptides, the major immunoreactive component of which has an apparent mol. wt similar to that of bovine insulin. These results suggest the existence of a peptide in the honeybee having both biological and structural similarities to vertebrate insulin.

INTRODUCTION

DIXIT and PATEL (1964) reported that royal jelly, a secretion of the hypopharyngeal, postcerebral, and mandibular glands of the honeybee, contains a peptide promoting glucose oxidation in vertebrate adipose tissue. Other reports of insulin-like factors in insects include those on the blowfly *Calliphora* (NORMANN, 1975), fruit fly *Drosophila* (SECOF and DEWHURST, 1974; MENESES and ORTIZ, 1975), and various Hymenoptera (ISHAY *et al.*, 1976). More recently we demonstrated the presence of insulin-like biological activity and immunoreactivity in gel-filtered extracts of the neuroendocrine system of the tobacco hornworm, *Manduca sexta* (TAGER *et al.*, 1976). We report here the identification of peptides with insulin-like hypoglycemic properties in honeybee royal jelly. These results further support the hypothesis that the vertebrate and invertebrate hypoglycemic factors are structurally related.

MATERIALS AND METHODS

Royal jelly was obtained from Sigma* and Dr. J. H. Law of the University of Chicago. *M. sexta* eggs were the gift of Dr. J. Reinecke of USDA, Fargo, North Dakota and larvae were reared on a semisynthetic diet (BELL and JOACHIM, 1976).

Royal jelly was prepared for gel filtration and biological assay by extracting an aqueous suspension of the lyophilized material (100 mg/ml) with an equal volume of diethyl ether. The denatured protein appearing at the solvent interface and the organic solvent were removed after centrifugation at 15,000 *g* for 30 min. The addition of 0.25 volume of acetic acid

to the residual material resulted in a soluble preparation and this was used directly for column chromatography. For biological assay, the protein in the acidic extract was precipitated by the addition of 10 volumes of ethanol to remove contaminating carbohydrate. The precipitate was lyophilized to dryness and redissolved in insect saline containing 5 mM sodium phosphate pH 6.8 and 0.1% bovine serum albumin (Sigma). The royal jelly extract (0.01 to 0.06 ml) was injected into larval *M. sexta* on the initial day of the fifth instar (2.0 ± 0.3 g) through a proleg of the sixth abdominal segment (TAGER *et al.*, 1976). After the animal had been incubated at 22°C for 90 min without feeding, haemolymph was collected into a test tube and frozen immediately.

Haemolymph trehalose was purified from other saccharides present in blood by gel filtration (0.1 ml) on a column of Bio-Gel P-2 (0.9 × 120 cm) (Bio-Rad Laboratories, LaJolla, California) equilibrated with 0.02 M Tris-(hydroxymethyl)-aminomethane, 0.1 M NaCl, pH 7.3 (TAGER *et al.*, 1976). The sugar was quantitated by the anthrone reaction (ROE, 1955).

The royal jelly extract was gel-filtered at 22°C on a column of Bio-Gel P-10 (0.9 × 60 cm) using a buffer containing 3 M acetic acid, 0.5 mg/ml bovine serum albumin and 0.05 M NaCl (TAGER *et al.*, 1976). For radioimmunoassay, aliquots (0.5 ml) of each 2-ml fraction obtained after gel filtration were dried in a vacuum dessicator to remove solvent and the residue was dissolved in 0.5 ml of 0.1 M Tris-(hydroxymethyl)-aminomethane, 0.05 M NaCl, 1 mg/ml bovine serum albumin. Insulin immunoassay was performed by standard techniques using a final assay volume of 0.65 ml and an incubation period of 24 hr at 4°C (TAGER *et al.*, 1976). Bound hormone was separated from free by centrifugation 30 min after precipitation of the former by the addition of 0.05 ml of normal

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

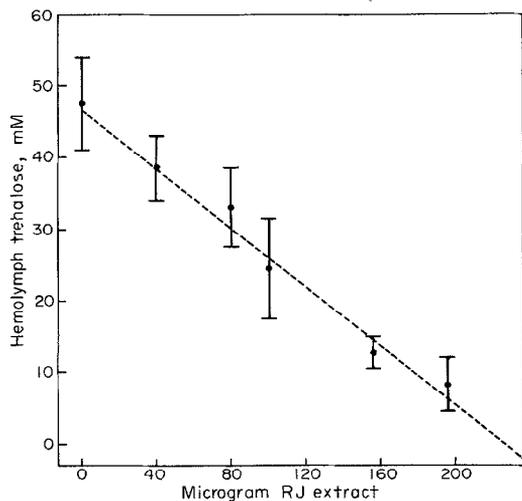


Fig. 1. Effect of injection of royal jelly protein extract from *Apis mellifera* on haemolymph trehalose concentration in larval *Manduca sexta*. Animals were injected with royal jelly extract or solvent used for extract solubilization 90 min before haemolymph was sampled for trehalose determination. The data point represents the mean value from 26 control and 3 to 5 extract injections and the error bar represents plus and minus the standard error of those values.

rabbit serum and 0.5 ml of 24% aqueous polyethylene glycol 6000 (J. T. Baker Chemical Co.). Anti-insulin serum, (125 I) insulin and (125 I) glucagon were from

Drs. A. H. Rubenstein and S. Kuku (the University of Chicago). All values reported were obtained by evaluation of experimental data against standard curves prepared using bovine insulin. The limit of detection of bovine insulin is 50 pg using this procedure.

Protein concentration was determined by the biuret method using albumin as the standard (LAYNE, 1957).

RESULTS AND DISCUSSION

Injections of the partially purified extract of royal jelly into young fifth instar *M. sexta* larvae resulted in a decrease in the level of hemolymph trehalose (hypoglycemia) as shown in the dose-response curve of Fig. 1. Concentrations of trehalose fell from a control value of 48 mM to 9 mM depending on the amount of royal jelly extract injected. This finding is consistent with and augments that of DIXIT and PATEL (1964) who demonstrated that the same secretion contains a promoter of carbohydrate utilization in mammalian adipose tissue.

In order to determine whether or not the royal jelly contains a peptide structurally related to the vertebrate hypoglycemic hormone insulin, an immunological examination specific for insulin-like polypeptides was performed on a protein extract of royal jelly that had been subjected to gel filtration on a column of polyacrylamide gel (Fig. 2). A rabbit antibody directed toward bovine insulin was used in the radioimmunoassay. Immunoreactive insulin was detected in

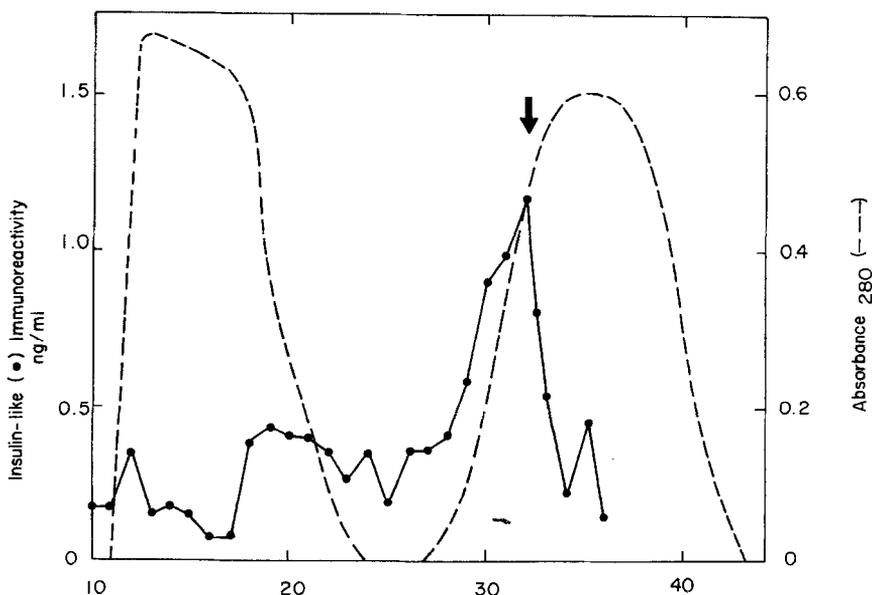


Fig. 2. Profile of insulin-like (—●—) immunoreactivity and absorbance at 280 nm (---) obtained after gel filtration of royal jelly from *Apis mellifera*. Lyophilized royal jelly equivalent to about 1 g of the fresh material was prepared for gel filtration by ether extraction as described in Methods. The conditions for gel filtration and the handling of samples for immunoassay are the same as those reported in TAGER *et al.* (1976). The solid arrow indicates the position taken by (125 I) glucagon, used as an internal column marker.

several peaks, the major one at fraction 32 representing 60% of the total activity. The elution volume of this major component is essentially that of bovine insulin, indicating the very similar mol. wt of these two peptides. The other reactive peptides were larger and might be related to precursor forms of the hypoglycemic factor. The amount of insulin-like peptide present in fractions 28 to 34 of Fig. 2 corresponds to about 15 ng equivalents of bovine insulin per gram of native royal jelly. DIXIT and PATEL (1964) reported about 14 times that amount based on biological activity, but a low immunological cross-reactivity or nonequivalent sample source might account for these apparently disparate results. Although ISHAY *et al.* (1976) were unable to detect immunoreactive insulin in the same secretion, they did not delipidate their samples or use an acidic buffer for solubilization before immunoassay. Insulin-like immunoreactivity was found however in various other hymenopteran tissues.

The function of the insulin-like peptide in royal jelly remains to be determined, especially with regard to whether or not it is indeed a hormone. Although the polypeptide has the biological activity of regulating carbohydrate metabolism in other animals, it has not been tested physiologically in the honeybee and we do not yet know if it is released into the hemolymph for delivery to peripheral tissues. Perhaps the insulin-like factor promotes growth and differentiation in the honeybee (cf. SEECOF and DEWHURST, 1974) or plays a nutritional role related to the high carbohydrate content of royal jelly which is a major foodstuff for larvae that develop into queen bees.

Insulin-like polypeptides have previously been reported in a number of other invertebrate species (FALKMER *et al.*, 1973) and have now been identified immunologically and biologically in three orders of Insecta: Hymenoptera (DIXIT and PATEL, 1964; ISHAY *et al.*, 1976; this work), Lepidoptera (TAGER *et al.*, 1976), and Diptera (SEECOF and DEWHURST, 1975; MENESES and ORTIZ, 1975; NORMANN, 1975). The

above results all contribute to the evidence for structural and functional similarities between the invertebrate hypoglycemic factor and the vertebrate counterpart.

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Key Word Index: Honeybee, Royal jelly, Insulin, Hypoglycemia, Immunoreactivity.