Insulin in Insects and Annelids

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SUMMARY

The fruitfly, *Drosophila melanogaster,* and the earthworm, *Annelida oligocheta,* were extracted with acid-ethanol by a classic method for recovering insulin from the pancreas. When each extract was filtered on a Sephadex G-50 column, a distinct peak of insulin immunoreactivity (equivalent to 0.1 to 2 ng of insulin/g wet weight) was recovered in the region typical of insulin. The material in this peak had reactivity in the insulin bioassay, measuring stimulation of glucose oxidation or lipogenesis by isolated rat adipocytes. The bioactivity was partially or largely neutralized by anti-insulin antibodies. In concordance with previous work showing the presence of material very similar to insulin in the blowfly and molluscs, we have confirmed the presence of insulin in insects and extended the observation to earthworms. These findings suggest that insulin is more widespread in invertebrates than was previously thought. In a companion study (Proc. Natl. Acad. Sci. USA 77:6184–88, 1980), we have demonstrated material similar to insulin in unicellular organisms. DIABETES *30*:70–76, January 1981.

Though insulin is classically synthesized in the β-cells of the pancreas, recent reports have described material that is very similar to insulin in gut-derived cells of primitive vertebrates and complex invertebrates, and in neural elements of mammals and insects.[1–10]

In the present study, we confirm the presence of material that is very similar to insulin in drosophila heads. In addition, we find similar material in extracts of drosophila bodies and in extracts of both the skin and internal structures of the earthworm. These findings suggest that insulin is much more widespread among the tissues of invertebrates than was previously thought, and not only in gut- or nervous system-derived cells. In addition, we have also shown, in a separate study, the presence of similar material in unicellular organisms, the ciliated protozoan *Tetrahymena pyriformis* and the unicellular fungus *Neurospora crassa.*[11]

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MATERIALS AND METHODS

Larvae of the fruitfly, *Drosophila melanogaster,* were maintained at room temperature on medium 4-24 (Carolina Biological Supply Company), which contained yeast, flour, sucrose, minerals, and vitamins. Adult flies were anesthetized with ether, the heads separated from the bodies, and stored at −70°C until extraction. The interior organs ("inside") of adult earthworms, *Annelida oligocheta* (Carolina Biological Supply Co.), were dissected out from the skin ("outside") and both were stored at −70°C.

Extraction of insulin. A standard extraction procedure was used[12] as previously described,[9,13] except that no albumin was added at any stage. The organisms were weighed and homogenized with a Brinkman polytron in 10 vol of ice-cold acid-ethanol (0.2 N HCl, 75% ethanol). The suspension was mixed overnight at 4°C. After centrifugation at 1500 × *g* for 20 min at 4°C, the precipitate was discarded, and the supernatant was concentrated by air evaporation at room temperature, resuspended in 5 vol of 0.05 M $(NH_4)_2CO_3$, and neutralized with concentrated NH_4OH. After centrifugation at 1500 × *g* for 20 min at 4°C, the precipitate was discarded, and the supernatant was lyophilized and reconstituted with distilled water.

The reconstituted extract was applied to a column of Sephadex G-50 (medium) and eluted with 0.05 M $(NH_4)_2CO_3$. Each effluent fraction was lyophilized and reconstituted to 1 ml with distilled water. The insulin content of each fraction was determined by radioimmunoassay at 1:10 (final dilution) of the sample. The fractions corresponding to the peak of insulin immunoactivity were pooled, lyophilized, and reconstituted with distilled water. The reconstituted pool was then tested for bioactivity and reassayed for immunoreactivity.

Insulin radioimmunoassay. Porcine insulin was purchased from Eli Lilly; rat insulin was a gift of Dr. R. E. Chance of Lilly Research Laboratories. Guinea pig anti-porcine insulin sera, designated 68 (gift of Dr. A. Kagan) and 619 (purchased from the Department of Pharmacology, Indiana University, Indianapolis), were both used in the radioimmunoassy, whereas only 619 was used to neutralize the insulin effect in the bioassays. The insulin radioimmunoassay was performed by standard methods using ^{125}I-labeled porcine insulin as the tracer and rabbit anti-guinea pig serum as the second antibody.[14,15] Both antisera, 619 and 68, gave identical results in the radioimmunoassays when tested with the various extracts.

Controls for RIA. To exclude the possibility that the apparent reactivity of the gel-filtered extracts in the immunoassay could be due to interference in the assay (e.g., degradation of ^{125}I-insulin or inhibition of the immunoprecipitation), two types of experiments were performed. In the first, at the end of 4 days of incubation the precipitates were separated from the supernatants, and the supernatants were added to TCA (5% final concentration); at least 92% of the radioactivity was precipitated in both the extracts and in the control (extract-free) samples. In the second, duplicate samples of extract (or of control) were incubated in the radioimmunoassay in the usual way. After 72 h, an excess (1:1000) of anti-insulin antibody was added to one sample of each extract and each control. Twelve hours later, the goat anti-guinea pig antibody was added to both samples to precipitate the antibody-bound insulin in the usual way. With excess antibody, more than 90% of the labeled insulin was precipitated in both the extract and control samples.

Insulin bioassays. Biologic activities of extracts were measured either as glucose oxidation, i.e., conversion of [U-^{14}C]glucose to $^{14}CO_2$,[16] or lipogenesis, i.e., incorporation of [3-^{3}H] glucose into toluene-extractable lipids[17] by isolated fat cells prepared from epididymal fat pads of young Sprague-Dawley rats. To show that the bioactive molecules were reactive with anti-insulin antibody, duplicate aliquots were mixed with a 1:100 dilution of guinea pig anti-porcine insulin antibody 619 or normal guinea pig serum before their addition to the bioassay. Normal guinea pig serum at this concentration had no effect on the bioassay.

RESULTS

Drosophila. When acid-ethanol extracts of drosophila were gel filtered on Sephadex G-50, insulin immunoreactivity was recovered in a discrete peak in the region typical of that for mammalian insulins (Figure 1A, Table 1) and was equivalent to a few ng of insulin/g wet weight. The effluent fractions that corresponded to the insulin peak, when diluted in the radioimmunoassay, gave results very similar to the mammalian insulins (Figure 1B). Results obtained with isolated heads were about twice those obtained with the rest of the drosophila (labeled "body" in Table 1). Aliquots of the gel-filtered material, when tested with isolated rat adipocytes, stimulated glucose oxidation as well as lipogenesis, two classical responses to insulin (Figure 1C, Table 1). The magnitude of the biologic effect of the extract was equivalent to its content of immunoreactive insulin, and the biologic effect was largely neutralized by the addition of anti-insulin antibody to the bioassay system.

Earthworm. When acid-ethanol extracts of earthworm were gel filtered on Sephadex G-50, insulin immunoreactivity (equivalent to a few hundred pg of insulin/g wet weight) was recovered in the insulin region (Figure 2A), though the peaks were not as neatly defined as those with drosophila. In addition, the total amount of immunoreactivity recovered after gel filtration varied widely from preparation to preparation (Table 2). The material recovered in the insulin region after gel filtration, on serial dilution in the immunoassay, gave results similar to those obtained with purified mammalian insulins (Figure 2B). Furthermore, aliquots from the gel-filtered material stimulated glucose oxidation and glucose incorporation into lipids in isolated rat adipocytes, and the

TABLE 1
Insulin extracts from *Drosophila melanogaster*

Batch	Wet weight (g)	Insulin (ng) recovered in effluent from G-50 Sephadex column: Column fractions (RIA)	Pooled peak (RIA)	Pooled peak (bioassay)	Antibody neutralization (% decrease)
1. Whole	4.0	10.5	9.1	7.2*	75%
2. Whole	2.7	13.2	10.5	13.5†	NT
3. Whole	2.0	<1.2	1.0	1.0†	90%
4. Head	0.03	1.2	0.5	NT	NT
Body	0.15	2.0	1.4	NT	NT

Results of four separate experiments are shown. Acid-ethanol extracts were filtered on a column of G-50 Sephadex, and the immunoreactivity of each fraction was determined (see Figure 1A and legend). "Column fractions" represent the sum of the immunoreactive insulin content of the individual effluent fractions that correspond in position to the peak of mammalian insulin. The fractions closest to the middle of this peak were pooled, lyophilized, and reconstituted in a small volume; an aliquot was measured in the radioimmunoassay (pooled peak—RIA) at one or more dilutions and the remainder was measured in the bioassay (pooled peak—bioassay). The bioactivity of some aliquots was also measured in the presence of anti-insulin antibody. The method for calculating the percent decrease in bioactivity in the presence of anti-insulin antibody is described in the legend to Figure 1. NT indicates that the sample was not tested.
* Indicates glucose oxidation.
† Indicates lipogenesis.

TABLE 2
Insulin extracts from earthworm (*Annelida oligocheta*)

Batch	Wet weight (g)	Insulin (ng) recovered in effluent from G-50 Sephadex column: Column fractions (RIA)	Pooled peak (RIA)	Pooled peak (bioassay)	Antibody neutralization (% decrease)
1. Outside	4	3.8	2.4	1.0*	45%
Inside	8	<1.2	1.0	4.0*	50%
2. Outside	42	12.5	9.0	6.0*	30%
Inside	22	<1.2	1.0	1.8*	50%
3. Whole	38	10.4	6.0	3.9†	NT
4. Whole	53	7.8	3.0	5.0†	50%

Results of four separate experiments, similar to those in Figure 2, are shown. The acid-ethanol extracts were reconstituted in a volume equal to about 5% of the total volume of the column. The data are expressed as described in the legend to Table 1.
* Indicates glucose oxidation.
† Indicates lipogenesis.

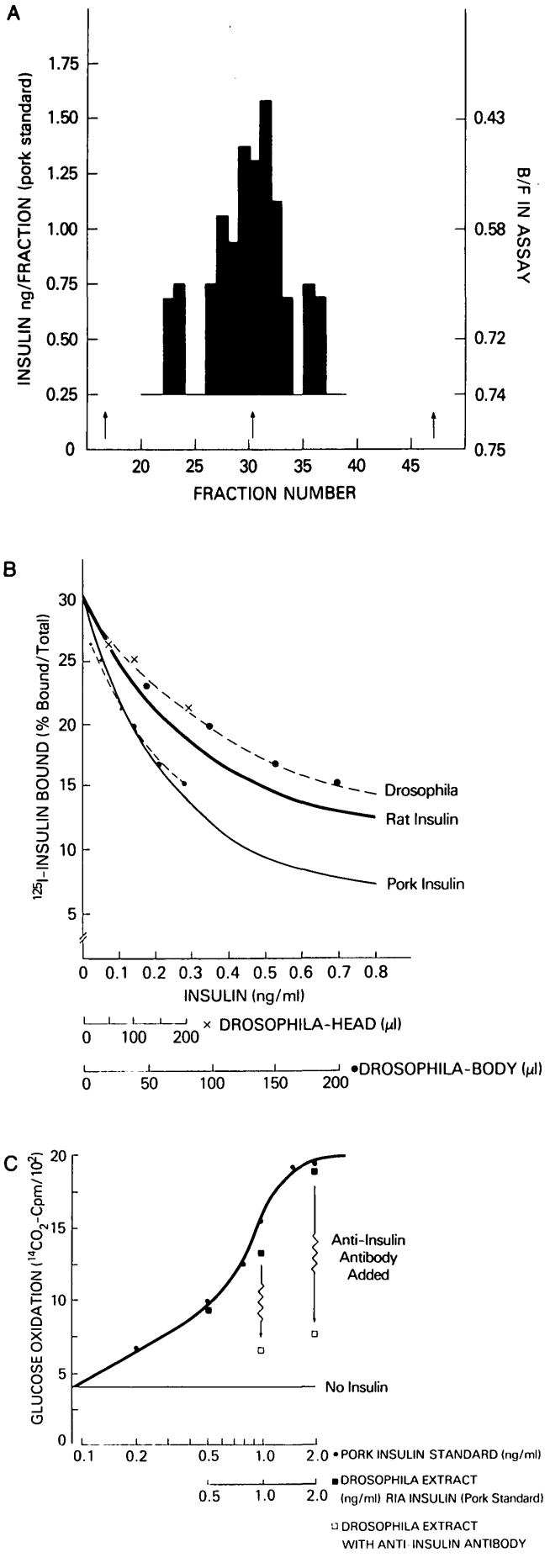

FIGURE 1. Drosophila extracts.

A. Gel filtration. An acid-ethanol extract of drosophila (batch 1 in Table 1) was filtered on a column of Sephadex G-50 (1.5 × 60 cm; 1-ml fractions), and the immunoreactive insulin was measured directly at a 1:10 final dilution in the assay. The horizontal line, equivalent to 0.25 ng insulin, indicates the sensitivity of the assay and the fractions that were tested. The arrow at the left indicates the void volume (^{125}I-thyroglobulin), the center arrow indicates the ^{125}I-insulin peak, and the arrow at the right marks the salt peak (^{125}I).

B. Radioimmunoassay. Extracts of head and body (batch 4 in Table 1) were each gel filtered and the five fractions from each column that corresponded to the peak of immunoactive insulin were separately pooled, lyophilized, and reconstituted in 0.5 ml of distilled water. An individual aliquot of each pool is represented by one point (X "head", • "body"). The solid lines represent the pork insulin and rat insulin standards, expressed as ng of insulin/ml. Since the lines for all purified insulins (pork, rat, etc.) appear to converge as the insulin concentration approaches zero, we set the scale for drosophila so that its line (heavy broken line, bold points) best approximates this convergence (to give an estimate of the absolute content of insulin). For comparison, we have included the same data on another line (light broken line, small points) where the drosophila data fit best with the pork insulin line to illustrate the closeness of cross-reactivity of the drosophila with pork insulin. Because the second line fits so closely to the pork insulin curve, we have simply expressed the insulin content of drosophila extracts in terms of the pork insulin standard curve.

C. Bioassay. After gel filtration (batch 1 in Table 1), the five fractions corresponding to the insulin peak were pooled, lyophilized, and reconstituted in 0.5 ml of distilled water. The reconstituted material was reassayed in the radioimmunoassay (RIA) and tested for biologic activity by measuring the conversion of (U-^{14}C) glucose to $^{14}CO_2$ by isolated rat adipocytes. The $^{14}CO_2$ produced is plotted as a function of the insulin concentration for the porcine insulin standard (●——●) or an equivalent amount of drosophila (■) insulin (determined by RIA against a pork insulin standard). The solid horizontal line in the graph represents the $^{14}CO_2$ produced in the absence of insulin. The open squares (□) indicate results with drosophila insulin in the presence of an excess of anti-insulin antibody.

To quantitate the degree of neutralization by anti-insulin antibody (as reported in Tables 1 and 2), the bioactivity of the sample assayed in the absence of antibody, measured against the pork insulin standard, was compared with the bioactivity obtained from the same sample in the presence of anti-insulin antibody, again measured against the pork insulin standard. For example, in this figure the drosophila extract, which had the equivalent of 2 ng/ml of RIA insulin when measured against the pork insulin standard, had bioactivity of 1.5 ng/ml of insulin in the absence of antibody. In the presence of anti-insulin antibody, the same sample had bioactivity equivalent to 0.3 ng/ml of pork insulin, which we designate as 80% neutralization. The drosophila extract that had 1 ng/ml of RIA insulin had the equivalent of 0.8 ng/ml of bioactivity on the pork insulin standard curve in the absence of antibody and had the equivalent of 0.2 ng/ml of pork insulin bioactivity in the presence of antibody yielding a 75% neutralization.

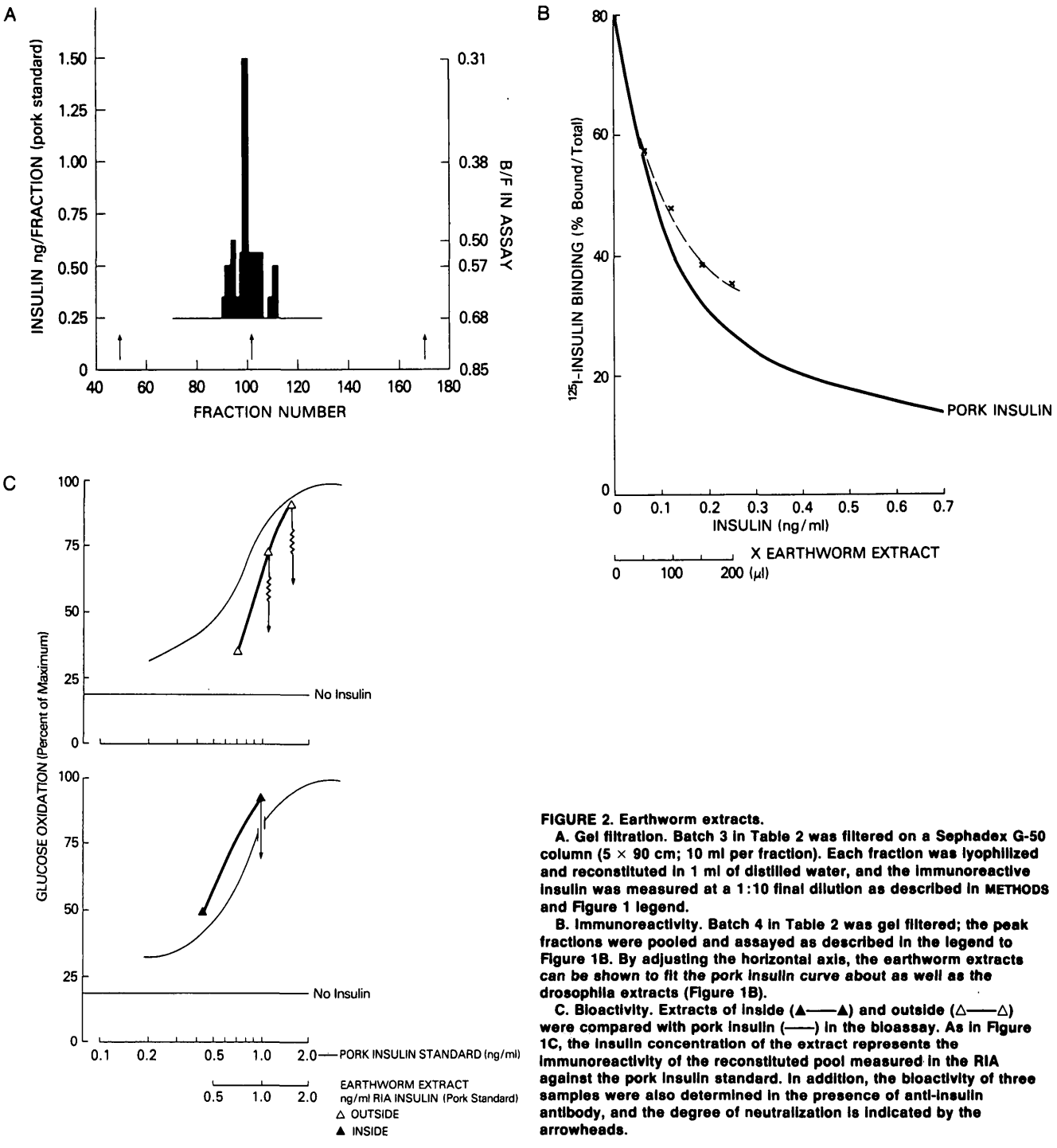

FIGURE 2. Earthworm extracts.

A. Gel filtration. Batch 3 in Table 2 was filtered on a Sephadex G-50 column (5 × 90 cm; 10 ml per fraction). Each fraction was lyophilized and reconstituted in 1 ml of distilled water, and the immunoreactive insulin was measured at a 1:10 final dilution as described in METHODS and Figure 1 legend.

B. Immunoreactivity. Batch 4 in Table 2 was gel filtered; the peak fractions were pooled and assayed as described in the legend to Figure 1B. By adjusting the horizontal axis, the earthworm extracts can be shown to fit the pork insulin curve about as well as the drosophila extracts (Figure 1B).

C. Bioactivity. Extracts of inside (▲——▲) and outside (△——△) were compared with pork insulin (——) in the bioassay. As in Figure 1C, the insulin concentration of the extract represents the immunoreactivity of the reconstituted pool measured in the RIA against the pork insulin standard. In addition, the bioactivity of three samples were also determined in the presence of anti-insulin antibody, and the degree of neutralization is indicated by the arrowheads.

magnitude of the effect was appropriate for the amount of immunoreactivity in the preparations (Figure 2C). Anti-insulin antibodies reduced the bioactivity substantially (Figure 2C, Table 2) but not as effectively as with drosophila extracts (see DISCUSSION). When the skin ("outside") was separated from the rest of the body ("inside"), there appeared to be somewhat more insulin in the skin.

DISCUSSION

The specificity of the insulin radioimmunoassay has been characterized very extensively. To react in the assay, a necessary (but not sufficient) condition is the presence of both A- and B-chain joined by disulphide bridges; isolated A- and B-chains (sulphonated or carboxymethylated) even from homologous species, alone or together, are nonreactive.[18–20] Even molecules that contain the overall structure of insulin and that can react with insulin receptors to produce the characteristic biologic effects of insulin in vivo and in vitro may be nonreactive in the radioimmunoassay; insulin-like growth factors I and II, whose A- and B-chain regions have 50% of their amino acids in common with pork insulin, are totally nonreactive in this RIA, and guinea pig insulin,

which has 65% homology with pork insulin, has trivial reactivity.[21] Even insulins with biopotencies that are nearly the same as pork insulin may be poorly reactive in the immunoassay, e.g., fish insulins and avian insulins with about 70% and 90% homology, respectively, are much less reactive in the pork insulin radioimmunoassay than in the bioassay for insulin (Figure 3). No material has been found that reacts specifically in this assay that is not very closely related structurally to intact insulin.

That our material reacts specifically (experiments to exclude nonspecific effects are described in MATERIALS AND METHODS) in the radioimmunoassay for pork insulin suggests that it is very similar to pork insulin or proinsulin. That the immunoreactivity is recovered in a peak in the same place as insulin suggests that its size-shape is very similar and unlikely to be proinsulin or proinsulin-like.

As suggested earlier in this DISCUSSION, the regions of the insulin molecule responsible for bioactivity are distinct from those required for immunoreactivity. That our material is reactive in the bioassay suggests that it contains the specific regions needed for bioactivity, i.e., regions on the insulin molecule that are needed for binding to receptor ("affinity") and regions required for activation of the target cell ("intrinsic activity"). That anti-insulin antibody neutralizes the bioactivity suggests that the immunoreactive site and bioactive site(s) are on the same molecular species.

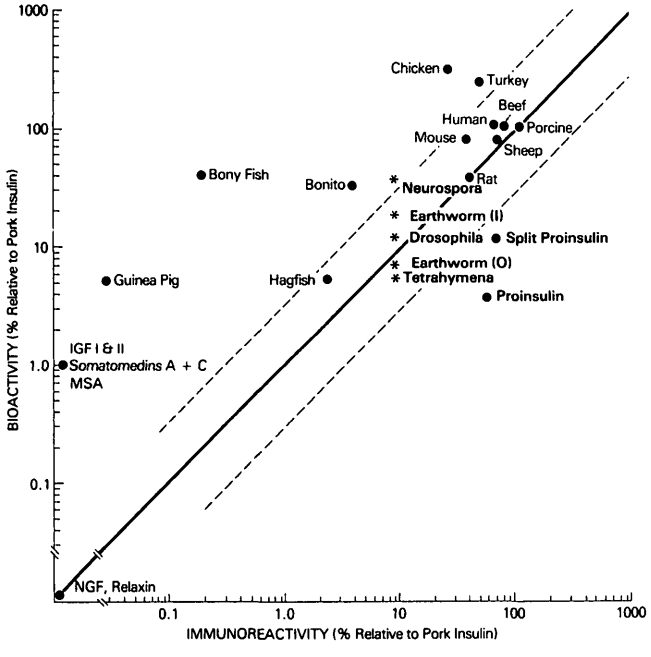

FIGURE 3. The bioactivity (relative to pork insulin) of insulins and insulin-related peptides are plotted as a function of their immunoreactivity (relative to pork insulin) in the pork insulin radioimmunoassay. The heavy diagonal line represents bioactivity/immunoreactivity equal to one. The upper dashed line represents bio/immuno equal to 3 and the lower dashed line represents bio/immuno equal to ⅓. NGF and relaxin, despite their structural similarities to insulin, are unreactive in both assay systems. The IGFs, somatomedins, and MSA have insulin bioactivity but are totally unreactive in this immunoassay. Note that all the naturally occurring insulins have bio/immuno > 1, while pork proinsulin, split proinsulin, and probably other intermediates have bio/immuno < 1. The extracts that we have studied are designated by (*). These points should more properly be drawn as lines parallel to the other diagonals through the designated points, because, in contrast to the other materials, we have only a ratio of the two activities with no measure of molar amounts for them. The extracts of the inside and outside of the earthworm are designated (I) and (O), respectively. The data for Tetrahymena and Neurospora are from ref. 11. Note that we may be overestimating the bio/immuno ratios of our extracts because we overestimate the bioactivity by using the total bioassay, uncorrected for the non-neutralized fraction, and because we are probably underestimating the immunoreactivity by using values read directly from the pork insulin standard curve.

There are substances other than insulins that can produce responses in the insulin bioassays (proteases, oxidizing agents, polyamines, heavy metals, etc.) but none of these co-migrate with insulin on Sephadex G-50, none react specifically in the pork insulin radioimmunoassay, and anti-insulin antibodies do not affect their bioactivity. Thus, we suggest that the material in the extracts is very similar to pork insulin.

In addition to the intestinal tract, insulin is present in mammalian brain cells and peripheral nerves, and there is evidence to indicate that at least some of this insulin is being produced locally by neural elements.[9,10] Because both insulin and specific receptors for insulin are widely distributed throughout brain, and because peripheral nerve stimulation seems to cause local release of insulin,[10,22] there is the possibility that insulin in neural cells may be acting as a neurotransmitter of neuromodulator.

A popular hypothesis that the peptide hormones of the intestinal tract originate phylogenetically and ontogenetically from nervous tissue is supported by the finding that extirpation of a particular nucleus of the brain of the blowfly markedly disturbed carbohydrate metabolism in this insect, that extracts of the head contained material very similar to mammalian insulins, and injection of the extracted material corrected the carbohydrate disturbance.[6] The authors suggest that these neurosecretory cells of blowfly brain serve in the same way as the β-cells of the vertebrate pancreas.

We have raised the possibility that, in addition to gut-derived and nerve-related cells, most cells make some insulin.[13] This is based on the finding that many cells in mammals have insulin at concentrations similar to those found in brain and several-fold higher than in plasma, and that the concentration of insulin in these cells, as in brain, changed little or not at all with extreme changes in levels of plasma hormone.[13,23] We also found insulin at similar concentrations in cells grown in tissue culture, and the level of cellular insulin was not affected by depletion or supplementation of insulin in the medium in which the cells were grown.

The suggestion that many (if not all) cells make some insulin is supported by studies in guinea pigs in which we detected two distinct insulins.[24] Typical guinea pig insulin was present in very large amounts in the pancreas and was the only type of insulin detected in the blood. The second insulin, which very closely resembled pork insulin but was quite different from guinea pig type-insulin, was present in brain and in a wide range of extrapancreatic cell types including many unrelated to nerve or intestinal tract.

In the present study, we confirm that insects contain material that is very similar to mammalian insulin. Further, we show similar material in annelids, which extends the distribution of insulin to another class of invertebrates. The concentration of insulin (ng immunoreactive/g wet weight) that we found in heads of drosophila appears to us to be very similar to that reported for heads of blowfly.[6] That the bodies of the drosophila had only moderately less insulin than

the heads suggests that insulin in insects is not restricted to a single nucleus of the nervous system nor to the nervous system in general. Likewise, that the inside and outside of the worm had similar amounts of the material suggests, again, a very broad distribution of insulin rather than a distribution restricted to one region of brain or gut. (This conclusion assumes that the efficiency of recovery is similar for the different parts, an assumption that is not yet proven.) Our earlier suggestion from previous data, and here from the flies and the worms, that many cell types (other than gut and nerve) make insulin is supported by our finding of material very similar to insulin in unicellular undifferentiated eukaryotes such as *Tetrahymena pyriformis*, a ciliated protozoa, and *Neurospora crassa*, a fungus.[11]

The equivalence between the immunoreactivity and bioactivity of the drosophila extracts, when related to a pork insulin standard, was surprising to us. Insulins isolated from the beta cells of most vertebrates typically have ratios of biologic to immunologic activities that are in excess of one and vary over a range of several hundredfold (Figure 3). The immunologic reactivity of some nonmammalian vertebrate insulins is orders of magnitude less than that of the mammalian insulins. Thus, our extract of material from drosophila is closer to mammalian insulin than are insulins from organisms that are closer phylogenetically to the mammals (e.g., fish and birds). A recent study from our laboratory, in which extracts from guinea pig tissues were used, suggests that the evolution of insulin may in some cases be nonallelic.[24] This may shed light on the discrepancy between the immunologic to biologic activity.*

The insulin bioactivity prepared from extracts of worms is only partially neutralized by anti-insulin antibody. This suggests that, in addition to insulin, the worm extract contained insulin-like growth factors analogous to the insulin-like growth factors and somatomedins described in mammalian systems (or contained substances that stimulated the rat adipocyte to release insulin-like factors). Several well characterized insulin-like growth factors (somatomedins A and C, insulin-like growth factors I and II, and MSA—multiplication stimulating activity) interact with insulin receptors to produce insulin-like effects, but are not reactive with anti-insulin antibodies in the radioimmunoassay, and their bioactivity is unaffected by anti-insulin antibodies though they are extremely similar to insulin in many aspects of their structure. Support for this speculation is provided by our observation that effluent fractions closer to the void volume on the Sephadex G-50 column (in the region typical of the insulin-like growth factor) show an even greater enrichment of bioactivity relative to immunoreactivity than fractions in the insulin region. Interestingly, other investigators have found that worms contain material that stimulates growth in hypophysectomized rats and material that reacts with growth hormone receptors in vitro.[25,26]

* Based on observations that a typical mammalian-type insulin is present in all invertebrates and most mammals; that the guinea pig has a typical mammalian-type insulin in addition to its very unusual pancreatic insulin;[25] and that many vertebrates have pancreatic insulins that differ from typical mammalian insulin, we propose that the gene for typical mammalian-type insulin arose very early and has been very well conserved throughout invertebrate and vertebrate evolution. By analogy to the guinea pig, we speculate that all species in which the pancreatic insulin differs widely from typical mammalian insulins are expressing a different allele in their B-cells and also possess genes for typical mammalian insulins that they may or may not express in extrapancreatic tissues.

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This paper is dedicated to Dr. Rachmiel Levine on the occasion of his 70th birthday.

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