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# Isolation of Material Displaying Insulin-Like Immunological and Biological Activity from the Brain of the Blowfly *Calliphora vomitoria*

By Hanne DUVE,* Alan THORPE† and Norman R. LAZARUS‡
**Institute of General Zoology, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark, †Department of Zoology and Comparative Physiology, Queen Mary College, University of London, Mile End Road, London E1 4NS, and ‡Department of Biochemical Research, The Wellcome Foundation, Dartford, Kent, U.K.*

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An insulin-like material from the brain of the blowfly *Calliphora vomitoria* was partially purified by acid alcohol extraction, gel filtration and ion-exchange cellulose chromatography. In addition, the $R_F$ value on polyacrylamide-gel electrophoresis was determined. The material was characterized by its ability to cross-react with bovine insulin antibody and by displaying diminished immunoreactivity on dilution. It displaced specifically bound $^{125}$I-labelled insulin from rat liver plasma membrane insulin receptors and displayed insulin-like biological activity on the isolated rat fat-cell. Within 30min of injection into *Calliphora*, made hypertrehalocaemic and hyperglucaemic as a result of median neurosecretory cell removal, it caused the concentrations of both sugars to return to normal. The hypothesis is put forward that the median neurosecretory cells are the source of the material.

Over the past 10 years several reports have claimed that insects possess a hormone with insulin-like activity and, although it has been suggested that the hormone is present in gut tissues and haemolymph (Ishay *et al.*, 1976), the most convincing evidence for its localization has come from studies on the neurosecretory system (Normann, 1975; Tager *et al.*, 1976; Chen & Friedman, 1977; Duve, 1978). Removal of the median neurosecretory cells from the blowfly *Calliphora erythrocephala* causes hyperglucaemia and hypertrehalocaemia, which can be normalized by injection of both median neurosecretory cell homogenates and brain extracts (Duve, 1978). In the blowfly *Phormia regina*, homogenates of brain tissue returned to normal the high concentration of haemolymph trehalose induced by removal of the corpora cardiaca. Furthermore, severance of the nerves linking the median neurosecretory cells with the corpora cardiaca/corpora allata complex also caused hypertrehalocaemia, presumably because it prevented the release of the hypotrehalocaemic hormone (Chen & Friedman, 1977).

The most convincing biochemical evidence for the presence of a peptide immunologically and biologically similar to bovine insulin has come from the work of Tager *et al.* (1976), who extracted 200 corpora cardiaca/corpora allata complexes from *Manduca sexta*.

These physiological and biochemical results initiated the present study to define the insulin-like material in *Calliphora*.

## Experimental and Results

### *Insects*

About 120000 pupae of *Calliphora vomitoria* obtained from a commercial culture (Blackwater Bait Co., Stone, Essex, U.K.) were kept in large polyethylene tanks for eclosion to take place. The flies were fed *ad libitum* on a diet of sugar and water for 6 days at 25°C, during which time it is known they accumulate neurosecretory granules in the median neurosecretory cells (Thomsen, 1965).

### *Tissue extraction*

We decided to extract heads only as this would provide median-neurosecretory-cell material without interference from the hyperglycaemic hormone known to reside in the corpora cardiaca/corpora allata complex in the thorax (Steele, 1961; Normann & Duve, 1969). The flies were anaesthetized with $CO_2$ and frozen at −20°C in polythene bags. When the bags were shaken vigorously, the heads detached and could be collected by appropriate sieves. A total of 460g of fly heads was obtained.

The extraction procedure was carried out at 4°C. To eliminate contamination by mammalian insulin during the extraction and purification procedure all materials and chemicals used were new.

The heads were homogenized by using a Polytron blender in a medium of 760ml of ethanol acidified to pH2.5 with 20ml of conc. HCl. Water (250ml) was added to the slurry to give a 74% (v/v) acid/alcohol

solution, which was left stirring overnight. The homogenate was squeezed through a layer of muslin and the residue re-extracted in a further volume of acid/ethanol. The filtrate was centrifuged at 4°C for 20min at 40*g* and the resulting supernatant filtered through a Hyflo bed. The re-extracted material was treated in the same way and the combined filtrates were evaporated to remove the alcohol, leaving 260ml of a clear brown solution.

*Column chromatography*

Gel filtration of the tissue extract was carried out on a Sephadex G-25 column (4cm×90cm) eluted with 3M-acetic acid (40ml/h) at 4°C. Fractions (10ml) were collected and a protein-absorbance profile was obtained by spectrophotometric absorbance measurements at 280nm (Fig. 1). Duplicate 100$\mu$l samples of the main protein-containing fractions (fraction nos. 60–100) were freeze-dried and tested for insulin immunoreactivity in a double-antibody radioimmunoassay procedure by using insulin-binding reagent (Wellcome Reagents, Hither Green, Kent, U.K.), $^{125}$I-labelled bovine insulin (The Radiochemical Centre, Amersham, Bucks., U.K.) and bovine insulin standards. The absorbance after fraction 100 was found to be due to various eye pigments and these fractions were discarded.

Fractions containing insulin immunoreactivity (fraction nos. 64–86; Fig. 1) were pooled and freeze-dried. This material was dissolved in 1M-acetic acid and applied at a flow rate of 9ml/h to a Sephadex G-50 (superfine) column (1.5cm×40cm) at 4°C. Fractions (1.5ml) were collected and a protein-absorbance profile was obtained. Duplicate 100$\mu$l samples of each fraction were freeze-dried and immunoassayed for insulin. Three peaks of immunoreactivity were found (Fig. 2). The peak between fraction nos. 22 and 32 was coincident with a bovine insulin marker applied to a similar but different column to avoid contamination of the fly material. Immunoreactive material in this peak, which demonstrated diminishing immunoreactivity in the bovine immunoassay on dilution, was pooled and further treated. The peak between fractions 33 and 40 showed anomalous dilution behaviour, which was ascribed to interfering substances present in the salt peak. This peak was discarded. The peak of immunoreactivity in fractions 16–21 i.e. the column void volume, demonstrated diminishing immunoreactivity on dilution. This material was pooled and further treated.

The pooled fractions (16–21 and 22–32) were freeze-dried, dissolved in 0.04M-citric acid buffer, pH3.3, and separately applied at a flow rate of 9ml/h to Whatman CM-52 cellulose ion-exchange columns (0.9cm×30cm) equilibrated with the same

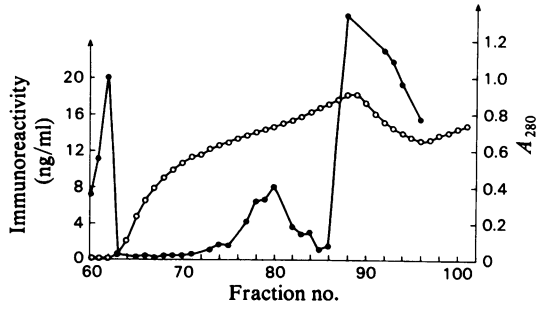


Fig. 1. *Profiles of insulin-immunoreactive material* (●) *and protein absorbance at* 280*nm* (○), *obtained after gel filtration of an extract of whole heads of C. vomitoria*

Heads (120000) of *C. vomitoria* were extracted with acid/ethanol and gel-filtered on a column (90cm×4cm) of Sephadex G-25 in 3M-acetic acid as described in the text. The flow rate was 40ml/h and the fraction size 10ml. Samples (100$\mu$l) of each fraction were freeze-dried and the resulting residues dissolved in buffer for immunoassay. Fractions containing insulin immunoreactivity (fraction nos. 64–86) were pooled, freeze-dried and applied to a Sephadex G-50 (superfine) column.

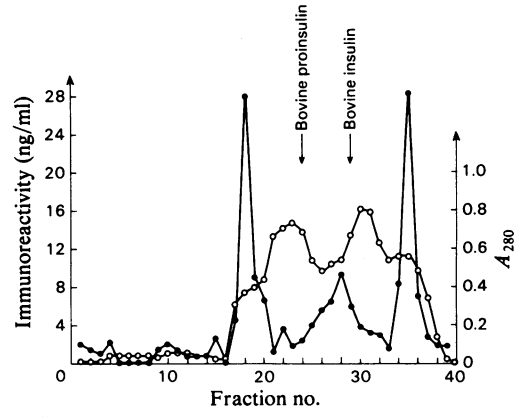


Fig. 2. *Profiles of insulin-immunoreactive material* (●) *and protein absorbance at* 280*nm* (○), *obtained after gel filtration*

Freeze-dried material (fraction nos. 64–86, Fig. 1) was dissolved in 1M-acetic acid and applied to a Sephadex G-50 (superfine) column (1.5cm×40cm) at a flow rate of 9ml/h (fraction size 1.5ml). Samples (100$\mu$l) of each fraction were freeze-dried and taken up in buffer for immunoassay. Three immunoreactive peaks were obtained. Peaks at fractions 16–21 and fractions 22–32 were freeze-dried for further purification. The peak at fractions 16–21 indicated the void volume and the peak at fractions 33–38 is the salt peak. The arrows indicate the elution positions for bovine proinsulin and bovine insulin obtained on a separate column to eliminate contamination.

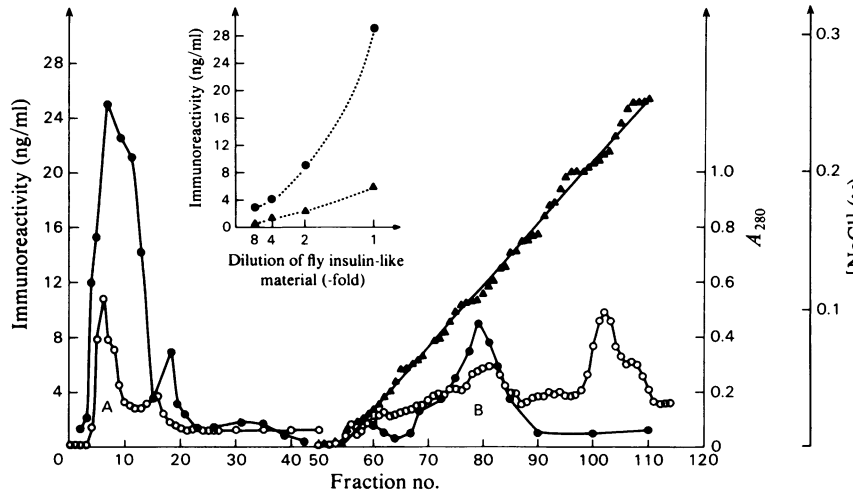


Fig. 3. *Profiles of insulin-immunoreactive material* (●), *protein absorbance at* 280*nm* (○) *and NaCl gradient* (0–0.3 M) (▲) *obtained after CM-52 ion-exchange chromatography*

Freeze-dried material (fractions 22–32, Fig. 2) was dissolved in 0.04 M-citric acid buffer, pH 3.3, and applied to an ion-exchange column (0.9 cm × 30 cm) at a flow rate of 9 ml/h (fraction size 3 ml). The inset shows the immunoreactive characteristics obtained on dilution of fractions 7 (●) and 79 (▲).

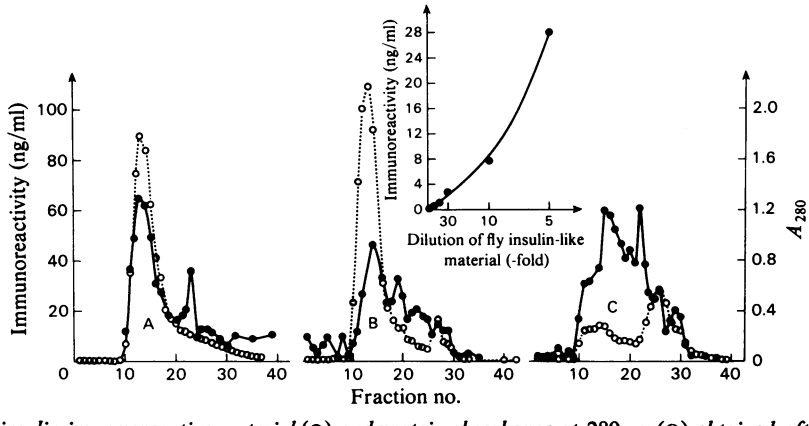


Fig. 4. *Profiles of insulin-immunoreactive material* (●) *and protein absorbance at* 280*nm* (○) *obtained after gel filtration on Sephadex G-25 in* 1 M-*acetic acid*

Peak A was obtained from the pooled freeze-dried fractions 3–15 (Fig. 3), peak B from pooled fractions 75–85 (Fig. 3) and peak C from ion-exchange chromatography of immunoreactive material from the void volume (fractions 16–21, Fig. 2). Column size was 0.9 cm × 30 cm at a flow rate of 1 ml/min (fraction size 1 ml). Samples of 50 $\mu$l were freeze-dried for insulin radioimmunoassay. The inset shows the immunoreactive characteristics obtained on dilution of fraction 17, peak C.

buffer at 4°C. Fractions (3 ml) were collected. Fig. 3 shows the protein and immunological profile obtained from the pool of fractions 22–32 (Fig. 2). When a stable absorbance baseline had been established after the initial breakthrough of protein material, an NaCl gradient (200 ml; 0–0.3 M) was applied. Immunoreactive material was detected in the protein peak obtained before the salt gradient was started. This material demonstrated diminishing immunological reaction on dilution and was pooled (peak A). The immunoreactive peak eluting between tubes 72 and 87 was also pooled (peak B). Fractions 16–21 from Fig. 2 were similarly chromatographed on CM-52 cellulose. One immunoreactive protein peak was

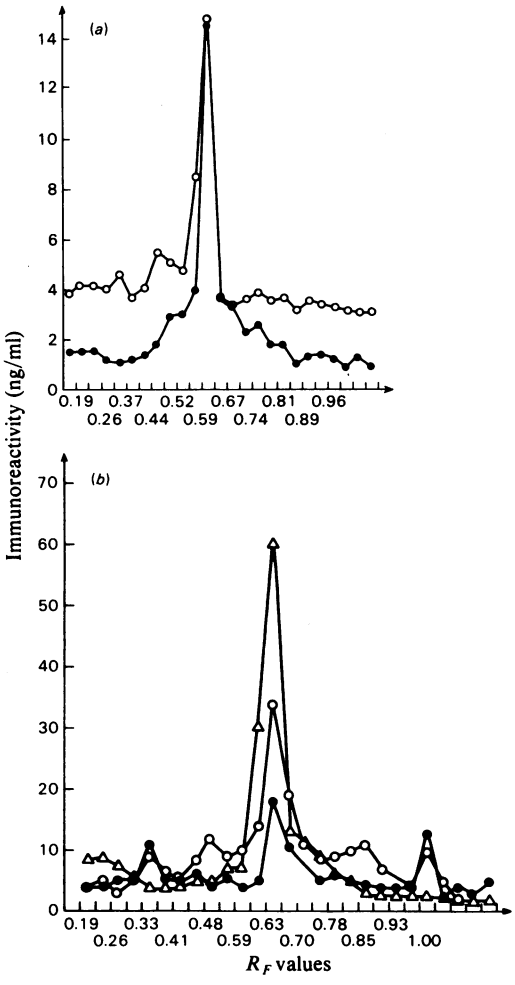


Fig. 5. *Profiles of insulin-immunoreactive material after polyacrylamide-gel electrophoresis of fly insulin-like extract*
(*a*) Profile obtained from fraction 16, peak C (Fig. 4) (●). The gel was sliced into 1.5 mm sections, extracted with acid/alcohol, freeze-dried and immunossayed as described in the text. The immunoreactive material was localized in a peak with an $R_F$ value of 0.58. A second profile (○) was produced by electrophoresing the gel slice corresponding to an $R_F$ value of 0.58 obtained by running a gel in parallel to that described above. (*b*) Profiles showing that fly insulin-like material (●), beef insulin (△) and a mixture of both materials (○) have the same $R_F$ value.

obtained, eluting in a position similar to peak B (Fig. 3). This material was pooled and labelled peak C. Each of the pooled peaks (A, B and C) was freeze-dried, separately dissolved in 1 M-acetic acid and applied to a Sephadex G-25 column (0.9 cm × 30 cm). Fractions (1 ml) were collected and assayed for protein and immunoreactivity (150 μl samples). Fractions from the main immunoreactive peaks showed decreasing immunological cross-reaction on dilution (see e.g. inset in Fig. 4) and were subsequently used for determination of physical characteristics and bioassays.

*Polyacrylamide-gel electrophoresis*

Fractions from the final Sephadex G-25 column were subjected to polyacrylamide-gel electrophoresis, pH 8.9. Selected 1 ml freeze-dried fractions from peaks A, B and C (Fig. 4) were dissolved in 20% sucrose buffer + Bromophenol Blue such that a 50–75 μl sample, when applied to the gel, would contain a maximum of 400–500 μg of protein. The running gel was modified to contain 14.2% acrylamide and 0.8% (w/v) bisacrylamide as ordinary acrylamide gels tended to dissolve in the acid/ethanol subsequently used to elute the immunoreactive protein band.

Gels were electrophoresed in triplicate, one being stained, one immunoassayed and one kept frozen (−80°C) for subsequent electrophoresis. The gels used for immunoassay and deep-freezing were cut serially into 1.5 mm slices. The gel slices for immunoassay were eluted in 200 μl of acid/ethanol overnight. The gel pieces were removed and the eluate freeze-dried and dissolved in 100 μl of Clarke–Lubb buffer and immunoassayed (Fig. 5*a*). When the position of the immunoreactive-protein band had been established, the appropriate slices from the frozen gel were electrophoresed on a new gel. As before, the gel was then cut into 1.5 mm slices and subjected to immunoassay. The immunoreactive band was shown to have

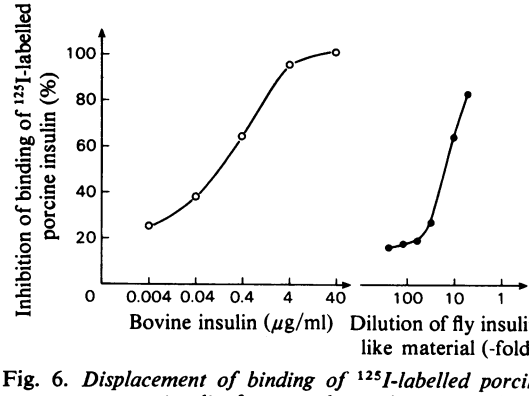


Fig. 6. *Displacement of binding of $^{125}$I-labelled porcine monocomponent insulin from rat liver plasma membrane insulin receptors by fly insulin-like material* (●) *and bovine insulin* (○)
Each point is the mean for triplicate determinations from fraction 17, peak C (Fig. 4). A value of 82% for inhibition of binding was obtained by a 5-fold dilution of fly insulin-like material.

the same $R_F$ value (0.58) as determined in the first gel. In a subsequent experiment an immunoreactive fraction and a sample of bovine insulin were electrophoresed both separately and together. The $R_F$ values of the immunoreactive bands were seen to be identical (Fig. 5*b*).

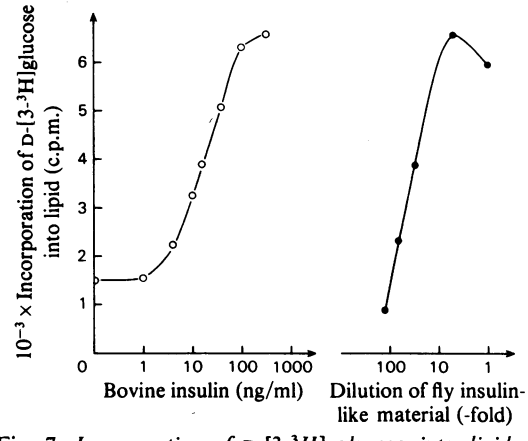


Fig. 7. *Incorporation of* D-[3-$^3H$] *glucose into lipid in isolated rat epididymal fat-cells*
The effect of fly insulin-like material (●; fraction 15, peak A, Fig. 4) and bovine insulin (○) is shown. At a 5-fold dilution, fly insulin-like material produced the same maximal biological activity as bovine insulin (100ng/ml).

*Displacement of specifically-bound $^{125}I$-labelled insulin from liver plasma membrane receptors*

$^{125}$I-labelled porcine monocomponent insulin was specifically bound to liver plasma membrane insulin receptors by the method of Baxter & Lazarus (1975), as modified from the method of Cuatrecasas (1971), and its displacement by fly immunoreactive material compared with bovine insulin (Fig. 6). Displacement by 80% was obtained with a 5-fold dilution of fly extract (fraction 17, peak C of Fig. 4). A comparison of the curves obtained shows that the two materials behave differently when displacing $^{125}$I-labelled insulin from its receptor.

*Incorporation of* D-[3-$^3H$]*glucose into lipid by isolated rat epididymal fat-cells*

Rat epididymal fat-cells were prepared by the method of Moody *et al.* (1974) and an immunoreactive freeze-dried fraction (15, peak A, Fig. 4) was

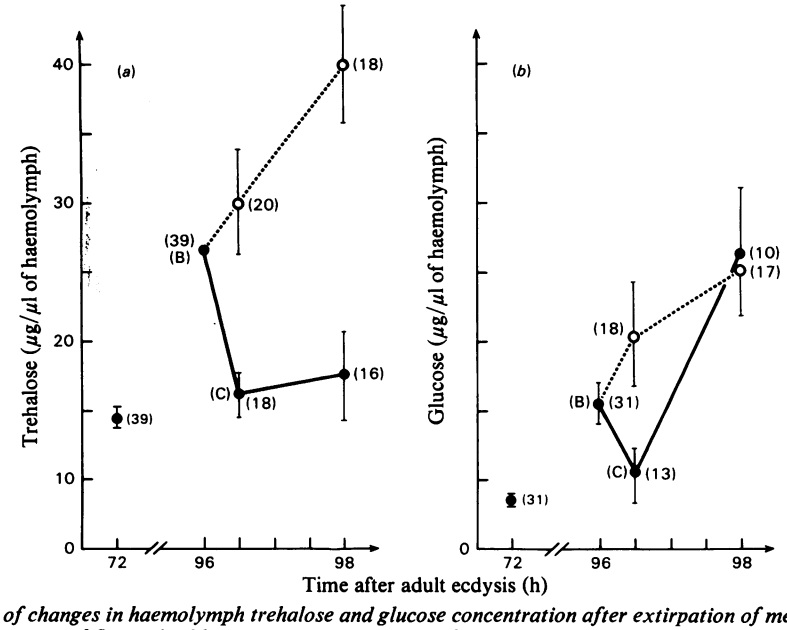


Fig. 8. *Time course of changes in haemolymph trehalose and glucose concentration after extirpation of median neurosecretory cells and injection of fly insulin-like material (approx. 1 ng of bovine insulin equivalents) into female blowflies*
Median neurosecretory cells were removed 72h after adult ecdysis. Fly insulin-like material (●) or carrier solvent (○) was injected at 96h when the flies were hypertrehalocaemic (*a*) and hyperglucaemic (*b*). A return to normal concentration was seen in both sugars after 30min. The hypotrehalocaemia persisted for 2h, whereas the hypoglucaemia was transient. Each point has been normalized as described by Duve (1978). The bars represent ± S.E.M. The numbers of insects used are shown in parentheses. When unnormalized values (B) are compared and tested against the values (C) in the Wilcoxon matched-pairs signed-ranks test (Siegel, 1956), values are significant for trehalose at $P<0.005$, and for glucose at $P<0.01$.

assayed against bovine insulin standards. A 5-fold dilution of fly extract gave the same maximal biological activity as 100ng of bovine insulin/ml (Fig. 7).

*Trehalocaemic and glucaemic assays in Calliphora in vivo*

The median neurosecretory cells were removed from *C. vomitoria* and the flies left to recover for 24h (Duve, 1978). Trehalose concentrations increased from a control value of 15$\mu$g/$\mu$l to 27$\mu$g/$\mu$l. Injection of insulin-like material (fractions 18 and 19, peak B, Fig. 4) dissolved in 0.001 M-HCl (approx. 1ng of bovine insulin equivalents) caused the trehalose to return to a normal concentration within 30min. This concentration was maintained for 2h. Injection of carrier solvent into control flies did not halt the developing hypertrehalocaemia (Fig. 8*a*). Similar but more transient effects were observed with glucose (Fig. 8*b*).

## Discussion

The results suggest that a material having insulin-like properties is present in the head of *C. vomitoria*. This conclusion is based on the following data. The material, which was isolated by an extraction and purification procedure designed for insulin, cross-reacted with bovine insulin antibody and exhibited an $R_F$ value identical with bovine insulin on polyacrylamide-gel electrophoresis. It displaced specifically bound $^{125}$I-labelled insulin from its liver plasma membrane receptor and displayed insulin-like biological activity on the isolated rat fat-cell. When injected into *Calliphora*, made hyperglucaemic and hypertrehalocaemic as a result of median-neurosecretory-cell removal, it caused the concentrations of both sugars to return to normal within 30min. In addition, evidence for the existence of such a hormone comes from already published physiological and biochemical studies (Normann, 1975; Tager *et al.*, 1976; Chen & Friedman, 1977; Duve, 1978).

The precise location of the hormone in *Calliphora* is unknown. We extracted the heads because the work of Duve (1978) had suggested that the hormone was located in the median neurosecretory cells. Tager *et al.* (1976) showed that the lepidopteran *Manduca sexta* possessed both insulin-like and glucagon-like peptides in the corpora cardiaca/corpora allata complex. The exact anatomical and physiological relationship of the median neurosecretory cells to the corpora cardiaca/corpora allata complex is not known in the Diptera. In most other insects including the Lepidoptera, axons from the median neurosecretory cells carry neurosecretory material to the corpora cardiaca and corpora allata for release. It is probable, therefore, that Tager and his co-workers were investigating the same material that we are reporting in the present paper, except that they isolated stored hormone and we have isolated newly synthesized hormone. It seems reasonable to postulate that in *Calliphora* insulin-like material originates from the perikarya of the median neurosecretory cells, from where it is either transmitted to the corpora cardiaca/corpora allata complex for storage, or to the aorta for release into the haemolymph. Supporting evidence for the localization of an insulin-like material within the perikarya of the median neurosecretory cells comes from immunofluorescence studies of *Calliphora* in which it was shown that material cross-reacting with antibovine insulin serum was present in some of the cells (Duve & Thorpe, 1979).

There are, however, references in the literature to the presence of insulin-like material in other insect tissues. Meneses & Ortiz (1975) showed that whole *Drosophila* extracts had insulin-like hypoglycaemic activity in mice. Dixit & Patel (1964) and, more recently, Kramer *et al.* (1977) have both shown that honey-bee royal jelly contains insulin-like hypoglycaemic and immunological activity. In these studies, however, the source of the insulin-like hormone has not been demonstrated and it could be that the hormone in these insects is produced in the neuroendocrine tissue.

The *Calliphora* insulin-like material was contaminated with protein. Aware that this protein might produce artefactual biological activity we designed experiments to overcome this contamination. The results on polyacrylamide-gel electrophoresis had shown that the immunoreactive material could be separated from the protein since it occurred in a slice that did not coincide with a stained protein band. The re-run of this slice on polyacrylamide-gel electrophoresis showed an immunoreactive band having an identical $R_F$ value with the original material, showing that the immunoreactivity resided in a very small fraction of the total protein. Further evidence that the contaminating protein was not responsible for biological activity came from the finding that peaks A, B and C demonstrated equivalence of immunological and biological activity despite a 10-fold difference in protein contamination (Fig. 4; peaks A, B and C).

In testing for biological activity we decided to use only the material that gave diminishing immunoreactivity on dilution. The fact that the dilution curves were non-linear suggests that *Calliphora*-immunoreactive material, although related to bovine insulin, is not immunologically identical with it (Fig. 4*c*).

Most insulins and insulin analogues when tested in the membrane-binding assays produce displacement curves having identical slopes. Some derivatives of insulin, e.g. desalanine,desasparagine-insulin and desoctapeptide-insulin, produce steeper competition curves for inhibition of $^{125}$I-labelled insulin binding

because of an overall change in their conformations (De Meyts *et al.*, 1978). Since *Calliphora* insulin-like material produces displacement curves similar to these derivatives, *Calliphora* material is probably conformationally different from bovine insulin.

*Calliphora* insulin-like material stimulated rat fat-cells to the same maximum value as bovine insulin as judged by the incorporation of [$^3$H]glucose into lipid. The response curves produced were different and although we are unable to explain this difference it must be remembered that the assay is composed of several components e.g. transport as well as many other metabolic reactions.

The assays on *Calliphora in vivo* are in agreement with previous experiments with median-neurosecretory-cell homogenates (Duve, 1978) and suggest a physiological role for the hormone in carbohydrate metabolism. The median neurosecretory cells (20–26 in total) have been shown to be diverse in both form (Thomsen & Lea, 1969; Panov, 1976) and function (Chapman, 1971). Among the functions known to be influenced by a hormone from the median neurosecretory cells in *Schistocerca* is increased protein synthesis during ovarian development (Hill, 1962). It could be that, as in vertebrates, protein and perhaps lipid anabolism are both controlled by an insulin-like material in addition to its effect on carbohydrates.

The insulin-like material characterized in *Calliphora* has marked similarities to mammalian insulin immunologically, physically and biologically, and it is noteworthy that these properties have been preserved during evolution. It may be thought surprising that insulin-like material is found in neurosecretory cells, but if the hypothesis that alimentary peptide-hormone-producing cells have a common origin in ontogeny in the neural ectoderm is correct (Pearse, 1969; Pearse & Takor Takor, 1976) then it follows that in early evolutionary forms insulin may be found in neural tissue. Furthermore, Barrington (1978) has suggested that some of the alimentary peptide hormones may have originated as neural transmitters or modulators. The finding of insulin-like material in insect neurosecretory cells is in accord with this idea.

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