Comparative Aspects of the Immunology and Biology of Insulin*

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Received June 16, 1967

Summary. Some immunological and physiological properties of insulin preparations from a wide range of vertebrate and invertebrate species have been compared with those of crystalline ox and cod insulin, and the yields of insulin from several of these islet preparations are given. — Highly potent guinea pig and horse antisera to ox insulin were unable to provoke hyperglycaemia in the marine Teleost *Cottus scorpius* (the sculpin) and in the Cyclostome *Myxine glutinosa* (the hagfish), respectively. — Crystalline ox and cod insulin provoked hypoglycaemia and death in both the sculpin and the hagfish, but only after large doses and several days' observation time. These findings contrast with the ease and fair rapidity of eliciting a hypoglycaemic response in the hagfish when the hagfish's own insulin was used. No marked quantitative differences were noted between the effects of ox, cod, and sculpin insulins in the sculpin, neither between ox and cod insulin in the hagfish. — The results indicate a species-specificity of insulin, both immunologically and hormonally.

Aspects comparatifs de l'immunologie et de la biologie de l'insuline

Résumé. On a comparé quelques propriétés immunologiques et physiologiques de préparations d'insuline obtenues à partir d'un grand nombre de vertébrés et d'invertébrés avec les propriétés de l'insuline cristalline de boeuf et de morue. La quantité d'insuline obtenue à partir de ces préparations insulaires est indiquée. — De très puissants sérums anti-insuline de boeuf obtenus chez le cobaye et le cheval se montraient incapables de provoquer de l'hyperglycémie chez le Téléostéen marin *Cottus scorpius* (le sculpin) et chez le Cyclostome *Myxine glutinosa* (la myxine). — L'insuline cristalline de boeuf et de morue provoquait de l'hypoglycémie et la mort aussi bien du sculpin que de la myxine mais seulement après de fortes doses et un temps d'observation de plusieurs jours. Au contraire, on obtenait avec facilité et avec une promptitude assez prononcée une réponse hypoglycémique chez la myxine en utilisant sa propre insuline. On n'a pas pu noter de différences quantitatives bien prononcées entre les effets de l'insuline de boeuf, de morue et de sculpin

chez le sculpin, ni entre l'insuline de boeuf et de morue chez la myxine. — Les résultats indiquent qu'il existe une spécificité d'espèce de l'insuline aussi bien immunologique qu'hormonale.

Vergleichende Untersuchungen zur Immunologie und Biologie verschiedener Insuline

Zusammenfassung. Einige immunologische und physiologische Eigenschaften der bei einer Reihe von Wirbeltieren und Wirbellosen gewonnenen Insulinpräparate wurden mit entsprechenden Eigenschaften kristalliner Insuline vom Rind und vom Dorsch verglichen. Gleichzeitig wird über die jeweiligen Ausbeuten an Insulin bei den Extraktionen berichtet. — Anti-Rinderinsulin-Seren vom Meerschweinchen und vom Pferd, die bei der Ratte eine starke Hyperglykämie hervorrufen, bewirken beim Seeskorpion oder Seeteufel (*Cottus scorpius*) aus der Klasse der Knochenfische und beim gemeinen Inger oder Schleimaal (*Myxine glutinosa*) aus der Klasse der Rundmäuler keine Hyperglykämie. Die Gabe kristalliner Rinder- bzw. Dorschinsulins bewirkte beim Seeskorpion und auch beim Schleimaal eine in einigen Fällen tödliche Hypoglykämie, jedoch erst bei sehr hoher Dosis und nach mehrtägiger Beobachtungszeit. Diese Befunde standen im Gegensatz zur ausgeprägten und rasch einsetzenden Hypoglykämie beim Schleimaal wenn homologes Insulin gegeben wurde. Rinder- und Dorschinsulin erwiesen sich beim Schleimaal als etwa gleich wirksam. Beim Seeskorpion zeigten Insuline vom Rind, vom Dorsch, und das homologe Insulin des Seeskorpions annähernd gleiche blutzuckersenkende Wirkungen. — Die Versuchsergebnisse zeigen, daß sich neben der immunologischen Spezifität verschiedener Insuline in einigen Fällen auch eine Speziespezifität der biologischen Wirkung nachweisen läßt.

Key-words: Insulin, invertebrates, Cyclostomes, Elasmobranchs, Teleost fish, Amphibians, Chicken, Ox, Guinea Pig, comparative endocrinology, comparative immunology, blood glucose, anti-insulin sera, passive cutaneous anaphylaxis test.

Introduction

Knowledge about those regions of the insulin molecule that govern its immunological properties and

* Presented in parts at the International Diabetes Federation Vth Congress in Toronto, July 20-24, 1964 (WILSON and FALKMER, 1964), at the 3rd Conference of European Comparative Endocrinologists in Copenhagen, August 2-5, 1965 (FALKMER and WILSON, 1965), and at the British Insulin Manufacturers' Colloquium on the Immunology of Insulin, London, September 16, 1965 (cf. COBB et al., 1966).

The investigation was supported by grants from the Nordic Insulin Fund.

The authors are indebted to Dr. A. KOTAKI for gifts of bonito and tunny insulins, and to Dr. P. J. MOLONEY for chicken insulin.

its biological action is not only of general interest in that field of hormone research dealing with the relationship of structure to biological activity of a hormone but is also of great importance in making synthetic insulin. Here, scientific investigations in the field of comparative endocrinology may be valuable. Recent work with isolated A- and B-chains including resynthesis of cod and ox insulins, preparation of cod-ox "hybrid" insulins (WILSON et al., 1962, 1966), studies with sulfated insulin (MOLONEY et al., 1964), and comparative studies of the amino acid composition of several types of vertebrate (WILSON and DIXON, 1961; FALKMER and WILSON, 1965; SMITH, 1966) and invertebrate (WILSON and FALKMER, 1965) insulins

have indicated that the activity of the insulin molecule is dependent on the correct, intact three-dimensional organization of the three SS-bridges (cf. CARPENTER, 1966; SMITH, 1966), and that the antibody-combining site mainly concerns the A-chain (WILSON et al., 1962), particularly the A₈₋₁₄ regions, but also loci within the sequences B₁₋₈ and B₂₄₋₃₀ (WILSON et al., 1967).

In regard to the biological activity of various types of insulins, no significant variation was previously found when the activity was measured by the standard mouse convulsion or blood glucose assays, not even with guinea pig and some fish insulins which differ markedly in amino acid sequences and in immunological properties (ERMISCH, 1965; SMITH, 1966). Recently, however, preliminary work with guinea pig insulin suggested that it may be only about a quarter as active as cattle insulin when measured in mouse convulsion and mouse blood glucose assays, although almost fully active when measured by guinea pig blood glucose assay (SMITH, 1966).

Against this background it was considered worth while to extend our previous immunological studies of insulins from lower vertebrates in order to compare the biological activities of various types of vertebrate insulins in non-mammalian test systems. Another reason for extending our previous studies was that it has been questioned whether insulin in lower vertebrates, such as marine teleosts, actually is involved in fat and carbohydrate metabolism and thus the regulation of the blood sugar, or whether its main role concerns protein metabolism (TASHIMA and CAHILL, 1964).

The present report gives the results of some immunological studies of vertebrate and invertebrate insulins, mainly fish insulins, and reports the variations in blood glucose in the marine teleost *Cottus scorpius* and the cyclostome *Myxine glutinosa*, evoked by administration of anti-ox insulin sera as well as by ox and cod insulins. The effects of the foreign insulins on the level of blood glucose are compared with those evoked by acid alcohol extracts from the isolated islet tissue of *Cottus* and *Myxine*, respectively.

Material and methods

Immunological Experiments

Acid alcohol extracts for obtaining insulin preparations were made from the pancreas, the principal islets, or parts of the digestive tract from the following species:

Mammals:	Ox (<i>Bos taurus</i>)
	Guinea Pig (<i>Cavia porcellus</i>)
Birds:	Chicken (<i>Gallus domesticus</i>)
Amphibians:	American Bullfrog (<i>Rana catesbeiana</i>)
Bony fish:	Sculpin (<i>Cottus scorpius</i>)
	Tunny (<i>Thunnus thynnus</i>)
	Bonito (<i>Katsuwonus pelamis</i>)
	Pollock (<i>Pollachius virens</i>)
	Cod (<i>Gadus callarias</i>)
	Catfish (<i>Ictalurus punctatus</i>)
	Bowfin (<i>Amia calva</i>)
Cartilaginous fish:	Spiny Dogfish (<i>Squalus acanthias</i>)
	Lemon Shark (<i>Negaprion brevirostris</i>)

Jawless fish:	Hagfish (<i>Myxine glutinosa</i>)
Tunicates:	Sea Squirt (<i>Ciona intestinalis</i>)
Echinoderms:	Purple Starfish (<i>Pisaster ochraceus</i>)
	Red Starfish (<i>Asterias rubens</i>)
Molluscs:	Octopus (<i>Eledone cirrata</i>)
	Oyster (<i>Ostrea lurida</i>)

Certain of these preparations were purified further (WILSON and FALKMER, 1965). Where enough material was available, the insulin content was assayed by the mouse convulsion test (YOUNG and LEWIS, 1947), otherwise the mouse hemidiaphragm assay (WARDLAW and MOLONEY, 1961) was employed. The tests for insulin-like material in extracts from the digestive tract of invertebrates were more comprehensive, including correlated biochemical, immunological, histological, and histochemical investigations as described previously (WILSON and FALKMER, 1965; WILSON et al., 1966). For the immunological studies guinea pig antisera to purified ox, chicken, and cod insulins were employed.

In pilot studies insulin preparations from the cod, catfish, and purple starfish were assayed by the double-antibody procedure of HALES and RANDLE (1963) (tests kindly performed by Dr. K. S. RASTOGI and Dr. J. CAMPBELL).

Passive Cutaneous Anaphylaxis Test

Depilated albino guinea pigs weighing 250–300 g were injected intracutaneously in sites 5 cm apart with 0.1 ml aliquots of undiluted guinea pig antisera to S-sulpho-ox-A-chain, S-sulpho-ox-B-chain, ox, chicken, and cod insulins (WILSON et al., 1966, 1967).

The guinea pigs were challenged intravenously 20–22 hours later with 0.5 ml of a saline solution containing the insulin preparation, together with 5 mg Pontamine sky blue 6 BX. The diameters of the blue spots appearing at the sites of the serum injections were measured 10–20 min later.

Blood Glucose Experiments

1. Administration of Anti-Ox-Insulin Sera

a) To *Cottus scorpius*: Details concerning the capture and maintenance of the fish, as well as the general outline of the blood glucose experiments have been given in preceding papers (FALKMER, 1961; FALKMER and MATTY, 1966a). The investigation was performed in December at a water temperature of 8°C. Freeze-dried guinea pig anti-serum to ox insulin was redissolved in glass-redistilled water to make a solution with an ox-insulin-inhibiting potency of 1 I.U. of insulin per ml, as determined by the mouse convulsion assay. The same amount of freeze-dried normal guinea pig serum was reconstituted with redistilled water to be used in the control experiments. Ten fasting adult sculpin of both sexes, with the same female predominance as previously (FALKMER, 1961) and with a mean body weight of 125 g (range 75–185 g), were injected intramuscularly with approximately 1 ml/kg body weight (range 0.6–1.3 ml/kg) of the guinea pig anti-ox-insulin serum solution. The blood glucose content was assayed by the glucose oxidase procedure at the following observation times: 0, 6, 12, 24, 48, 72, and 96 hours after the injection. After the last sampling of blood the fish were killed, the principal islets excised and fixed in Bouin's fluid. After alcohol dehydration and clearing in xylene and methylbenzoate, the islets were embedded in paraffin. Sections, approximately 5 µ thick, were stained with van Gieson's stain and Maske's modification of the aldehyde fuchsin procedure (FALKMER, 1961). The control experiments with the solution described above of normal guinea pig serum were performed on 11 fasting adult sculpin with a similar sex and weight distribution (mean 135 g; range 95–195 g). The blood glucose investigations and

the histological examinations were identical to those of the experiments with anti-ox-insulin serum.

b) *To Myxine glutinosa*: Details concerning the capture and maintenance of the animals, as well as the blood sampling, haematocrit, and glucose assay procedures have been given in preceding papers (FALKMER and WINBLADH, 1964a and b; FALKMER and MATTY, 1966b). The experiments were performed in October and November at a water temperature of 10°–14°C and at a water salinity of 33 per thousand. All animals were fasting.

Freeze-dried, horse anti-ox-insulin serum (lot No. 2712) was reconstituted with redistilled water giving an estimated ox-insulin-inhibiting capacity in the mouse convulsion assay of 1.6 I.U. of insulin per ml. Approximately the same concentration (per mg dry substance) of similarly redissolved freeze-dried, normal horse serum was used for control experiments. Ten adult hagfish of both sexes, weighing between 25–40 g, were injected intramuscularly with 1 ml per fish of the solution of horse anti-ox-insulin serum. Two animals were killed at each of the following time intervals after injection: 1, 4, 8, 20, and 44 hours. Exactly analogous experiments with the solution of normal horse serum were performed on 10 hagfish of the same sex and size distribution. In another experiment 15 adult hagfish of both sexes with a mean body weight of 44 (range 30–60) g were injected intramuscularly with 2 ml of the same solution of horse anti-ox-insulin serum as above, and 5 animals killed at each of the time intervals 8, 20, and 44 hours after the administration. Fifteen adult hagfish of both sexes and of the same size were used for analogous control experiments with the solution of normal horse serum. When the animals were killed, the islet tissue was excised for light microscopical examination as described above for the corresponding sculpin experiments. In some cases pieces of islet tissue were also fixed in osmium tetroxide for electron microscopical investigations, using the same methods as previously described (FALKMER and WINBLADH, 1964a and b).

The potencies of the anti-ox-insulin sera were also tested in pilot experiments in rats of both sexes weighing about 200 g. Animals were injected with a series of doses from 0.1–20 U per animal, and the blood glucose level was checked 20 min after each injection.

2. Administration of Ox Insulin

In all types of experiments crystalline ox insulin (Vitrum AB, Stockholm K; lot No. 624¹) with a potency of 25.9 I.U. per mg air dry substance was used. It was dissolved in either 0.01 M HCl or 0.1 M NaCl-NaOH buffer pH 9.0 (PEARSE, 1960). From preceding investigations it was known (FALKMER, 1961; FALKMER and MATTY, 1966b) that these solvents, at the volumes and concentrations used did not affect the blood glucose levels of the sculpin and the hagfish. Only a single dose was given.

a) *To Cottus scorpius*: The dosages were: 1000, 100, 20, 5, and 1 I.U. per kg body weight. Six adult fish of both sexes, weighing between 80 and 250 g, were injected at each dosage. Both fed and fasting fish were used, and before the experiments the available lot was evenly distributed with regard to size, sex, and feeding state into 6 groups. The dose level used in each group was determined by lot. The water temperature was approximately either 10°C or 15°C. At the dosage 1 I.U./kg the experiments were performed at both temperatures; for the other dose levels the water temperatures are given in Tables 3 and 4 and in the diagrams. The experiments were performed in June and July. Blood glucose assay was performed 0, 1, 2, 3, 4, 5, and 6 days after the injection, applying previously described procedures (FALKMER, 1961; FALKMER and MATTY, 1966a). No morphological investigations were made.

b) *To Myxine glutinosa*: Previous investigations (FALKMER and MATTY, 1966b) were supplemented by identically performed experiments with the dose levels 100 and 20 I.U./kg. The water temperature was 10°C (adjusted by a cooling device). The experiments were made in July. The animals were of the same sex and size distribution as previously used. No morphological examination of the islet tissue was made this time.

3. Administration of Cod Insulin

In all experiments with cod insulin, a preparation (lot 10/3c/F4) of the same type as described previously (WILSON and DIXON, 1961) was used. Its potency was 15 I.U./mg (95 % confidence limits: 12–20 I.U./mg). It was dissolved (4 mg/ml) in 0.01 M HCl and further diluted with physiological saline to a concentration suitable for the dosage desired. The stock solution was kept at + 4°C.

a) *To Cottus scorpius*: The dosages were 100 (range 69–125), 20, 5, and 1 I.U. per kg body weight. The general outline of the investigations followed that of the corresponding ox insulin experiments as closely as possible. They were performed in June, July, and December.

b) *To Myxine glutinosa*: The general outline of the cod insulin investigations in the hagfish also followed that of the corresponding ox insulin experiments as closely as possible, and were performed at the same time. The dosages were 100, 20, and 5 I.U./kg.

4. Administration of Crude Cottus and Myxine Insulin

All the details concerning the preparations of hagfish insulin have been reported previously (FALKMER and MATTY, 1966b). The general outline of the sculpin insulin experiments was almost exactly the same. Principal islets from 91 adult sculpin of both sexes were dissected, making up a total amount of tissue of 492 mg wet weight, and a crude sculpin insulin preparation was obtained by the same procedure as that used for the hagfish islets (FALKMER and MATTY, 1966b). The potency of the sculpin and hagfish preparations was assayed by the mouse convulsion procedure (see Results). The experiments were performed in June and December at the water temperatures 15°C and 8°–10°C. Three series of fish were used, each containing 6 adult sculpin of both sexes with a mean body weight of 100 g (range 80–150). The crude sculpin insulin preparation was injected intramuscularly at dose levels estimated to be approximately 20, 5 and 1 I.U. per kg body weight. Blood glucose determinations were made at the same time intervals as in the experiments with administration of ox and cod insulin. No morphological examination was performed at the end of the investigations, as this has been made previously (FALKMER, 1961).

Results

Content and Immunology of Insulin from Various Types of Islet Tissue

The yields of insulin activity in the acid alcohol extracts of tissues supposed to contain insulin-producing cells are given in Table 1. A summary of previously obtained results of inhibition experiments with guinea pig anti-ox-insulin and anti-cod-insulin sera are also included in the Table.

A faint but definite insulin activity was found in the Sea Squirt and the Starfish where pseudoisocyanin-positive cells occur in the digestive tract epithelium. The insulin-like activity of the Sea Squirt was abolished

¹ Kindly supplied by Mr. R. BERGMAN, Vitrum AB.

($P < 0.001$) by a 66-fold excess of guinea pig antiserum to ox insulin. A more detailed report of these results is presently being prepared.

So far, possible insulin-producing cells have not been found in the digestive tracts of the octopus or

phylogenetic order (NORMAN and GREENWOOD, 1963) and neutralizability within the group of Teleosts. There was relatively little cross-reactivity between ox and cod insulins, but insulin from a low-order teleost (Bowfin) was found to be readily neutralizable by both

Table 1. *Potencies and immunological behaviour of insulins extracted from various species*

Group of animals	Species	Wt. of tissue (g)	Wt. of Zinc salt (g)	Potency & 95% C.L. I.U./mg	Yield I.U./g tissue	Amount of Antiserum which neutralizes 1 mU insulin activity in mouse diaphragm test	
						Antiserum to ox insulin (mU)	Antiserum to cod insulin (mU)
Mammals	Ox Lot GRP (Purified)			29.4 (27.1—31.8)		1	60
	Guinea Pig	351	0.045	~ 0.4 ²	0.06	> 250	
Birds	Chicken	45.4	5.31	8.2 (6—11)	1.0	1—2	
	Chicken (Purified)			29.6 (24—36)		1—2	
Amphibians	Frog	70	0.049	~ 0.40 (0.27—0.57)	0.26	1—2	
Bony Fish	Sculpin	0.50	0.0091	9.6 (5.3—17.4)	68	4	1—2
	Tunny (Purified)			7.2 (4.6—11.0)		27	9
	Bonito (Purified)			11.5 (8.5—16.0)		9	3
	Pollock (Purified)	30	0.028	~ 8 ²	7.5	> 20	1—2
	Pollock (Purified)			30.8 (14—78)			
	Cod	296	2.01	6.7 (5.2—8.7)	47	> 20	1
	Cod (Purified)			20 (16.7—24)		30	1
	Catfish	59	0.246	0.62 (0.46—0.82)	2.5	50	50
Cartilaginous Fish	Bowfin	13	0.133	0.02 (0.012—0.034)	0.2	2	2
	Dogfish	58	0.168	0.13 (0.09—0.20)	0.4	20	30
	Lemon Shark	75	0.515	0.87 (0.4—2.2)	0.6		40
Jawless Fish	Hagfish W 1	0.63	0.0017	0.80 (0.36—1.99)	2.1	50	30
	F 1	0.42	0.0034	0.62 (0.23—1.6)	4.8		
	F 2	0.34	0.0089	1.13 (0.64—1.95)	30.0		
Tunicates	Sea Squirt	29	0.307	0.0056 (0.0045—0.0087)	0.06	130	n.d.
Echinoderms	Starfish	228	1.13	0.0011 (0.0009—0.0019)	0.06	56	n.d.
	Starfish (partly purified)			0.029 (0.018—0.046)			
Molluscs	Octopus	6	0.104	0.0011 (0.0008—0.0016)	0.02	> 200	n.d.
	Oyster	349	0.77	None detected			

¹ Potency of ox, guinea pig, frog, chicken, pollock, cod and catfish insulin preparations by mouse convulsion assay (YOUNG and LEWIS, 1947); other preparations by 4-point mouse hemidiaphragm assay (WARDLAW and MOLONEY, 1961).

² Only performed as 3-point mouse hemidiaphragm assays.

oyster, although an acid-alcohol extract from the octopus enhanced glycogen deposition in the mouse diaphragm assay of insulin activity (Table 1).

It is obvious from Table 1 that with the notable exception of the guinea pig, high-order species appeared to have neutralizable insulins, whereas the majority of the fish insulins tested were immunologically unlike ox insulin. However, there was no relationship between

antisera, suggesting that it had antibody-combining sites in common with both insulins. The most interesting finding, however, was that the hormonal activity of *all* the insulins could be neutralized specifically by both antisera, providing that enough of the latter was added. The only exception was guinea pig insulin, which was not neutralized by at least a 250 fold excess of anti-ox-insulin serum.

Preliminary results (Table 2) with some insulin preparations in the PCA test (cf. WILSON et al., 1966, 1967) are largely in agreement with those from the hormone-inhibition test and show that insulins from widely differing species may have antigenic deter-

minants in common. Thus, 1 mU catfish insulin gave a PCA reaction only with antiserum to chicken insulin, and was also neutralized more readily by this antiserum than by antisera to ox or cod insulins. Guinea pig

insulins are antigenically like ox insulin; catfish and sea squirt insulins are like chicken insulin, whereas sculpin and pollock insulins are like cod insulin.

Table 2. *Passive cutaneous anaphylactic reaction produced by various insulins in guinea pigs injected with three insulin antisera*

Insulin for Challenge	Dose per G. Pig		Diam. of Blue Spot (mm) with Antiserum ¹ to:-		
	μ g	Milli-units	Ox Insulin	Chicken Insulin	Cod Insulin
Ox	1	30	50	35	0
Guinea Pig	60	25	0	0	0
Chicken	1	30	25	35	30
Sculpin	0.7	7	0	0	31
Tunny	1	7	35	0	25
Bonito	1	12	28	0	32
Pollock	0.25	2	28	0	25
	0.05	0.4	0	0	28
Cod	1	20	5	n.d.	30
Catfish	2	1	0	28	0
Bowfin	400	4	20	n.d.	15
Lemon shark	50	2	25	25	22
Sea Squirt	1000	3	0	25	0

¹ In no case did antisera to the isolated A- and B-chains of ox insulin give a reaction.

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Fig. 1. Mean blood glucose values (Δ - Δ) of 10 *Cottus scorpius* (the sculpin) at various time intervals after an intramuscular injection of anti-ox-insulin guinea pig serum at a dosage found to make a 200 g rat hyperglycaemic. Ten sculpin injected with the same amount of normal guinea pig serum were used as controls (curve of mean values: $\circ$ - $\circ$). The heavy black line gives the mean blood glucose value of untreated fish and the dashed line limits the upper range of 2.58 times the standard deviation

insulin did not yield a PCA reaction with any of the guinea pig antisera, indicating that the animal is highly tolerant to its own insulin. All of the immunological findings taken together indicate that most of the mammalian insulins, and the chicken and frog

Fig. 2. Mean blood glucose values (Δ - Δ) of 10 *Myxine glutinosa* (the hagfish) at various time intervals after an intramuscular injection of anti-ox-insulin horse serum at a dosage found to make 200 g rat hyperglycaemic. Ten hagfish injected with the same amount of normal horse serum were used as controls (curve of mean values: $\circ$ - $\circ$). Same signs and symbols as in Fig. 1

ox-insulin serum in the double-antibody procedure. The biological potencies of the three solutions supplied were 10 and 1 U/ml (mouse convulsion assay) for cod and catfish insulin and 0.02 U/ml (mouse hemidiaphragm assay) for starfish insulin. Thus, these insulins cannot be assayed by this tracer technique.

Blood Glucose Experiments

Administration of Anti-Ox-Insulin Serum

In the pilot investigations on rats 1 U of the guinea pig anti-ox-insulin serum used was found to make a 200 g rat hyperglycaemic as early as 90 min after the injection. Previously published comparisons between the guinea pig and horse antisera used in this investigation (MOLONEY and APRILE, 1960) showed that the horse anti-ox-insulin serum was diabetogenic although less avidly than that from the guinea pig.

The results of the experiments with the sculpin are given in Fig. 1, and those of the first type of the experiments with the hagfish in Fig. 2. On the basis of previous findings (FALKMER, 1961; FALKMER and MATTY, 1966a) one sculpin of the control group with

marked hyperglycaemia, apparently connected with clumsy blood samplings, skin ulcers, and anemia, was excluded from the diagrams. Although the ranges of the individual observation time values have been omitted from the diagrams, it is obvious from the given mean values that the endogenous insulin in the sculpin and the hagfish was not readily neutralized *in vivo* by the two types of antiserum.

The second type of the experiments with the hagfish showed that not even the double dose of antiserum in a larger series caused any obvious blood glucose rise. The mean values and the range of both the blood glucose and haematocrit values were almost exactly the same as in the control group, and were within the normal range at all three observation times.

Neither the light microscopical nor the ultrastruc-

Table 3. Review of the Blood Glucose Effects in the Sculpin on Various Time Intervals after Administration of Various Types of Insulin

Hours	Ox Insulin (I.U./kg)												Cod Insulin (I.U./kg)										Sculpin Insulin (I.U./kg)						
	1 Fed 10°C		1 Fasting 15°C		5 Fed 10°C		20 Fasting 15°C		100 Fasting 15°C		1000 Fed 15°C		1 Fed 10°C		1 Fasting 15°C		5 Fed 10°C		20 Fed 10°C		100 Fasting 10°C		1 Fasting 15°C		5 Fasting 15°C		20 Fasting 10°C		
after																													
Insulin:	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	"Hypo"	"Normo"	
Injection	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	Dead Live	
(At start of the experim.)			6		6		6		6		6		6		6		6		6		6		6		6		6		5
0—48			6		6		6	1	2	3	3		6		6		6		6		6	1	5		2		4	3	2
49—72			6		6		6	2	2	1	1	1	1	5		6	1	5		6		6	4	1		2	3	1	2
73—96			1	1	4		2	1			1	1	1	4		1	4				1		2	3		3			
97—120			6		5		1	5	1				2	2	3	3		4	4	2	2	1	2		1	2			
121—144			1	5	1	1	3		5	1			1	1		6		4		6		5		2					
(At end of the experim.)			1	5	2	1	3		1	5	5	1		4	2	2	3	1				6	1	1	4		6		5

The term "Hypo" (hypoglycaemia) signifies a blood glucose value that is both significantly outside the range of 2 times the Standard Deviation of the initial blood glucose level of the animals and less than 10 mg/100 ml (cf. FALKMER 1961). - In the term "Normo" (normoglycaemia) isolated fish with obviously hyperglycaemic blood glucose levels are also included. The figures in the columns give the number of fish. - A long hyphen in a broad major column signifies that no observations were made at the time interval of that column.

Table 4. Review of the Blood Glucose Effects in the Hagfish on Various Time Intervals after Administration of Various Types of Insulin

Hours after Insulin Injection	Ox Insulin (I.U./kg)												Cod Insulin (I.U./kg)												Hagfish Insulin (I.U./kg)											
	20 10°C				100 10°C				1000 13°C				2000 13°C				3000 13°C				5 10°C				20 10°C				100 10°C				5 10°C			
	"Hypo"		"Normo"		"Hypo"		"Normo"		"Hypo"		"Normo"		"Hypo"		"Normo"		"Hypo"		"Normo"		"Hypo"		"Normo"		"Hypo"		"Normo"		"Hypo"		"Normo"		"Hypo"		"Normo"	
	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live		
(At start of the experiment)				6				6				4				4				6				6				6					6			
0—48												4		3		1		4															2		3	
49—72			1	5		2		4		2		2		3		1		3		1			6				6			1	5		3		2	
73—96						5		1		3		1																				3		2		
97—120			2		3					3		1										6	1	1	2	2			1	2	2		1		4	
121—144			2		3																2	4		1		2		1		2						
145—168		1	2	1	1	1	1		4												4		3				2		1							
(At end of the experiment)		1	2	2	1	1	1		4		3		1		3		1				2	4	1	3	2			2	3	1			1		4	

All hagfish were fasting. - The term "Hypo" (hypoglycaemia) signifies a blood glucose value that is both significantly outside the range of 2 times the Standard Deviation of the initial blood glucose level of the animals and less than 10 mg/100 ml (cf. FALKMER and MATTY, 1966b). - In the term "Normo" (normoglycaemia) isolated fish with obviously hyperglycaemic blood glucose levels are also included. The figures in the columns give the number of hagfish. - A long hyphen in a broad major column signifies that no observations were made at the time interval of that column.

tural investigations of the sculpin and hagfish islet tissue disclosed any significant deviations from the normal in any of the groups at any of the observation times.

Fig. 3. Individual blood glucose fluctuations of 6 *Cottus scorpius* injected intramuscularly with 20 I.U./kg of crystalline ox insulin. The heavy black line gives the mean initial blood glucose level of the animals and the dashed lines limit the range of 2 times the standard deviation. The sign † indicates death of a fish.

Fig. 4. Individual blood glucose variations of 6 *Cottus scorpius* injected intramuscularly with 1000 I.U./kg of crystalline ox insulin. The heavy black line gives the mean for untreated fish and the dashed line limits the upper range of 2.58 times the standard deviation. Otherwise same signs and symbols as in Fig. 3.

Administration of Various Types of Insulin

Tables 3 and 4 and Figs. 3–8 clearly show that hypoglycaemia and death could be evoked in both types of fish with all the three types of insulin tested.

In the sculpin this hypoglycaemia was often associated with imbalance and uncoordinated swimming movements alternating with a comatous state (cf. FALKMER, 1961). No such effects were noted in the hagfish (cf. FALKMER and MATTY, 1966b).

Fig. 5. Individual blood glucose variations of 6 *Cottus scorpius* injected intramuscularly with 20 I.U./kg of crystalline cod insulin. Same signs and symbols as in Fig. 3.

Fig. 6. Individual blood glucose variations of 6 *Cottus scorpius* injected intramuscularly with 100 I.U./kg of crystalline cod insulin. Same signs and symbols as in Fig. 3.

In the experiments with the sculpin the results do not, however, allow any detailed quantitative comparison between the effects of the various types of insulin used, as it is obvious that the feeding state of the fish and the temperature of the water influenced the blood glucose response to at least the same extent as did the insulin itself (cf. Figs. 3 and 4). As found previously (FALKMER, 1961; FALKMER and MATTY,

1966a and b), hypoglycaemia and death were both fairly easily elicited in fasting fish at higher temperatures, whereas fed fish at lower temperatures were markedly resistant to even tremendous doses of ox and cod insulin (Tables 3 and 4). In general, however,

Fig. 7. Individual blood glucose fluctuations of 6 *Cottus scorpius* injected intramuscularly with an acid-alcohol extract of sculpin islet tissue, constituting a crude insulin preparation corresponding to a dosage of 1 I.U./kg of sculpin insulin. Signs and symbols are analogous to those of Fig. 3

Fig. 8. Similar type of experiment as in Fig. 7 but using a dose of about 20 I.U./kg of crude sculpin insulin. Signs and symbols are analogous to those of Fig. 3

increasing the insulin dosage increased the frequency of hypoglycaemia and death in both fish, and in the sculpin no marked differences were noted between ox, cod, and sculpin insulins.

In the experiments with the hagfish, where only fasting fish at more strictly controlled temperatures were used, the hagfish's own insulin was markedly more effective than either ox or cod insulin in evoking hypoglycaemia. No marked difference between ox and cod insulin was noted in the hagfish either. The transient hypoglycaemia produced in the hagfish by 5 I.U. hagfish insulin per kg occurred earlier and was more pronounced than that caused by either 20 I.U. cod insulin or 100 I.U. ox insulin per kg.

A rough comparison of cod and ox insulins in the mouse hemidiaphragm assay at 37°C and 17°C showed that the relative potency of purified cod insulin versus crystalline ox insulin standard was 0.9 (0.7–1.4) at 37°C and 0.6 (0.2–2.2) at 17°C. Thus, the two hormones had apparently similar temperature coefficients with respect to their effects on glycogen synthesis in the mammalian system.

Discussion

The observation that also the sea squirt seems to contain insulin-producing cells, whereas so far no convincing evidence for insulin production has been obtained in the Molluscs, conforms with our present working hypothesis that insulin mainly occurs in the Deuterostomes (Fig. 9). Here, however, further in-

Fig. 9. Much simplified phylogenetic tree showing some groups of animals where tests have been made on the occurrence of insulin

vestigations are in progress, as this question may be of great phylogenetical interest.

The high yield of insulin from sculpin islets is in good conformity with the old reports of McCORMICK and NOBLE (1924) and VINCENT et al. (1925), and with the quantitative morphological studies of the islet tissue of this species (FALKMER, 1961), which showed "giant" pure islets with about 25% β -cells without any admixture of traversing strands of acinar tissue. There is also a fairly good correlation between the insulin content and the morphology of the islet tissue in both

the cod and the hagfish (cf. FALKMER and WINBLADH, 1964a). The relatively low yield of insulin in most other species investigated can also be easily understood from the histological appearance of the islets. This is especially true of the shark pancreas, which is of the "mammalian" type (cf. FERNER and KERN, 1964), and of the digestive epithelium of the pyloric coeca of the starfish and the digestive tract of the sea squirt where the apparently insulin-producing cells only constitute a small fraction (WILSON and FALKMER, 1965).

Blood Glucose Experiments

Administration of Anti-Ox-Insulin Serum

Potent anti-ox-insulin sera were unable to provoke hyperglycaemia in the sculpin and the hagfish, indicating that the endogenous insulin of these two species was not readily neutralized by the antisera *in vivo*. The absence of any light microscopical or ultra-structural changes in the islets was in conformity with this stability of the level of blood glucose. These results contrasted with the ease of producing hyperglycaemia with the same antisera in the mouse and rat, but were in agreement with the immunological differences between the fish insulins and mammalian insulins.

Administration of Various Types of Insulin

The present results, together with those previously obtained (FALKMER, 1961; FALKMER and MATTY, 1966b), clearly show that both ox and cod insulin affect the blood glucose level in both Teleosts and Cyclostomes. The fact that TASHIMA and CAHILL (1964) failed to obtain any hypoglycaemia in the marine teleost *Opsanus tau* (the toadfish) with ox insulin at a dosage of 100 I. U./kg i. v. may be explained by several interfering factors. Firstly, it is well known that the intravenous path of injection is ineffective in fish, probably due to sensitive vasoregulatory reflexes and poor mixing conditions in the single circuit circulation system of Teleosts (cf. FALKMER, 1961; LAZAROW, 1964). Secondly, the present investigation has shown that the blood glucose level of fed fish (the toadfish were kept in cribs in the open sea) is fairly resistant to ox insulin, even when tremendously high doses were applied (Fig. 4). Thirdly, it is essential to follow the blood glucose level of each individual fish separately by repeated blood samplings *in vivo* in order to detect transient hypoglycaemias occurring in isolated fish. However, even when all these precautions are taken into consideration (intramuscular path of administration without lengthy manipulations, fasting fish and long observation times with regular blood sampling), it is, to judge from the present investigations in the sculpin, correct to state that the blood glucose level of Teleosts seems markedly resistant to administration of ox insulin (cf. FALKMER, 1961). It is interesting to note that this also holds true for cod insulin,

which differs markedly from ox insulin both in immunological behaviour and in amino-acid sequence (WILSON and DIXON, 1961). The sculpin's own insulin was also surprisingly inefficient at short observation times at the lowest dose (Fig. 7). It is thus still possible that insulin is involved only to a small extent in the blood sugar regulation of Teleosts.

The finding that in the hagfish the crude native insulin preparation was much more effective in producing hypoglycaemia than either of the two foreign crystalline insulins, may be due to a real species-specificity of the hormone or to inadequate assessment of the potencies of the crude insulin preparation in the mammalian test systems used. However, if the latter explanation has some bearing, it may also be an expression of a species-specificity of the hormone, like that for guinea pig insulin referred to in the Introduction. Moreover, it is seen from Table 1 that fairly large fluctuations could occur in the insulin yields of different preparations.

Thus the main results of the present investigations are that insulin apparently induced hypoglycaemia in both Teleosts and Cyclostomes, as it does in higher species; and that there is a species-specificity of insulins from a wide range of animals, both immunologically and with regard to its hormonal action as measured by its effects on the blood glucose level. This species-specificity not only manifests itself between different groups of animals but also within the large animal groups. Similar reports have previously been given for other protein hormones, e.g. thyrotropin (cf. BARRINGTON, 1964). Moreover, MACARTHUR and FISHER (personal communication) recently found in a study on the action of frog, fish, and ox insulins on frog muscle tissue, that bullfrog insulin evoked a significantly faster increase in respiration of leopard frog muscle tissue than did cod or ox insulins.

The present results do not permit any speculations concerning the influence of temperature upon the activity of sculpin and hagfish insulins (cf. BARRINGTON, 1964). However, we have found that cod insulin has the same potency relative to ox insulin at 17°C and at 37°C in the mouse hemidiaphragm assay. The fish and mammalian hormones, therefore, appear to have similar temperature coefficients in the mammalian test system.

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