Insulin Biosynthesis in the Bullhead, *Ictalurus nebulosus*, and the Effect of Temperature

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Insulin biosynthesis in the brown bullhead, *Ictalurus nebulosus* (Le Sueur), was studied by measuring the incorporation *in vitro* of [^3H]leucine into proteins of the principal islet. The tissue was incubated for 6–15 h in Krebs–Ringer bicarbonate buffer with [^3H]leucine, supplemented with amino acids and glucose. Proteins, precipitated with trichloroacetic acid and extracted with acid ethanol, were separated by gel-filtration on Biogel P-30 in 3M-acetic acid. Three major components were found after incubation of the islets at 22°C. On the basis of the results of sulphitolysis, biological activity and the demonstrated precursor–product relationship, components I and II were identified as proinsulin and insulin respectively. The third component was not identified. At 12°C, [^3H]leucine was incorporated only into proinsulin. No radioactivity was found in insulin or the unidentified component III at 12°C as was found after incubation at 22°C. When the temperature was lowered from 22° to 12°C after 3 h of a 15 h incubation, decreased conversion of proinsulin into insulin resulted at the lower temperature compared with the control tissue maintained at 22°C. When the temperature was raised from 12° to 22°C at 3 h of a 15 h incubation, conversion of proinsulin into insulin occurred. No conversion occurred in the control tissue with the temperature maintained at 12°C. No qualitative difference in the incorporation of [^3H]leucine into proinsulin and its conversion into insulin at 12° and 22°C could be demonstrated between islet tissue from fish acclimated to less than 12°C or to 22°C. The results suggest that the enzyme(s) responsible for converting proinsulin into insulin in the bullhead may be temperature sensitive with low activity at 12°C.

Elevated blood glucose concentrations have been observed in some species of fish acclimated to low temperatures. Nace *et al.* (1964) reported that the blood glucose concentration of the toadfish, *Opsanus tau*, at 20°C in summer was about 20 mg/100 ml, but in the winter at –1°C, it was more than 100 mg/100 ml. Umminger (1971a) found that the blood glucose concentration of *Fundulus heteroclitus* at 11°C was 59 mg/100 ml, but at 0.1°C it was nearly 1100 mg/100 ml.

The insulin–glucose feedback relationship is not as clearly established in fish as it is in mammals. However, glucose has been shown to stimulate the release of insulin from incubated toadfish islets (Watkins *et al.*, 1968; Patent & Foa, 1971). Insulin *in vivo* generally causes a decrease in the blood glucose concentration, although the decrease occurs more slowly than in mammals (Falkmer, 1961). Thus the observations on elevated blood glucose concentrations in these fish suggest that insulin deficiency may occur at low temperatures. Insulin deficiency at low temperature could result from a decrease either in the synthesis or the secretion of the hormone. High

blood glucose concentrations could also result from a decreased activity of insulin.

Insulin synthesis in fish has been studied by using the principal islets of cod (Grant & Reid, 1968) and anglerfish (Trakatellis & Schwartz, 1970; Noe & Bauer, 1971). These studies showed that, as in mammals (Steiner *et al.*, 1969), proinsulin is a precursor in the synthesis of insulin in fish. However, the effect of temperature on the synthesis of proinsulin and its conversion into insulin in fish is not known.

The present paper reports our findings on the biosynthesis of insulin in the bullhead, *Ictalurus nebulosus*, and on the possible regulatory effect of temperature on the synthesis of proinsulin and its conversion into insulin in this fish.

Materials and Methods

Experimental animal

The principal islet of the brown bullhead, *Ictalurus nebulosus*, is composed of a central endocrine region surrounded and infiltrated by exocrine tissue. β -Cells are the commonest endocrine cell type (Murrell &

Nace, 1959). The bullheads were obtained from the St. Lawrence River near Gananoque, Ontario, Canada. They were kept in 600-litre aquaria supplied with running, aerated, dechlorinated water at 3–8°C, on a light-cycle the same as natural daylight. The fish were not fed and were used between 2 and 12 weeks after they were obtained.

Chemicals

Amino acids, bovine serum albumin and puromycin were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. Trasylol (aprotinin) was supplied by FBA Pharmaceutical Ltd., Montreal, P.Q., Canada. Aquasol, DL-[4,5-³H]leucine (specific radioactivity 5 Ci/mmol), L-[³H]alanine (specific radioactivity 3 Ci/mmol), L-[³H]aspartic acid (specific radioactivity 2 Ci/mmol) and [³H]glycine (specific radioactivity 5 Ci/mmol) were supplied by New England Nuclear Corporation, Boston, Mass., U.S.A. Sephadex G-75 was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden, and Biogel P-30 by Bio-Rad Laboratories, Richmond, Calif., U.S.A. Trypsin TPKC (L-1-chloro-4-phenyl-3-tosylbutan-2-one)-treated was supplied by the Worthington Biochemical Corp., Freehold, N.J., U.S.A. Crystalline bovine insulin was a gift from the Connaught Medical Research Laboratories, Toronto, Canada, and bonito insulin was a gift from Dr. A. Nikki, Nagoya, Japan. All other chemicals were reagent grade.

Incubation method

The principal islet (15–30 mg) was removed, weighed, cut into six pieces and placed in 1 ml of Krebs-Ringer bicarbonate buffer (Umbreit *et al.*, 1964) in vials (5.5 cm × 1.8 cm) fitted with rubber stoppers. The buffer was supplemented with 18 naturally occurring amino acids, excluding leucine, at 0.2 mg/ml, glucose at 0.4 mg/ml, Trasylol at 1000 kallikrein inhibitor units/ml and [³H]leucine at 20 μ Ci/ml. The vials were gassed with O₂ + CO₂ (95:5) and shaken at 100 cycles/min in a water bath at 12° or 22°C.

Extraction and separation of proinsulin and insulin

At the end of the incubation period, 1 ml of a solution containing 2 mg of crystalline ox insulin, 1 mg of leucine and 10 mg of bovine serum albumin in 6 mM-HCl was added to the vial. Proteins were precipitated in 10% (w/v) trichloroacetic acid and then homogenized. The precipitate was washed three times with 10% (w/v) trichloroacetic acid and then extracted overnight at 4°C in 10 ml of acid ethanol (1.5 ml of conc. HCl in 100 ml of 75% (v/v) ethanol). The pH was adjusted to 7 and the extract was refrigerated for 6 h, centrifuged to remove any precipitate and dried *in vacuo* at 37°C.

The residue was redissolved in 2 ml of 3 M-acetic acid, centrifuged and the supernatant applied to a column (Pharmacia, 1.5 cm × 90 cm) of Biogel P-30 and eluted with 3 M-acetic acid. The eluent was collected in 80 1.6 ml fractions. The position of marker bovine insulin was located by measuring the E_{275} of the fractions. A sample of each fraction was added to 10 ml of Aquasol and counted for radioactivity in a liquid-scintillation counter (Nuclear-Chicago, Mark I). Counting efficiency was 50%.

Identification of insulin and proinsulin

Fractions containing the radioactive components were pooled and dried *in vacuo* at 37°C. The components were then characterized by a number of methods. Sulphitolysis was performed by the method of Varandani (1966). Biological activity in the fractions containing the maximum amounts of radioactivity for components I, II and III was measured by the rat hemidiaphragm assay (Shaw & Chance, 1968), by utilizing the increased glycogen produced in the diaphragm in the presence of insulin, and the rat epididymal fat-pad assay (Renold *et al.*, 1960) by utilizing the increased conversion of [¹⁴C]glucose into ¹⁴CO₂ in the presence of insulin. The experimental design of these assays followed the three-point method of Sheps *et al.* (1960).

Results

Incorporation of [³H]leucine into islet proteins

Incubation of the bullhead islet tissue with [³H]-leucine resulted in three principal radioactive components, soluble in acid ethanol, designated as I, II and III in Fig. 1. Since bullhead insulin does not cross-react well with antibodies to mammalian insulin, bullhead insulin could not be identified by immunological methods. The identification of the radioactive components as insulin or proinsulin was therefore carried out as described below.

Radioactive component III appeared to be like bovine insulin in size as indicated by its elution position (Fig. 1). Sulphitolysis of component III resulted in only one radioactive component unaltered in size from the control (Fig. 2). Since the amino acid composition of bullhead insulin is unknown, it was considered possible that one of the peptide chains might contain no leucine. Islets were incubated as described with 20 μ Ci each of [³H]alanine, [³H]-aspartic acid and [³H]glycine. Again the resulting ³H-labelled component III was eluted as one peak after sulphitolysis.

Since component III was not composed of two polypeptide chains joined by disulphide bonds, it was not insulin. Component III was not further characterized.

Fig. 1. Incorporation of [^3H]leucine into acid-ethanol-soluble proteins

Two islets (45 mg) were incubated at 22°C for 13 h with 20 μCi of [^3H]leucine. The extract was eluted on a column (1.5 cm $\times$ 90 cm) of Biogel P-30 with 3 M-acetic acid; 1.6 ml fractions were collected. ●, Radioactivity; ····, $E_{275}^{1\text{cm}}$ of bovine insulin.

Fig. 2. Sulphitolysis of component III

Pooled, dried component III was dissolved together with 20 mg of bovine insulin. Half of this mixture was eluted as the untreated control (b) and the other half was subjected to sulphitolysis (a). Elution was on a column (2.5 cm $\times$ 40 cm) of Sephadex G-75 with 50% (w/v) acetic acid; 1.6 ml fractions were collected. ●, Radioactivity; ····, $E_{275}^{1\text{cm}}$.

Radioactive component II appeared to be larger than bovine insulin since it was consistently eluted six fractions earlier on Biogel P-30 (Fig. 1). However, in the presence of 7 M-urea, component II was eluted in the same position as bonito insulin (Fig. 3) or bovine insulin. The mechanism by which urea caused a change in the elution position of component II relative to that of bonito or bovine insulin was not determined. It may have resulted from a change in molecular conformation or from disruption of non-covalent interactions between insulin and the Biogel P-30 matrix. Such non-covalent interactions between certain peptides and Sephadex matrix have been

known to cause the abnormal elution behaviour of these peptides (Janson, 1967). The amino acid composition of bonito and other fish insulins differs considerably from bovine insulin (Dayhoff, 1969) and such differences could contribute to a difference in non-covalent interaction and thus in elution position. Indeed Noe & Bauer (1971) reported that anglerfish insulin was eluted three to six fractions before bovine insulin. Consequently we concluded that component II, since it was eluted with bovine and bonito insulin

in the presence of urea, was about the same size as bovine insulin.

Sulphitolysis of component II resulted in two peaks of radioactivity very similar to the profile of the sulphonated A and B chains of bovine insulin

(Fig. 4). Tryptic hydrolysis produced no change in the elution position of component II.

Peak fractions, eluted from Biogel P-30, containing components I, II and III were tested in the rat hemidiaphragm and rat epididymal fat-pad bioassays. The amount of insulin-like activity was more than 20-fold greater in the fraction containing component II than in those containing components I and III, as measured in both bioassays (Fig. 5).

Component I was eluted in a position similar to that of bovine proinsulin indicating that they may be about the same size. Sulphitolysis of component I resulted in no change in the elution position of the single radioactive component. Tryptic hydrolysis of component I resulted in several radioactive components (Fig. 6a), one of which was eluted in the same position as component II. This product, after sulphitolysis, was eluted from Sephadex G-75 like the products of sulphitolysis of component II (Fig. 6b). Thus it was concluded that component I was a single-chain polypeptide, hydrolysable by trypsin to a product similar to component II.

The precursor-product relationship between components I and II was further established by the demonstration that incorporation of [^3H]leucine into component I occurred before the appearance of radioactivity in component II. Incorporation of [^3H]leucine into component I was decreased when [^3H]leucine was removed during an incubation (Fig. 7a) or when puromycin was added (Fig. 7c). Incorporation of [^3H]leucine into component II continued to increase under these conditions. The addition of puromycin (or cycloheximide), inhibitors of protein synthesis, inhibits subsequent proinsulin synthesis, but not the conversion of proinsulin already present into insulin (Steiner *et al.*, 1969).

Component II fitted the criteria for bullhead insulin. It was similar in size to bovine and bonito insulin when eluted from Biogel P-30 in the presence of 7M-urea. It was a double-chain protein, joined by disulphide bonds. It was found in the fraction with the highest insulin-like biological activity. Component I fitted the criteria for bullhead proinsulin.

Fig. 3. Gel filtration of ^3H -labelled component II and bonito insulin with urea

Pooled, dried component II was redissolved in 3M-acetic acid together with 7mg of bonito insulin and divided into two aliquots. One half (a) was eluted on a column (1.5 cm × 90 cm) of Biogel P-30 equilibrated with 3M-acetic acid. The other half (b) was eluted on a similar column equilibrated with 3M-acetic acid containing 7M-urea. ●, Radioactivity; ····, $E_{275}^{1\text{cm}}$ of bonito insulin.

Fig. 4. Sulphitolysis of component II

The proteins were treated as described for Fig. 2. (a) Component II after sulphitolysis and (b) control mixture. ●, Radioactivity; ····, $E_{275}^{1\text{cm}}$.

It was similar in size to bovine proinsulin, was not a double-chain protein and had much less insulin-like activity. Components I and II exhibited a precursor-product relationship similar to that established for mammalian insulin and proinsulin. Therefore it was concluded that component I was bullhead proinsulin and component II was bullhead insulin.

Effect of temperature on the formation of bullhead proinsulin and insulin

Islet tissue from fish acclimated to less than 12°C was incubated at 12°, 22° and 37°C. The incorporation of [^3H]leucine into proteins soluble in acid ethanol at these temperatures is shown in Fig. 8. At 37°C incorporation of [^3H]leucine was very poor. At 22°C, as shown above, [^3H]proinsulin,

Fig. 5. Bioassay of components I, II and III

Insulin-like activity in the fractions containing maximum amounts of radioactivity for components I, II and III were measured by the rat hemidiaphragm (a) and epididymal fat-pad assays (b). ●, Standard bovine insulin; □, fish islet components. Vertical bars indicate S.E., and each point represents the mean of three determinations for (a) and four determinations for (b). For details see the Materials and Methods section on identification of insulin and proinsulin.

[^3H]insulin and [^3H]labelled component III were formed. At 12°C, only [^3H]proinsulin appeared. Little or no incorporation of [^3H]leucine into insulin and component III was observed.

Poor incorporation of [^3H]leucine at 37°C was not unexpected as 37°C is not a 'physiological' temperature for fish acclimated to 3–8°C. The upper lethal temperature for a fish is considered to be the upper lethal temperature for its excised tissue (Fry & Hochachka, 1970). For the bullhead, this temperature is 37°C (Brett, 1944). Smith (1973) for example, has shown that four mitochondrial enzymes from 14 species of fresh-water fish are not stable when incubated for 30 min at 37°C.

The difference between incorporation of [^3H]leucine at 12° and 22°C required further investigation. It was considered possible that the enzymic system converting proinsulin into insulin might be temperature sensitive. Such a sensitivity would explain the observed effects of temperature on the incorporation

Fig. 6. Characterization of component I

(a) Elution profile of the tryptic hydrolysate of pooled component I. The pooled, dried protein was redissolved in 1 ml of 0.2M-Tris-HCl buffer, pH8, and incubated with 5 μ g of trypsin at 37°C for 30 min. The hydrolysate was eluted through Biogel P-30 with 3M-acetic acid. (b) Pooled fractions from tryptic hydrolysate [indicated by the bar in (a)] were subjected to sulphitolysis after the addition of bovine insulin. ●—●, Radioactivity; ····, $E_{275}^{1\text{cm}}$.

of [^3H]leucine into proinsulin and insulin. Several experiments were performed to test this hypothesis. First, the islets were incubated for 4 h at 22°C with [^3H]leucine. The medium was replaced with a medium containing unlabelled leucine and the incubation was continued for 12 h at 12°C. Less radioactivity was transferred from proinsulin to insulin than in the control system, which was incubated at 22°C for 16 h.

Fig. 9 shows the incorporation of [^3H]leucine into bullhead insulin and proinsulin when the temperature was increased during an incubation. At 22°C (Fig. 9a), considerable conversion of proinsulin into insulin occurred whereas little or no conversion occurred at 12°C (Fig. 9b). These observations strongly suggest that the enzymic conversion of proinsulin into insulin in the bullhead islet was temperature sensitive with low activity at 12°C, a temperature close to the acclimation temperature of the fish.

Incorporation of [^3H]leucine at 12° and 22°C into islet tissue from fish acclimated to 22°C for 6–20 weeks was qualitatively the same as it was for tissue from fish acclimated to less than 12°C.

Effect of glucose and amino acid concentration

No difference in the incorporation of [^3H]leucine, or proinsulin conversion into insulin, could be demonstrated when glucose was omitted from the medium or increased from the normal physiological concentration to 400 mg/100 ml. Glucose did not stimulate synthesis or conversion at 12° or 22°C and it did not seem to be required.

The amino acid concentration (with the exception of leucine, which was not tested) did not appear to affect proinsulin synthesis or conversion of proinsulin into insulin.

Discussion

Our results demonstrate that when the principal islet tissue of the bullhead was incubated with [^3H]leucine, three radioactive components were recovered after gel filtration of the acid-ethanol extract. Two of these components, components I and II, were identified as proinsulin and insulin respectively by physical, chemical and biological characterization. Because of the lack of cross-reactivity conclusive identification by immunological methods must await the availability of sufficient quantity of pure bullhead insulin for the production of antiserum. Nevertheless it is apparent that biosynthesis of insulin in the bullhead occurred in two phases as has been demonstrated for other vertebrates: synthesis of the single-chain precursor proinsulin and enzymic conversion of proinsulin into insulin.

Glucose had no marked quantitative effect on proinsulin synthesis. This agrees with the results of

Fig. 7. Demonstration of the precursor-product relationship between components I and II

(a) Islet tissue (72mg) was incubated with $50 \mu\text{Ci}$ of $[^3\text{H}]$ leucine for 6h at 22°C , followed by 6h incubation with non-radioactive leucine. (b) Islet tissue (68mg) was incubated with $50 \mu\text{Ci}$ of $[^3\text{H}]$ leucine for 12h at 22°C . (c) Islet tissue (45mg) was incubated with $20 \mu\text{Ci}$ of $[^3\text{H}]$ leucine for 3h at 22°C . Puromycin ($20 \mu\text{g}$) was added and the incubation continued for a further 12h. (d) Islet tissue (45mg) was incubated with $20 \mu\text{Ci}$ of $[^3\text{H}]$ leucine for 15h at 22°C .

Grant & Reid (1968) and Noe & Bauer (1971) who found that increasing the concentration of glucose in the medium did not increase the incorporation of labelled amino acids into acid-ethanol-soluble proteins. Noe & Bauer (1971) omitted glucose claiming that it actually inhibited protein synthesis. In mammals glucose stimulates proinsulin synthesis. Permutt & Kipnis (1972) showed that incorporation of $[^3\text{H}]$ leucine into insulin was increased eightfold when the glucose concentration in the incubation medium was increased from 2.8 to 15.3 mM. However,

in foetal calf islet glucose failed to stimulate proinsulin synthesis (Tung & Yip, 1969).

Grant & Reid (1968) found radioactive intermediates in the conversion of proinsulin into insulin. Yamaji *et al.* (1972) also were able to isolate an intermediate from anglerfish islet tissue. We did not attempt to look for intermediates since not enough tissue was available.

Temperature had a profound effect on the enzymic conversion of proinsulin into insulin. At 12°C , only a few degrees above the temperature at which the

Fig. 8. Effect of temperature on the incorporation of [^3H]leucine into acid-ethanol-soluble proteins

Four islets were incubated with $50\mu\text{Ci}$ of [^3H]leucine for 7 h at 37°C ($\square$), 22°C ($\bullet$) and 12°C ($\circ$). Tissue weights were 47, 51 and 65 mg respectively. Extracts were eluted on Biogel P-30 with 3 M-acetic acid.

fish were acclimated, little or no conversion of [^3H]proinsulin into insulin *in vitro* could be demonstrated. At 22°C , more than 10°C higher than the acclimation temperature of the fish, incorporation of [^3H]leucine into insulin was observed (Fig. 8). Labelled proinsulin was not converted into insulin in islet tissue maintained at 12°C for more than 15 h. When tissue was incubated at 12°C with [^3H]leucine and then further incubated at 22°C without [^3H]leucine, conversion of radioactive proinsulin into insulin occurred. In the control incubation maintained at 12°C no conversion was observed. We have established that conversion of proinsulin into insulin did not occur in the incubation medium, but occurred in the granule fraction of the tissue (Moule, 1972).

Such a temperature effect has not been demonstrated in the previous studies on insulin synthesis in fish possibly because relatively higher temperatures

Fig. 9. Effect of increasing the temperature during an incubation

(a) Two islets (34.5 mg) were incubated for 3 h with $20\mu\text{Ci}$ of [^3H]leucine at 12°C followed by 9 h at 22°C in fresh medium with unlabelled leucine. (b) Two islets (31 mg) were incubated for 15 h with $20\mu\text{Ci}$ of [^3H]leucine at 12°C . The medium was replaced at 3 h with one containing unlabelled leucine. $\bullet$ — $\bullet$, Radioactivity; $\cdots$, $E_{275}^{0.1}$ of bovine insulin.

were used. Biosynthesis of insulin in anglerfish islet tissue was measured at 20°C (Trakatelis & Schwartz, 1970; Noe & Bauer, 1971). Grant & Reid (1968) found that incorporation of labelled amino acids into total acid-ethanol-soluble proteins of cod islet was maximal at 15°C and consequently used this temperature for their experiments. The cod appears to prefer temperatures of 1 – 5°C with 20°C as the upper limit of its range (Wise, 1958). The bullhead, although able to tolerate temperatures of 0 – 37.5°C , lives in Ontario

at 4°C for nearly half the year (Brett, 1944). The incubation temperature of 12°C is in the lower part of the bullhead's temperature range. It is possible that the temperature effect on the conversion of proinsulin into insulin is manifested only at the lower part of the fish's temperature range. Thus the cod islet might also show similar temperature dependence at temperatures much lower than 15°C, closer to the preferred temperatures.

That proinsulin was not converted into insulin at 12°C could have been due to an absence of the enzyme or its low activity at that temperature. The results of increasing or decreasing the incubation temperature suggest that the enzyme was present in the islet preparation but had low activity at 12°C. It is quite possible that other factors could stimulate the conversion of proinsulin into insulin at 12°C. In our system, neither glucose nor amino acids acted as a stimulus. It is also possible that the enzyme was temperature sensitive and had a low activity at 12°C. If this is so, then there may be little insulin present in the bullhead at temperatures of 12°C or less. In fact we found no insulin activity in the fractions corresponding to proinsulin and insulin in extracts of bullhead islet in Spring and Winter, whereas there was a very marked insulin activity in these fractions obtained in the Summer (Moule, 1972). Said & Al-Hussaini (1966) also observed a lack of insulin activity in pancreatic extract from *Tilapia* sp. and *Mugil* sp. in Autumn and Winter, whereas they could demonstrate such activity from extracts obtained in Spring and Summer. These observations are suggestive of a seasonal difference in the insulin content of the pancreas in these fish.

The phenomenon of an increase in blood glucose in the toadfish (Nace *et al.*, 1964; Silver & Shenk, 1968) and in *Fundulus heteroclitus* and *Fundulus grandis* (Umminger, 1971a,b,c) at low temperature is noteworthy. Umminger suggested that either a decrease in insulin production or an increase in glucagon production could account for the cold-induced hyperglycaemia. A temperature-sensitive enzymic conversion of proinsulin into insulin would result in a decrease in insulin production. Fry & Hochachka (1970) reviewed the metabolic changes in fish acclimated to different temperatures and proposed that changes in hormonal balance must occur. Although the temperature-sensitive enzymic conversion of proinsulin into insulin may play a role in hormonal balance, it remains to be demonstrated that serum insulin or proinsulin present in the bullhead at low temperature reflect the results we have obtained *in vitro*.

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