Biosynthesis of an Insulin Precursor by Islet Tissue of Cod (*Gadus callarias*)

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(Received 26 June 1968)

1. At 15°, slices of cod islet tissue incorporated [U-¹⁴C]proline into proteins soluble in acid-ethanol at a linear rate for 6 hr. 2. Initially, all the radioactivity was associated with a polypeptide that had a molecular weight of about 10 000 and was appreciably more basic than cod insulin. After 1 hr. there was also a significant and progressive increase in the radioactivity of insulin and of fractions intermediate in molecular size and basicity between the polypeptide and insulin. 3. *O*-Ethyl *O*-*p*-nitrophenyl phenylpropylphosphonate markedly decreased the radioactivity both of the intermediate fractions and of insulin, but had no significant effect on the biosynthesis of the polypeptide. In contrast, puromycin inhibited the incorporation of radioactivity into all the fractions. 4. The polypeptide had an activity of less than 0.2 international unit/mg. in the epididymal-fat-pad bioassay. Treatment with low concentrations of trypsin caused a progressive increase in the formation of an insulin-like material, judged by bioassay and ion-exchange chromatography of the digest. 5. Gel filtration of the polypeptide after oxidative sulphytolysis indicated that it was a single polypeptide chain. 6. The results suggest that the polypeptide is an insulin precursor whose formation is inhibited by puromycin and that the steps involved in the conversion of precursor into product are sensitive to *O*-ethyl *O*-*p*-nitrophenyl phenylpropylphosphonate.

A degree of species variation occurs in the amino acid sequences of insulins (Smith, 1966), and this is particularly marked in certain fishes (Grant & Reid, 1968a). At the same time, all insulins are comprised of two polypeptide chains: an A chain, containing an intrachain disulphide bond and 21 amino acid residues, connected by two interchain disulphide bonds to a B chain, containing either 29 or 30 amino acid residues. The evidence available suggests that a conventional RNA-directed mechanism is concerned in the biosynthesis (Humbel, 1965) by the rough fraction of microsomes (Bauer, Lindall, Dixit, Lester & Lazarow, 1966). In these and other studies on the mode of formation, storage and release of insulin, the islet tissue of certain fishes was used in preference to preparations of the mammalian pancreas since the former is usually free of acinar tissue and is largely composed of β -cells of the islets of Langerhans.

A unique feature of insulin is the formation at some point in the biosynthetic sequence of the single intrachain and the two interchain disulphide bonds. Chymotrypsin (Hartley, 1964), immunoglobulins (Fleischman, Pain & Porter, 1962), insulin and possibly fibrinogen (Blombäck, Iwanaga & Wallén, 1967) appear to be the only proteins that

contain interchain disulphide bonds, and two quite distinct biosynthetic mechanisms may be envisaged (Wang & Carpenter, 1965). One mechanism would be the formation of a single-polypeptide-chain precursor that contains in the overall amino acid sequences the correct sequences that eventually become the chains of the product. The configuration of the formed precursor would determine the correct alignment and formation of the disulphide bonds (Givol, Lorenzo, Goldberger & Anfinsen, 1965). Evidence for the existence of this type of insulin precursor has been obtained with preparations from rat and human tumour islets (Steiner & Oyer, 1967; Clark & Steiner, 1968) and with material isolated from crystalline commercial insulin (Yip & Lin, 1967; Chance & Ellis, 1968). The conversion of the precursor by trypsin into an 'insulin-like' product has been demonstrated (Steiner, Cunningham, Spigelman & Aten, 1967; Chance & Ellis, 1968). The alternative mechanism would be the separate and parallel synthesis of each polypeptide chain with a subsequent directive mechanism for disulphide bond formation such as a template, an enzyme (Varandani, 1967) or the information contained in the primary sequence of each chain (Katsoyannis, 1966). The separate

synthesis of each chain of angler-fish insulin has been inferred by Humbel (1965) as the explanation for the unequal labelling of the three prolyl residues observed in insulin after islet tissue slices had been incubated with [^3H]proline for 15 or 30 min. The interpretation of these results may, however, be complicated by the possibility that β -cells contain more than one type of prolyl-t-RNA,* each containing anticodons specific for one of the four proline codewords. For example, there are two prolyl-t-RNA species in *Escherichia coli* (Pearson & Kelmers, 1965), and two different arginyl-t-RNA species transfer arginine into different positions of rabbit haemoglobin in a way that suggests that the three arginine residues of the α -chain correspond to three different codons (Weisblum, Cherayil, Bock & Soll, 1967).

The present work is concerned with the biosynthesis of insulin by slices of cod islet tissue. The results indicate that a precursor polypeptide is formed by a puromycin-sensitive pathway and that the precursor is converted into insulin by an enzyme that contains a 'unique' serine at the active centre. A brief account of some of this work has already been given (Grant & Reid, 1968*b*).

MATERIALS AND METHODS

Materials. Puromycin was obtained from Sigma (London) Chemical Co., London, S.W. 6. Actinomycin D was a gift from Dr D. C. Burke. NEP was a gift from Dr W. N. Aldridge (see Becker, Fukuto, Boone, Canham & Boger, 1963). L-[U- ^{14}C]Proline (specific radioactivity 270 $\mu\text{C}/\mu\text{mole}$) and D-[1- ^{14}C]glucose (specific radioactivity 41.5 $\mu\text{C}/\mu\text{mole}$) were obtained from The Radiochemical Centre, Amersham, Bucks.

$\text{Na}_2\text{S}_4\text{O}_6$ was prepared from $\text{Na}_2\text{S}_2\text{O}_3$ and iodine, as described by Gilman, Philips, Koelle, Allen & St John (1946). Urea was deionized by passing an 8M solution of AnalaR-grade urea over a bed of Amberlite MB-1 cation- and anion-exchange resin. All other chemicals and solvents were of AnalaR grade obtained from British Drug Houses Ltd., Poole, Dorset, and were used without further purification.

Sephadex G-50 and G-75 and DEAE-Sephadex A-25 (fine particle size) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. CM-cellulose (medium mesh) was obtained from Sigma (London) Chemical Co.

Trypsin (batch TRTPCK, 7FB) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Chymotryptic activity of the trypsin was minimized by treatment with L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one as described by Kostka & Carpenter (1964). Ox insulin (six times recrystallized, 22.2 i.u./mg.; batch no. CB 14245LL) was obtained from Boots Pure Drug Co. Ltd., Nottingham. Pig glucagon (twice crystallized, batch no. B66) was obtained from Novo Industri, Copenhagen, Denmark. Lysozyme (crystalline, prepared

from egg white; batch no. ED1990) and ox plasma albumin (fraction V) were obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. Ribonuclease from ox pancreas (five times crystallized, batch no. 26B-1370) and cytochrome *c* from horse heart (batch no. 115B-7660) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. A tissue-culture medium (Eagle) without added antibiotics, batch no. M1687, was obtained from Burroughs Wellcome and Co., London.

Animals. Live cod (about 3–6 kg.) were obtained by seine-net from Aberdeen bay in the period September–May. They were maintained at 15° in a sea-water aquarium for 24–48 hr. before slaughter.

Incubation medium. A Krebs–Henseleit bicarbonate-buffered salt solution, pH 7.4, containing 0.14M-Na⁺ (Umbreit, Burris & Stauffer, 1957) was used in all experiments unless otherwise stated. Before use the buffer was equilibrated by gassing it with O₂+CO₂ (95:5) for 10 min.

Isolation of islet tissue. Cod were killed by a blow on the head and the encapsulated aggregate of islet tissue (Brockman body) was dissected free from vascular connexions and the gall bladder. Each islet (70–120 mg. wet wt.) was immediately immersed in incubation medium at 4° and stored for not longer than 30 min. before use.

Incubation procedures. (a) Normal procedure. Each islet was removed from the cold incubation medium, placed on a pad of filter paper and cut into four pieces by two cuts, the second at right angles to the first, with a stainless-steel razor blade. One slice was added from each of eight islets to each of four pre-weighed incubation flasks (5 cm. $\times$ 2.5 cm., 35 ml. capacity) containing 1.6 ml. of incubation medium and [U- ^{14}C]proline (1–10 μC , 270 $\mu\text{C}/\mu\text{mole}$) at 4°. This served to minimize the effects of individual variation between animals and to make the results of repeated experiments reasonably reproducible. The flasks were reweighed, stoppered (Suba-Seal, size 53) and gassed for 5 min. with O₂+CO₂ (95:5) by hypodermic needles (20 gauge) inserted through the membrane of the stopper. Incubations were carried out with gentle shaking (72 cye./min.) in a metabolic bath (H. Mickle Ltd., Gomshall, Surrey), normally at 15°, and the reaction was stopped by the addition of 1.6 ml. of 20% (w/v) trichloroacetic acid from a hypodermic syringe with a needle inserted through the membrane of the stopper.

(b) Addition of inhibitors. A solution of actinomycin D (100 μl .) or puromycin (100 μl .) in incubation medium or a solution of NEP in acetone (10 μl .) was added at the beginning of the experiment or after incubation for 1 hr. Similar additions of incubation medium or of acetone were made to control flasks.

Isolation of total protein and acid-ethanol-soluble protein. At the end of an incubation period, trichloroacetic acid was added as described above and the suspension was kept for 30 min. at 4°. The flask contents were quantitatively transferred to a 15 ml. centrifuge tube, the suspension was centrifuged and the precipitate of insoluble protein was resuspended in 5 ml. of 7% (w/v) trichloroacetic acid. The residual crude protein was extracted three times with 3 ml. portions of acid-ethanol (ethanol-N-HCl-water, 39:9:4, by vol.). The extracts were combined and adjusted to about pH 3–4 by the addition of M-trisodium citrate. Ethanol was removed *in vacuo* at 35°, and lipids were removed by an extraction with an equal volume of methylene chloride. Trichloroacetic acid was added to the residual aqueous

* Abbreviations: t-RNA, transfer RNA, m-RNA, messenger RNA; NEP, *O*-ethyl *O*-*p*-nitrophenyl phenyl-propylphosphonate.

solution to give a final concentration of 5% (w/v). The suspension was kept overnight at 5°. The precipitate (protein soluble in acid-ethanol but insoluble in trichloroacetic acid) was repeatedly washed with ether-acetone (1:1, v/v), and finally with dry ether.

Gel filtration. (a) Proteins soluble in acid-ethanol were dissolved in 1.0 M-acetic acid and applied to a column (80 cm. $\times$ 1.3 cm.) of Sephadex G-50 previously equilibrated with the same solvent. Elution with M-acetic acid was maintained at a rate of 10 ml./hr. by the use of an LKB proportionating pump.

(b) With the conditions given above, a column of Sephadex G-50 was used to prepare a calibration curve relating the logarithm of the molecular weight of a polypeptide to its elution volume (Andrews, 1965). The purified materials used, with their molecular weights in parentheses, were: *S*-aminoethyl A chain of cod insulin (2300); *S*-aminoethyl B chain of cod insulin (3300); glucagon (3500); ox insulin (5750); cytochrome *c* (12400); ribonuclease (13700); lysozyme (14400); trypsin (23800).

(c) Gel filtration on Sephadex G-75 was carried out as described by Grant & Reid (1968a).

Ion-exchange chromatography. Gradient elution from columns of DEAE-Sephadex A-25 and of CM-cellulose were carried out as described by Grant & Reid (1968a).

Recovery of fractions. Pooled fractions containing either acetic acid or ammonium formate buffer were freeze-dried. Residual ammonium formate was subsequently removed by sublimation with an infrared lamp at a pressure of less than 0.05 mm. Hg.

Measurement of extinction. Extinctions were read on a Unicam SP.500 instrument with quartz cells of either 1 cm. or 4 cm. path length.

Measurement of radioactivity. A low-background (<2 counts/min.) flow counter [Omni-Guard; Tracerlab (G.B.) Ltd., Weybridge, Surrey] was used. The overall efficiency for ^{14}C was about 30%. Samples were counted at 'infinite thinness' on circles of lens tissue mounted on 1 in.-diameter planchets. Solvents were removed by exposure to an infrared lamp. Not less than 2000 counts were collected in the repeated assay of any one sample.

Oxidative sulphytolysis. This procedure, which converts cod insulin into the *S*-sulpho A and B chains, was carried out essentially as described by Reid, Grant & Youngson (1968). Cod insulin (5 mg.) and a radioactive polypeptide (polypeptide P1, Fig. 5a) was dissolved in 0.5 ml. of 0.2 M-potassium phosphate-8 M-urea buffer, pH 7.5; Na_2SO_3 (12 mg.) and $\text{Na}_2\text{S}_4\text{O}_6$ (7 mg.) were added and the reaction was allowed to proceed at 37° for 3 hr. The reaction mixture was acidified by the addition of acetic acid, and the *S*-sulpho A and B chains of insulin were separated by application of the entire reaction mixture to a column of Sephadex G-75 previously equilibrated with aq. 50% (v/v) acetic acid (Fig. 5b).

Electrophoresis. Disc (70 mm. $\times$ 6.5 mm.) electrophoresis in 7.5% polyacrylamide gel was carried out by using the discontinuous buffer system described by Ornstein & Davis (1964).

Quantitative amino acid analysis. Hydrolysis with acid and amino acid analysis of the hydrolysate were carried out as described by Grant & Reid (1968a).

Tryptic-digestion and insulin-assay procedures. A polypeptide (polypeptide P1, Fig. 5a) was dissolved in 0.1 M-ammonium carbonate buffer, pH 8.1, and incubated with

trypsin at 37°. Enzyme/substrate ratios were in the range 1:250–1:100 in different experiments. The digestion was stopped by the addition of acetic acid and the solution was freeze-dried.

The polypeptide or enzymic digests were dissolved in potassium phosphate buffer, pH 7.4, containing 1% (w/v) ox plasma albumin, and the insulin-like activity of the solutions was measured in the rat epididymal-fat-pad bioassay procedure described by Renold *et al.* (1960), with crystalline cod insulin (11.5 i.u./mg.; Grant & Reid, 1968a) as standard.

RESULTS

Cod insulin contains one residue of proline in the A chain and three in the B chain (Grant & Reid, 1968a). ^{14}C was readily incorporated by slices of islet tissue incubated with $[\text{U-}^{14}\text{C}]$ proline into acid-ethanol-soluble proteins. The rate of incorporation was negligible at 30°, but was approximately linear for 6 hr. at 15° (Fig. 1). No significant change in the rate of incorporation of $[\text{U-}^{14}\text{C}]$ proline into islet tissue slices was found when the following changes were made in the standard incubation medium: increase in the concentration of salt from 0.14 M- to 0.25 M- Na^+ ; addition of glucose (1.5 or 3.0 mg./ml.); addition of bovine plasma albumin (2 mg./ml.); replacement of the standard medium by a tissue-culture medium (Eagle). In contrast, actinomycin D (0.5 $\mu\text{g.}/\text{mg.}$ wet wt. of islet tissue) effected a slight but consistent increase in the rate of incorporation.

At 15°, it was found that only about 30% of the total radioactivity in the protein (material insoluble in trichloroacetic acid) could be extracted into acid-ethanol.

Fig. 1. Effect of temperature on the rate of incorporation of radioactivity into acid-ethanol-soluble protein of islet-tissue slices (150 mg.) incubated with $[\text{U-}^{14}\text{C}]$ proline (1 μC ; 270 $\mu\text{C}/\mu\text{mole}$).

Labelling patterns in polypeptides and fractions isolated from acid-ethanol extracts

Gel filtration. In comparable experiments, slices of islet tissue were incubated with $[U-^{14}C]$ proline at 15° for various times, and acid-ethanol extracts were filtered on Sephadex G-50 (Fig. 2). The extinctions on the trace (Figs. 2a and 2b) showed a maximum at fraction 45 and this peak corresponded to insulin. After 1 hr., the insulin was unlabelled and the radioactivity was almost entirely associated with a peak (peak P1) with a maximum at fraction 40 (Fig. 2a). The low extinctions suggested that polypeptide P1 either was present in low concentration or contained few aromatic amino acid residues. Experiments, described below, with a calibrated column of Sephadex G-50 indicated that insulin behaved as a monomer (mol.wt. about 5700) under these conditions, and that the molecular weight of peak P1 is about 10000.

Longer periods of incubation (Fig. 2c) resulted both in a progressive shift in the fraction containing

the maximum radioactivity towards the retention volume of insulin and in a progressive increase in the radioactivity. These results showed that polypeptide P1 is the first polypeptide to be biosynthesized by islet tissue and demonstrated the subsequent formation of compounds intermediate in molecular size between polypeptide P1 and insulin. For this reason a more distinct separation was attempted by a procedure that utilized differences in properties other than molecular size.

Chromatography on DEAE-Sephadex A-25. Material with molecular weight in the approximate range 3000–12000 was obtained from each experiment by gel filtration (Fig. 2c). Each pooled fraction was subsequently fractionated, together with unlabelled insulin, on DEAE-Sephadex A-25 (Fig. 3). By this procedure radioactive polypeptide P1 was well separated from insulin (Fig. 3a), and the results suggested that the former is appreciably more basic than the latter. The radioactivity associated with polypeptide P1 from these experiments in which islet tissue was incubated with

Fig. 2. Fractionation of the proteins soluble in acid-ethanol from slices of islet tissue (200–240mg.) after incubation with $[U-^{14}C]$ proline ($10\mu C$, $270\mu C/\mu mole$) on a column (80cm. $\times$ 1.3cm.) of Sephadex G-50 in 1.0M-acetic acid. The flow rate was 10ml./hr. Fractions (1.8ml.) were collected. ----, $E_{276}^{cm.}$; —, radioactivity. Incubation was for: (a) 1 hr.; (b) 6 hr.; (c) $\circ$, 1 hr.; $\bullet$, 2 hr.; $\square$, 4 hr.; $\blacksquare$, 6 hr. In each experiment, the fractions within the horizontal bar were pooled for subsequent chromatography on DEAE-Sephadex.

Fig. 3. Elution diagrams of pooled radioactive fractions from a column (27cm. $\times$ 0.9cm.) of DEAE-Sephadex A-25. The pooled fractions had been isolated by gel filtration (Fig. 2c) of acid-ethanol extracts of islet tissue previously incubated with $[U-^{14}C]$ proline for (a) 1 hr.; (b) 3 hr.; (c) 6 hr. Fractions (2.2ml.) were collected. Gradient elution was with 0.01–1.0M-ammonium formate, pH9.5. The flow rate was 30ml./hr. Crystalline cod insulin (5mg.) was added to each pooled fraction before chromatography. ----, $E_{276}^{cm.}$; —, radioactivity.

[U-¹⁴C]proline for 1, 4 and 6 hr. (Figs. 3*a*, 3*b* and 3*c* respectively) did not increase after 4 hr., but the 4 hr.- and 6 hr.-incubation experiments indicated a progressive formation of radioactive intermediates and of insulin.

The most retarded radioactive fraction from the 6 hr. incubation experiment was tentatively identified as insulin. This was confirmed by further chromatography of the combined material (fractions 60–70) on CM-cellulose, as described by Grant & Reid (1968*a*). Under these conditions polypeptide P1 and the intermediates were retained on the column. In an exactly comparable experiment a radioactive peak corresponding to insulin was isolated without the addition of unlabelled insulin. The radioactive material was identified as insulin from its biological activity in the rat fat-pad bioassay and from its amino acid analysis. The latter was in agreement with that obtained from cod insulin isolated by the same procedure from about 70 g. of cod islets (Grant & Reid, 1968*a*).

Effects of NEP and of puromycin

Although there was inhibition at higher concentrations, 0.2 μ g. of NEP/mg. of islet tissue had little effect on the incorporation of [U-¹⁴C]proline into acid-ethanol-soluble protein. Slices of islet tissue were incubated for 60 min. with [U-¹⁴C]proline to label polypeptide P1, NEP (0.2 μ g./mg. of islet tissue) was added and the incubation was continued for a further 140 min. The acid-ethanol-soluble protein was gel-filtered on Sephadex G-50, 5 mg. of crystalline cod insulin was added to the pooled radioactive fractions (cf. Fig. 2*c*) and the mixture was fractionated on DEAE-Sephadex (Fig. 4*a*). The results show that NEP had little effect on the biosynthesis of the first radioactive peak (polypeptide P1), but almost completely abolished the incorporation of radioactivity into insulin and the intermediate fractions. In an exactly comparable experiment (Fig. 4*b*), puromycin (1 μ g./mg. of islet tissue) inhibited the incorporation of radioactivity into all the fractions, but the effect on polypeptide P1 was most pronounced. The peak of radioactivity associated with polypeptide P1 was even smaller than that of the control incubated for only 1 hr. In a separate experiment, puromycin was added at the beginning of a 4 hr. incubation period of cod islets with [U-¹⁴C]proline. When compared with a control, the formation of polypeptide P1 was inhibited by about 70%, and there was no detectable incorporation of radioactivity into either insulin or the intermediate fractions.

These results are consistent only with the concept that polypeptide P1 is formed by the conventional mechanism of protein synthesis and that the polypeptide intermediates and insulin are

Fig. 4. Effects of NEP and of puromycin. Elution diagrams from a column (27 cm. $\times$ 0.9 cm.) of DEAE-Sephadex, of radioactive polypeptides, obtained as described in the text, after incubation of islet tissue (150 mg.) with [U-¹⁴C]proline in the presence and absence of inhibitors. Fractions (2.2 ml.) were collected. Gradient elution was with 0.01–1.0 M ammonium formate, pH 9.5. The flow rate was 30 ml./hr. ----, $E_{276}^{1\text{cm}}$; —, radioactivity. (a) $\circ$, 60 min. incubation; $\bullet$, 200 min. incubation, no addition; $\square$, NEP added after 60 min., incubation continued for a further 140 min. (b) $\circ$, 60 min. incubation; $\bullet$, puromycin added after 60 min., incubation continued for a further 140 min.

formed from polypeptide P1 by a mechanism that is sensitive to NEP.

Properties of polypeptide P1

A sample of radioactive polypeptide P1, obtained by the combination of fractions from several experiments, was rechromatographed on DEAE-Sephadex. The material was mixed with 10 mg. of unlabelled crystalline cod insulin and portions of the mixture were gel-filtered on Sephadex G-75, before (Fig. 5*a*) and after (Fig. 5*b*) oxidative sulphitolysis in urea. Polypeptide P1 was not significantly affected by oxidative sulphitolysis, but cod insulin was converted into the S-sulpho A and B chains. The S-sulpho A chain was eluted after the S-sulpho B chain, and this was followed by a rise in the extinction value due to the elution of urea and buffer salts (Fig. 5*b*).

Unlabelled polypeptide P1 was prepared from the acid-ethanol-soluble protein from 70 g. of cod islet tissue, by gel filtration and chromatography on DEAE-Sephadex. Labelled polypeptide P1 was added and the mixture was chromatographed again on DEAE-Sephadex to yield a single symmetrical

Fig. 5. Gel filtration, on a column (80 cm. $\times$ 1.3 cm.) of Sephadex G-75, of a mixture of unlabelled cod insulin (5 mg.) and radioactive polypeptide P1: (a) without treatment; (b) after oxidative sulphytolysis. ----, E_{276}^{cm} ; —, radioactivity. The solvent was aq. 50% (v/v) acetic acid. The flow rate was 10 ml./hr. Fractions (2.3 ml.) were collected.

Table 1. Comparison of the amino acid composition of fraction P1 and of cod insulin

Two separate amino acid analyses of a sample of fraction P1 were performed as described in the Materials and Methods section. Each sample was hydrolysed for 24 and 72 hr. The results were corrected for amino acid decomposition, and the mean values were calculated on the assumption that there are three residues of histidine/molecule of P1. The amino acid composition of cod insulin is that given by Grant & Reid (1968a). —, Not determined.

Amino acid	Amino acid composition (residues/mol., to the nearest integer)		
	Fraction P1	Cod insulin	(a-b)
Asp	9	8	1
Thr	2	0	2
Ser	4	1	3
Glu	12	3	9
Pro	—	4	—
Gly	5	4	1
Ala	6	2	4
Val	3	3	0
Cys	6	6	0
Met	2	1	1
Ile	2	2	0
Leu	8	5	3
Tyr	3	3	0
Phe	3	3	0
Lys	5	1	4
His	3	3	0
Arg	4	2	2

peak as judged by the radioactivity and extinction at 276 m μ of the fractions. The overall yield was 3 mg.; this was in general agreement with that calculated from amino acid analysis of the solution and from the assumption that the molecular weight was 10000. The latter value was based on the retention volume of polypeptide P1 and of insulin on a column of Sephadex G-50 calibrated with other polypeptides of known molecular weight. The overall yield of polypeptide P1 corresponded to about 1% of that of insulin isolated by the same procedures.

Electrophoresis of polypeptide P1 in polyacrylamide gel at pH 8.9 showed one intensely stained band together with one or possibly two trace components. The amino acid analysis of polypeptide P1 is given in Table 1. Good agreement was obtained between the results of duplicate analyses, but it is considered that a complete reliance should not be placed on the results given in Table 1, because of the relatively small amount of material available, which had been found to contain traces

Fig. 6. Release of insulin-like activity in the epididymal-fat-pad bioassay procedure after treatment of polypeptide P1 (20 μ g.) with trypsin (enzyme/substrate ratio about 1:250) in 0.1 M-ammonium bicarbonate buffer, pH 8.1, at 30°. Details of the bioassay procedure are given in the Materials and Methods section. All measurements were carried out in triplicate, and the vertical bars define the maximum and minimum values given by the distal and proximal sections of the fat pad respectively. The graph line is drawn through the arithmetic means of the three values.

of other polypeptide components. Amide groups were not determined, but, because of the basic nature of polypeptide P1 relative to insulin (Fig. 2), it is presumed that most of the additional acidic amino acids in polypeptide P1 are in the form of either glutamine or asparagine.

Digestion of polypeptide P1 with trypsin. Chromatography on DEAE-Sephadex A-25 after the digestion of polypeptide P1 with trypsin (enzyme/substrate ratio about 1:250) for 30 min. showed a marked change in the elution pattern. The radioactivity associated with polypeptide P1 virtually disappeared and was replaced by peaks of radioactivity corresponding to insulin and the intermediate fractions. This apparent conversion of polypeptide P1 into an insulin-like material was also shown more directly by bioassay. In this procedure (Renold *et al.* 1960), insulin stimulates the release of $^{14}\text{CO}_2$ when pieces of rat epididymal fat pad are incubated with $[1-^{14}\text{C}]\text{glucose}$. Bioassay of the tryptic digest of polypeptide P1 (Fig. 6)

is a stoichiometric inhibitor of enzymes that contain a unique serine residue as part of the catalytic centre (Becker *et al.* 1963); indeed trypsin was found to convert the purified precursor polypeptide into an insulin-like molecule as judged by bioassay and chromatography of the digest (Fig. 6). These conclusions are in general agreement with the first real evidence for the existence of an insulin precursor or proinsulin in β -cells (Steiner & Oyer, 1967; Steiner *et al.* 1967), except that the conversion of precursor into product in cod islet tissue appears to involve a number of compounds that are intermediate in molecular size and basicity between the precursor and insulin (Figs. 1 and 2). The identity of these intermediates has not been established, but the observations that they are formed before insulin (Fig. 2b), that their formation is inhibited by NEP (Fig. 3) and that a tryptic digest of polypeptide P1 contains compounds of similar chromatographic behaviour strongly suggest that they are obligatory intermediates in the conversion of P1 into insulin:

showed a progressive increase of insulin-like activity. After digestion for 40 min., no further increase was detectable in this bioassay, and in three separate bioassays with an increased amount of trypsin (enzyme/substrate ratio about 1:100). On the assumption that one mole of polypeptide P1 yields one mole of insulin, a comparison of these results with those obtained with standard cod insulin solutions permits the approximate calculation that 35–70% of polypeptide P1 had been converted into insulin after a 40 min. digestion with trypsin.

The yield of $^{14}\text{CO}_2$ from the appropriate controls, either polypeptide P1 alone or polypeptide P1 with added inactivated trypsin, was not significantly different from the blank value (no addition). At the same time, the bioassay of larger amounts of polypeptide P1 (up to $2\mu\text{g.}$ of polypeptide/incubation) indicated that the maximum biological activity of this insulin precursor was less than 0.2 i.u./mg.

DISCUSSION

The main conclusions that may be drawn from these studies are that a single-polypeptide-chain precursor of insulin is formed in cod islet tissue by a puromycin-sensitive pathway, and that this precursor is converted into insulin by a mechanism that is inhibited by NEP. This organic phosphonate

After labelling goose-fish islets with ^{14}C -labelled amino acids, Bauer *et al.* (1966) found that the insulin-storage-granule fraction contained a high proportion of labelled protein (insoluble in trichloroacetic acid) as well as insulin-like polypeptide (insoluble in trichloroacetic acid but soluble in acid-ethanol). As only about 30% of the total radioactivity of the protein from cod islet tissue was extracted by acid-ethanol, the possibility cannot be completely discounted that the proinsulin is a protein and that the polypeptides studied in the present work, including polypeptide P1, are merely intermediates in the conversion into insulin. The observation that NEP has little effect on the biosynthesis of the large polypeptide (polypeptide P1), but completely inhibits its conversion into insulin (Fig. 4), makes the concept of a proinsulin protein rather improbable.

Ultrastructural (Williamson, Lacy & Grisham, 1961) and fractionation studies (Randall & Shaw, 1963; Bauer *et al.* 1966) suggest that insulin (probably as the precursor) is synthesized on the ribosomes, passes across the membrane of the endoplasmic reticulum into the cisternae and is further transported within smooth-vesicular structures. The final storage form is concentrated within membrane-bound storage granules. In this sense there are many similarities, in the synthesis and secretory activities, with pancreatic β - and acinar

cells as well as with other cells that specialize in the synthesis and secretion of exportable proteins. At the same time, the conversion of precursor into insulin in the β -cell may be analogous to the transport of macromolecules in the endosomal system originally described by de Duve (de Duve & Wattiaux, 1966), since insulin and not the precursor is secreted into the circulation. There is ample evidence for the induction of biological activity by limited proteolysis in living cells (Ottesen, 1967). Peptide chains are split only at those bonds where the surrounding sequence of the chain meets the specificity requirements of the enzyme and where the configuration of the chain is sufficiently flexible or exposed to permit the formation of the enzyme-substrate complex. After cleavage of these 'available' bonds the rate of subsequent hydrolysis is much decreased. In this connexion, the precursor contains nine basic amino acid residues/molecule compared with three for insulin (Table 1), and it is known that insulin is readily degraded by chymotrypsin, but resistant to trypsin (Butler, Dodds, Phillips & Stephen, 1948) except under rather special conditions (Carpenter, 1966).

The optimum temperature for the incorporation of [U - ^{14}C]proline into acid-ethanol-soluble protein of cod islets was 15° (Fig. 1), and there is an interesting parallel between the inhibitory effects of higher temperatures and the lethal effect of environmental temperature on the intact animal (about 25°). The locus of the thermosensitive step in cod islets has not been studied, but in the synthesis of protein by cell-free extracts of the psychrophilic (cold-loving) *Micrococcus* the reactions concerned with amino acid activation and attachment to t-RNA are thought to be the most thermosensitive (Malcolm, 1968) and certain bacterial mutants contain temperature-sensitive aminoacyl-t-RNA synthetases (Eidlic & Neidhardt, 1965).

Actinomycin D inhibited RNA synthesis in cod islets (Track, Jarrett & Keen, 1968), but had a stimulatory effect on protein synthesis. This suggests that m-RNA had a long half-life (Soeiro & Amos, 1966) and that the primary control of protein synthesis in the present experiments with cod islet tissue is unlikely to be at the transcription stage.

The support of the Medical Research Council to K. B. M. R. is gratefully acknowledged. We are indebted to Mr A. Youngson, who performed the amino acid analyses.

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