The Incorporation of Tritium-Labelled Amino Acids into Insulins in Rat Pancreas *in vitro*

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It has been widely recognized that a system in which isolated pancreas is incubated *in vitro* with labelled substances might serve as a useful model in which to study the biosynthesis of insulin, and slices of ox or calf pancreas (Pettinga & Rice, 1952; Vaughan & Anfinsen, 1954; Light & Simpson, 1956), or fish islet tissue (Humbel, Renold, Herrera & Taylor, 1961), have been used. Incorporation of amino acids into insulin in rat pancreas, however, has not so far been studied *in vitro*. There appeared to be great advantages in using rat pancreas for this purpose. Thus knowledge of the physiology of insulin in the rat is considerable. Moreover the rat is a convenient small animal in which to alter the endocrine or metabolic state before removal of the pancreas.

With the recent availability of tritium-labelled amino acids of high specific activity it was decided to examine their incorporation into insulin in rat pancreas. While this work was in progress it became known that two insulins of differing structure were present in rat pancreas (Smith, 1964). The complete amino acid sequences of these insulins have now been established (L. F. Smith, personal communication). An account of the labelling of these insulins, and of methods for their separation from other pancreatic proteins, is given below. Preliminary accounts of certain aspects of this work have already been published (Taylor, Smith & Gardner, 1962; Smith, Gardner, Mallory & Taylor, 1962).

MATERIALS AND METHODS

Animals. Male rats of a Wistar strain were used throughout. These were bred at King's College Hospital, and were fed on a standard rat diet (M.R.C. diet 41 B). The weight of rats at slaughter was usually 140–160 g., and animals were not starved beforehand.

Insulin. Ox insulin for use as carrier material was a recrystallized specimen, provided by The Wellcome Research Laboratories.

Radioactive amino acids. DL-[4,5-³H₂]Leucine of specific activity 5400 c/mole, and generally labelled DL-[³H]isoleucine of specific activity 316 c/mole, were obtained from The Radiochemical Centre, Amersham, Bucks.

Incubation medium. The basal medium for incubation of either pancreas segments or rat diaphragm was a bicarbon-

ate-buffered salt mixture, gassed with O₂+CO₂ (95:5), pH 7.4 at 37° (Gey & Gey, 1936). This contained D-glucose (2.5 g./l.). For pancreas incubations, the basal medium was mixed with normal human serum (25 ml./l.) to serve as a convenient source of intermediary metabolites and precursors for protein synthesis. The radioactive amino acids were added as supplied in a small volume of water.

In experiments in which the uptake of [³H]leucine from the medium was followed, the final concentration of radioactive leucine in the medium was 10 mc/l. In most other experiments, the concentration of radioactive amino acid was 50 mc/l.

Measurement of radioactivity. Tritium was determined in a Packard Tri-Carb automatic two-channel liquid-scintillation spectrometer (model 314 EX). A filter-paper technique derived from that described by Wang & Jones (1959) was used. It was found that a straight-sided, thin-walled, ½ in. × 2 in. glass specimen tube with polyethylene cap (Hoslab Ltd.) fitted very conveniently into the standard 20 ml. screw-cap counting vial and was held centrally with little lateral freedom. A 1 cm. × 3 cm. slip of filter paper bearing the sample to be counted was inserted into the specimen tube and covered with approx. 3 ml. of fluor mixture [4 g. of 2,5-diphenyloxazole and 100 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 l. of sulphur-free toluene]. The sample was therefore supported approximately at the centre of the counting vial, as recommended for liquid-scintillation counting of tritium on filter paper (Davidson, 1961). Each sample was counted at least twice to minimize any variation due to the positioning of the paper strip relative to the photomultiplier tubes and a total of at least 1000 counts was taken. Quenching of fluorescence did not occur to any significant extent in any of the samples.

The distribution of tritium-labelled material on paper chromatograms or electrophoretograms was determined by cutting the dried papers lengthwise into strips 3 cm. wide, dividing these strips transversely into 1 cm. sections and counting the individual sections as described above.

In uptake and incorporation experiments duplicate 20 µl. samples of incubation medium or of the acid-ethanol-soluble fraction of the tissue were carefully applied to clean 3 cm. × 1 cm. slips of filter paper in the specimen tubes. The tubes were placed in an oven at 80° for 1 hr. and then counted as described. Samples prepared in this way gave a count rate of approx. 25000 counts/min./µc. In a particular sample, with a mean count rate of 250/min., measured in eight successive determinations of 10 min. each, and with a normal cycle of operation, the standard error of the mean was ± 7. Generally, Whatman no. 1 filter

paper was used, although the counting efficiency did not appear to be appreciably lower with the thicker paper no. 3MM.

pH values. Those given for ethanolic solutions are apparent values, as measured with a glass electrode and calomel reference electrode which had been calibrated against aqueous standard buffer solutions.

Incubation procedure. Rats were killed by a blow on the head, and as much blood as possible was drained from each carcass through an incision in the jugular vein. The tail and main portion of the pancreas were then carefully dissected from the spleen and stomach and removed with the minimum of trauma to a Petri dish of ice-cold basal incubation medium containing glucose (see above). The pancreas from two or three animals (about 1.5 g. of tissue) was then cut with scissors into pieces weighing approx. 50 mg., drained free of surplus medium and transferred to a 500 ml. Erlenmeyer flask containing 20 ml. of full incubation medium. The flask was then gassed with $O_2 + CO_2$ (95:5) for 5 min., sealed with a rubber bung and incubated with gentle shaking in a water bath at 37° for 3–4 hr. In experiments in which the medium was periodically sampled, the flasks were gassed again before resealing. Usually two or three flasks were set up for each experiment.

After incubation, the pieces of tissue were removed from the flasks and washed carefully by decantation with two changes of cold medium containing a large excess of the appropriate unlabelled DL-amino acid (1 mg./ml.). Residual medium was drained off with filter paper and the tissue was then rapidly frozen by dropping into acetone–solid CO_2 . If possible, the isolation of insulin was begun immediately; otherwise the frozen pancreas was stored at -10° . In some instances the tissue was freeze-dried.

Extraction of crude insulin. A crude insulin-containing fraction of the tissue was prepared by a procedure based on that of Best, Haist & Ridout (1939). At the suggestion of Dr L. F. Smith (cf. Smith, 1964), ethanol– H_3PO_4 was used in place of ethanol–HCl for the initial stages of the extraction procedure. The volumes of fluid given are those used for 3 g. wet wt. of tissue (as in a typical experiment). The frozen tissue was ground in a mortar with 10 ml. of 80% (v/v) ethanol containing sufficient H_3PO_4 to bring the final pH of the mixture to 3. (In experiments where the tissue had been freeze-dried, 12 ml. of 65%, v/v, ethanol containing H_3PO_4 was used instead.) The resulting slurry was allowed to stand for at least an hour with occasional stirring at room temperature, or overnight at 4°. After centrifugation the insoluble fraction was treated with 3–5 ml. of 65% (v/v) ethanol which had been adjusted to pH 3 with H_3PO_4 . If necessary the supernatant and washings were filtered to remove floating fatty material; they were then combined and 200 μ g. of crystalline ox insulin was added in a small volume of dilute HCl and the pH of the extract was adjusted to 8 with aq. 5N- NH_3 soln. The precipitate which formed was removed by centrifugation and absolute ethanol (1.5 vol.) and diethyl ether (2.5 vol.) were added to the supernatant. The mixture was left to stand overnight at 4° and the crude insulin that was precipitated was separated by centrifugation. It contained insulin and other proteins, and also some salt and fatty material which might have interfered with the subsequent purification. The saline and fatty impurities were easily removed by the following procedure (Fenton, 1961). The

precipitate was dissolved in 4–5 ml. of 0.2N-HCl, and saturated picric acid (2 vol.) was added with mixing. After standing for 30 min., the precipitated picrate was separated by centrifugation and dissolved in 0.5 ml. of acidified acetone (acetone containing 0.2%, v/v, of conc. HCl). An excess (50 ml.) of the acidified acetone was then added with stirring and the mixture was allowed to stand for 30 min. After centrifugation, the crude insulin hydrochloride was washed with 10 ml. of ether and allowed to dry. Further purification of insulin was carried out by paper chromatography followed by paper electrophoresis as described in the Appendix to this paper (Taylor & Smith, 1964). This electrophoresis also separated the two rat insulins.

Separation of labelled A-chain derived from rat insulins. Insulins purified by two-dimensional paper chromatography and electrophoresis were in some experiments oxidized by spraying with performic acid, and the A-chain made was purified by electrophoresis as described in the Appendix. After electrophoresis, the paper was divided into 1 cm. $\times$ 3 cm. sections for determination of radioactivity.

Isolation of labelled A- and B-chains of rat insulins after addition of carrier ox insulin. In some experiments, insulins were purified to the chromatography stage and, after elution from paper, a large excess of crystalline ox insulin was added to improve the yields of the separated chains. Labelled insulins derived from six rat pancreases were eluted from paper, after chromatography, by being left overnight with 5 ml. of 0.01N-HCl. Then 75 mg. of crystalline ox insulin was added and further purification carried out by isoelectric precipitation at pH 5.4. In this procedure, the insulin was dissolved in 2% acetic acid containing an excess of unlabelled DL-leucine or DL-isoleucine, and the pH was adjusted to 5.4 with aq. 0.85% NH_3 soln. (Vaughan & Anfinsen, 1954). Usually six precipitations were necessary to reduce the specific radioactivity to a constant amount.

Oxidation of insulins was carried out by the method of Sanger (1949), with performic acid prepared by the addition of 0.5 ml. of 30% (v/v) H_2O_2 to 9.5 ml. of A.R. 98–100% formic acid (Atfield & Morris, 1961). The mixture was kept for 2 hr. before use. A- and B-chains of insulin were fractionated by the method of Sanger (1949), with certain modifications. After reaction with performic acid, the oxidized products of insulin were diluted with an equal volume of water and the mixture was evaporated to dryness *in vacuo*. The product was then stirred with 2 ml. of 0.1M ammonium acetate, pH 6.5, whereupon the B-chain remained undissolved and the A-chain went into solution.

In further purification of the A-chain, the pH of the solution was adjusted to 4.5 by addition of 0.1N-acetic acid and the resultant precipitate spun off. The supernatant was then evaporated almost to dryness *in vacuo*, and A-chain was further purified by electrophoresis in 20% formic acid on Whatman no. 3MM paper. For this purpose water was added to the A-chain to make a volume of about 0.5 ml. and the whole transferred to paper for electrophoresis by streaking across the mid-line of a sheet 36 cm. $\times$ 18 cm. Electrophoresis was carried out in 20% formic acid as described in the Appendix. After electrophoresis the position of the A-chain was ascertained by spraying the edges of the paper with NN' -diethylpseudocyanine chloride (see Appendix). The area of paper that corresponded with A-chain was then cut out, and the peptide was eluted from the paper with a few millilitres of water through a sintered-

glass funnel. The solution was dried *in vacuo*, and the peptide was weighed. It was then dissolved in 0.2 ml. of water, and a suitable quantity (usually 0.05 ml.) removed for determinations of radioactivity.

In making B-chain, the method of Sanger (1949) was not modified, though the product finally obtained was further purified by paper electrophoresis. The residue left after removal of the A-chain was washed with 0.01 N-acetic acid, and dissolved in 1 ml. of 0.1 N-HCl to which 8 ml. of ethanol was added. After allowing it to stand, the gelatinous precipitate which formed was spun off and the supernatant retained. The precipitate was washed once with 4 ml. of 80% (v/v) ethanol and the washings were combined with the supernatant. The solution was then evaporated almost to dryness *in vacuo* and B-chain precipitated with an excess of A.R. acetone, into a weighed tube. The acetone was removed *in vacuo* and, after drying, the quantity of B-chain present was obtained by weighing. The B-chain was then dissolved in water (usually 1 ml.) and appropriate small quantities (0.2 ml.) were streaked on Whatman no. 1 paper for electrophoresis in 20% formic acid in a vertical tank as before. Virtually all the material present on the paper which stained with bromocresol green possessed the same mobility as a B-chain marker spot, derived from ox insulin. Appropriate areas of paper were cut out and counted.

Hydrolysis of tritium-labelled insulins. For this purpose, extracts from six pancreases which had previously been incubated with the [^3H]leucine or [^3H]isoleucine were pooled and further purified by paper chromatography and electrophoresis. The labelled insulins (corresponding with both rat insulins) were eluted from paper by allowing to stand with 2 ml. of 0.01 N-HCl. After drying *in vacuo*, 5 mg. of crystalline ox insulin was added as carrier, and the mixture hydrolysed with 0.1 ml. of constant-boiling-point HCl for 24 hr. at 110° in a sealed tube.

Excess of acid was then removed *in vacuo* and the residue dissolved in 0.1 ml. of aq. 10% (v/v) propan-2-ol for separation of the constituent amino acids by high-voltage electrophoresis by the method of Atfield & Morris (1961). This method has already been shown by Atfield & Morris to give good separation of the constituent amino acids of insulin. Electrophoresis was carried out with a formic acid-acetic acid buffer, pH 1.81, as used by these authors. The basic amino acids ran off the paper and were disregarded. The position of other amino acids was demonstrated by staining both sides of the strip with cadmium-ninhydrin reagent, and sections of paper 1 cm. wide corresponding with the stained zones were cut out and counted.

Incorporation of tritium into an acidic-ethanol-soluble fraction of pancreas. In experiments in which the incorporation of labelled amino acids into the acidic-ethanol-soluble fraction of pancreas was examined, a modified extraction procedure based on that described by Grodsky & Tarver (1956) was used. The tissue, incubated, washed and frozen as described above, was ground in a mortar with trichloroacetic acid (10%, w/v; 5 ml./g. of tissue). The resulting slurry was centrifuged and the precipitate washed five times with 3 ml. portions of 5% (w/v) trichloroacetic acid. This precipitate was then treated with 5 ml. of ethanol-0.8 N-HCl (3:1, v/v), followed by a further 2.5 ml. of the same solution. The extracts were combined and 0.02 ml. samples taken for radioactivity determinations. Insulin is known to be present in extracts of pancreas made essentially by this procedure (Grodsky & Tarver, 1956).

RESULTS

Uptake of tritium-labelled leucine by segments of rat pancreas. The time-course for the uptake of radioactivity by segments of rat pancreas is shown in Fig. 1. There was a rapid uptake of leucine in the first hour and a subsequent slowing off in the rate in the next 3 hr. of incubation. During the first 3 hr. of incubation uptake appeared to have been uninfluenced by the presence of 2,4-dinitrophenol in the medium. Under the conditions under which these experiments were performed about half of the DL-leucine present appeared to have been taken up.

Incorporation of tritium into an acidic-ethanol-soluble fraction of rat pancreas. When incorporation of tritium from [^3H]leucine into the acidic-ethanol-soluble fraction of rat pancreas was studied, there was a rapid accumulation of label in this fraction within the first hour of incubation (Fig. 2). The rate of incorporation then proceeded more slowly during the next 3 hr. 2,4-Dinitrophenol markedly inhibited incorporation.

Distribution of radioactivity in amino acids obtained by the hydrolysis of tritium-labelled insulins. An investigation of the manner in which radioactivity was distributed between the amino acids of

Fig. 1. Effect of 2,4-dinitrophenol on the uptake of [^3H]leucine by segments of rat pancreas *in vitro*. Tritium remaining in the medium was measured. Each point is the mean of results obtained in two separate experiments. $\circ$, Control; $\triangle$, incubated with 2,4-dinitrophenol (0.25 mM).

Fig. 2. Effect of 2,4-dinitrophenol on the incorporation of tritium into an acidic-ethanol-soluble fraction of rat pancreas during incubation *in vitro* with $[^3\text{H}]$ leucine. The tissue was ground with trichloroacetic acid, the precipitate was treated with ethanol-HCl and the radioactivity of the soluble portion was then determined. Each point is the mean of results obtained in at least two separate experiments. $\circ$, Control; $\triangle$, incubated with 2,4-dinitrophenol (0.25 mM).

Fig. 3. Radioactivity of amino acids obtained on hydrolysis of rat insulins, labelled by incubating segments of pancreas with ^3H -labelled amino acids. The insulins were extracted with acidic ethanol and purified by chromatography and electrophoresis on paper. The insulins were eluted from the paper, combined and hydrolysed with 5.5N-HCl. The hydrolysate was fractionated by high-voltage electrophoresis on Whatman no. 3MM paper. Amino acids were located by staining guide strips with cadmium-ninhydrin reagent. An appropriate region of the paper was counted after division into sections 1 cm. wide. (a) Pancreas incubated with DL- $[^3\text{H}]$ leucine; (b) pancreas incubated with DL- $[^3\text{H}]$ isoleucine.

insulin previously labelled by incubation of pancreas with either $[^3\text{H}]$ leucine or $[^3\text{H}]$ isoleucine is depicted in Fig. 3. After hydrolysis and separation of the amino acids by electrophoresis, the greater part of the radioactivity appeared to be still associated with either leucine or isoleucine respectively. Minor amounts of radioactivity appeared to be associated with zones which corresponded with glutamic acid and alanine when $[^3\text{H}]$ leucine was used, and with aspartic acid in addition when $[^3\text{H}]$ isoleucine was used. In the experiment with isoleucine a little radioactivity was associated also with material which ran on electrophoresis a little faster than glutamic acid. This area did not stain red with the ninhydrin reagent and the radioactivity may represent an incompletely hydrolysed fragment of insulin.

Incorporation of $[^3\text{H}]$ leucine into A-chain of rat insulin 2. Rat insulin 2 is the insulin with the same electrophoretic mobility as ox insulin (see Appendix, Fig. 1).

The distribution of tritium in acidic peptides made by the performic acid oxidation of rat insulin 2, labelled by incubation of pancreases with $[^3\text{H}]$ leucine, is shown in Fig. 4. Radioactivity was broadly spread across the paper as far as a zone which corresponded with the A-chain marker. When, however, an area of a similar mobility to that of the marker was cut out, sewn on to a fresh strip of paper and run a second time in 20% formic acid, a single peak of radioactivity was obtained. Incorporation into A-chain appears to have been abolished when pancreases were incubated with 2,4-dinitrophenol.

Incorporation of tritium into A- and B-chains purified by addition of carrier ox insulin. In Table 1 are shown the specific activities of A- and B-chains of insulin made after adding a large excess of carrier ox insulin to a sample of crude rat insulin prepared from pancreas incubated with either $[^3\text{H}]$ leucine or $[^3\text{H}]$ isoleucine. Final purification was achieved by paper electrophoresis. It will be seen that there was a substantial incorporation of tritium from $[^3\text{H}]$ leucine into materials with the same electrophoretic mobilities as both A- and B-chains of ox insulin. This was not the case when the insulins were labelled by incubating pancreas with $[^3\text{H}]$ isoleucine. In this instance only A-chain was labelled and little or no radioactivity appeared in the B-chain.

Under the conditions of these experiments only a proportion of the radioactive material associated with the B-chain before electrophoresis migrated with B-chain from ox insulin (or to the expected position of the B-chain of rat insulin 1). Some of this radioactivity was associated with material which remained at the origin and which did not stain with bromocresol green.

DISCUSSION

Incorporation into crude protein fractions of rat pancreas. The usefulness of surviving mammalian pancreas incubated *in vitro* as a system in which to study protein synthesis has now been inferred by many workers (Hokin, 1956; Rychlik & Šorm, 1956; Campagne & Gruber, 1962; Dickman, Holtzer & Gazzinelli, 1962). In the experiments which are reported here there was a rapid uptake of leucine from the medium during a 4 hr. period, with a simultaneous incorporation into an acidic-ethanol-soluble fraction of pancreas. Available evidence indicates that segments of rat pancreas incubated under similar conditions are able to survive for several hours (Campagne & Gruber, 1962).

In accordance with the observations of other workers who have used similar systems, incorporation into a crude extract known to contain insulin was markedly inhibited in the presence of 2,4-dinitrophenol. It is suggested that incorporation into protein in this crude fraction is energy-dependent.

Incorporation into insulins. A major difficulty in studying the incorporation of labelled amino acids into insulin in pancreatic tissue lies in the separation of the very small quantities of radioactive insulin from the large quantities of other radioactive proteins labelled simultaneously. This problem appears to be one of special complexity in adult mammalian pancreas, where insulin represents less than 0.1 % of the proteins and where substances such as enzymes or zymogens are probably being synthesized with great rapidity. Purification procedures for insulin may have to be appropriately elaborate.

Early in this work it was found that unidimensional chromatography of crude rat insulins on paper resulted only in a partial purification and recourse was had to more elaborate purification methods. The method initially chosen was electrophoresis in a second dimension on paper after chromatography, as described by Taylor & Smith (1964). When this was done two insulin-containing zones were obtained which appeared to be identical with the two insulins already demonstrated to be present in rat pancreas by Smith (1964). From each of these insulins A-chain was prepared, which because of its strong negative charge is readily separable from contaminating materials by electrophoresis at the pH prevailing in 20 % formic acid (Harris, Sanger & Naughton, 1956). When A-chain was made from one of the labelled rat insulins which had been separated by chromatography and electrophoresis, and was purified by repeated electrophoresis in 20 % formic acid, a peak of radioactivity was obtained corresponding in position with A-chain markers from ox or pig

insulin run under identical conditions (Fig. 4). A-chain separated by this technique did not appear to be radiochemically pure, however, although

Fig. 4. Radioactivity of A-chain and other acidic material made by performic acid oxidation of rat insulin 2, labelled by incubating segments of pancreas with DL-[^3H]leucine. Insulins were extracted with acidic ethanol and purified by chromatography and electrophoresis on paper. A rectangle of paper containing rat insulin 2 (see Fig. 1 of Taylor & Smith, 1964) was cut out, sewn on to a fresh piece of chromatography paper and sprayed with performic acid. The products of oxidation were separated by electrophoresis in 20 % (v/v) formic acid. The marker was A-chain derived from ox insulin (50 $\mu\text{g.}$) oxidized on the paper in the same manner, run alongside the oxidized rat insulin and located by spraying with N,N' -diethylpseudocyanine. An appropriate region of the paper was counted after division into sections 1 cm. wide. Each histogram represents the results obtained in two separate experiments; the position assumed by the marker is indicated diagrammatically over each histogram. (a) Acidic oxidation products of labelled rat insulin 2 after electrophoresis once; (b) similar to (a), but insulin derived from pancreas incubated with 2,4-dinitrophenol (0.25 mM); (c) similar to (a), but A-chain further purified by re-electrophoresis by the same method.

Table 1. *Specific radioactivity of A- and B-chains of rat insulins labelled by incubating segments of pancreas with ^3H -labelled amino acids*

A separate incubation was carried out for each experiment. Insulins were extracted with acidic ethanol. After purification by paper chromatography 75 mg. of carrier ox insulin was added and the insulin further purified by repeated precipitation at pH 5.4. A- and B-chains of insulin were made by performic acid oxidation and further purified by paper electrophoresis in 20% (v/v) formic acid. B-chain derived from rat insulin 2 (i.e. that with the same mobility as B-chain from the added ox insulin) was counted directly on the paper and its specific activity expressed in terms of weight of crude B-chain originally isolated. Purified A-chain was eluted from the paper and its weight and radioactivity were determined.

Labelled amino acid used	Expt. no.	Sp. activities (counts/min./mg.)		
		Crude B-chains (before electro-phoresis)	B-chain from insulin 2 (after electro-phoresis)	A-chain (after electro-phoresis)
DL- ^3H Leucine	1	280	60	47
	2	196	48	17
DL- ^3H Isoleucine	3	—	4	19
	4	59	0	—
	5	61	2	35

much of the apparent contamination remaining after electrophoresis was possibly due either to incompletely oxidized insulin, which migrates more slowly to the anode, or to material which had become irreversibly absorbed to the paper (cf. Harris *et al.* 1956). Since recovery of labelled A-chain by this technique in any case appeared to be rather low, it was decided in subsequent experiments to isolate the A- and B-chains by the method of Sanger (1949) after addition of carrier insulin.

In these later experiments, a large excess of carrier ox insulin was added after chromatography and the insulin further purified by isoelectric precipitation at pH 5.4 (Vaughan & Anfinsen, 1954), before oxidation by the method of Sanger (1949). Nevertheless, B-chain made by this method still exhibited considerable contamination with other radioactive material, though it ran as a single spot on paper electrophoresis. This is illustrated in Table 1, where with ^3H leucine only a small part of the total radioactivity present in the B-chain preparation before electrophoresis moved in association with the bromocresol green-staining spot on electrophoresis. Similar contamination before electrophoresis was also apparent in B-chain prepared from insulin in pancreas segments that had been incubated with ^3H isoleucine, although here little or no radioactivity was associated with the B-chain zone after electrophoresis. The nature of this radioactive contamination is uncertain. It is possible that much of the contamination was due to trace amounts of smaller-molecular-weight substances, which could have been of higher specific activity than the insulin itself and which might have been firmly bound to the insulin or its derived

B-chain peptide. Such materials might remain very firmly attached to B-chain unless rather drastic procedures such as electrophoresis in 20% formic acid are employed in the purification of the peptides. Since the amounts of these contaminants were probably very small, it is unlikely that they would have been revealed by routine methods of chemical analysis applied to either the insulin or its component peptides. A similar difficulty has been reported by Decken & Campbell (1962) in the purification of serum albumin labelled by incorporation of radioactive amino acids into a ribosomal fraction of rat liver. These findings underline the inadequacy of some current procedures for the isolation of proteins, particularly when the incorporation of amino acids of high specific activity is being studied.

It is also clear from Table 1 that B-chain of insulin prepared from pancreas previously incubated with ^3H isoleucine may be obtained virtually free from radioactivity by electrophoresis in 20% formic acid. On the other hand, B-chain prepared by an identical procedure from pancreas which had been incubated with ^3H leucine was still radioactive. These results are entirely in accord with the structures proposed for rat insulins (L. F. Smith, personal communication), in which isoleucine is absent from B-chain, though present in A-chain. They also suggest that electrophoresis under these conditions adequately purifies B-chain. The converse experiment, involving the distribution of an amino acid not represented in A-chain, was not carried out. Nevertheless, because of its unique properties, it seems unlikely that A-chain prepared by the method of Sanger (1949) and then further purified by electrophoresis in formic acid

would be appreciably contaminated. It is therefore reasonable to assume from these results that both A- and B-chains, made after the addition of carrier, have been isolated in a state of radiochemical purity and that radioactivity associated with them represents incorporation into insulin.

It is of interest to know the disposition of radioactivity between the two chains of rat insulins, since Vaughan & Anfinsen (1954) have postulated an unequal labelling of the two chains of ox insulin after incubation of slices of ox pancreas *in vitro* with ^{14}C -labelled amino acids. From Table 1 it might be inferred that, of the two chains, the B-chain was being labelled preferentially with leucine. On the assumption that radioactivity is due only to leucine, random labelling of the chains should have resulted in twice the radioactivity being present in the B-chain by comparison with the A-chain, since there are known to be two leucyl residues present in the A-chain and four in the B-chain (L. F. Smith, personal communication). However, since there are two B-chains derived from rat insulins, and the relative contribution of one or the other to the total B-chain is not known, firm conclusions cannot be drawn with regard to the labelling pattern in these experiments.

Relationship of incorporation to biosynthesis. Although incorporation into A-chain of insulin appears to be an energy-dependent phenomenon, since it is abolished by addition of 2,4-dinitrophenol to the incubation medium, it is by no means certain that this process represents actual biosynthesis. No net synthesis has been demonstrated in this work. Again there is no complete certainty that amino acids taken up from the medium are necessarily always incorporated into their expected positions in the sequences of either chain. However, the experiments with 2,4-dinitrophenol argue against some non-specific type of association of leucine with the insulin or its A-chain since uptake of $[^3\text{H}]$ leucine was unimpaired in the presence of this agent. The virtual absence of tritium in the B-chain when pancreas segments were incubated with $[^3\text{H}]$ isoleucine also favours an effect which is specific. If some unspecific type of association of the radioactive isoleucine with the insulin had taken place it might be expected that both chains would have been labelled. There is, for example, no evidence from these experiments that insulin is being labelled by reactions with heavy-metal ions (Grodsky & Tarver, 1957), by transpeptidation reactions (Zioudrou, Fujii & Fruton, 1958; Suttie, 1962) or on terminal sites of insulin by other mechanisms (Cornwell & Luck, 1958).

As a working hypothesis, it is suggested that incorporation of labelled amino acids into insulins in these experiments *in vitro* is a specific process mediated by generally accepted mechanisms for

protein synthesis. Final verification that amino acids are always incorporated into their expected sites in this system must await a detailed analysis of the distribution of radioactivity in smaller peptide fragments of labelled insulin.

After the completion of this paper, results which are broadly similar to those indicated above were reported by Humbel (1963) for the incorporation of ^{14}C -labelled amino acids into insulins of teleost fish islet tissue *in vitro*. As with rat pancreas incubated under similar conditions $[^{14}\text{C}]$ isoleucine was incorporated predominantly into A-chain.

SUMMARY

1. Segments of rat pancreas have been incubated *in vitro* with radioactive amino acids to study incorporation into insulins.

2. When $[^3\text{H}]$ leucine is incubated with pancreas segments in a bicarbonate-buffered medium containing glucose there is a substantial uptake of amino acid from the medium, with a rapid incorporation of tritium into an acidic-ethanol-soluble fraction of the pancreatic proteins. Incorporation into this fraction appears to be inhibited by addition of 2,4-dinitrophenol (0.25 mM) to the medium.

3. A-chain made from rat insulins appears to be labelled when isolated from rat-pancreas segments which had been incubated with $[^3\text{H}]$ leucine or $[^3\text{H}]$ -isoleucine. Incorporation of labelled leucine into A-chain is inhibited by addition of 2,4-dinitrophenol to the medium.

4. When rat-pancreas segments are incubated with $[^3\text{H}]$ leucine, B-chain from one of the insulins is also labelled, though virtually no label appears in the B-chain isolated from pancreas which had been incubated with $[^3\text{H}]$ isoleucine, in accordance with the postulated structures for rat insulins.

5. These results argue against some non-specific form of incorporation of the amino acids into rat insulins when amino acids are incubated with rat pancreas *in vitro*.

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APPENDIX

The Purification of Insulins in Crude Extracts of Rat Pancreas by Two-Dimensional Chromatography and Electrophoresis on Paper

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In most recent methods for the small-scale purification of insulin from pancreas, an initial extraction with an acidified organic solvent is followed by further purification by chromatographic methods. Such separations may be carried out on columns, with a variety of supporting media (Porter, 1953; Mendiola & Cole, 1960; Davoren, 1962; Epstein & Anfinsen, 1963; Smith, 1964), or alternatively by paper chromatography (Light & Simpson, 1956; Grodsky & Tarver, 1956; Taylor, Humbel, Steinke & Renold, 1961). Paper-chromatographic methods offer the advantage of relative simplicity, and an account is given below of attempts to apply a paper-chromatographic technique to crude extracts of rat pancreas. Additional purification after paper chromatography has been carried out by electrophoresis in a second dimension. In this method a sewing technique similar to those used for two-dimensional chromatography (Stöckli, 1954), or for two-dimensional ionophoresis (Richmond &

Hartley, 1959; Naughton & Hagopian, 1962), was used.

Paper chromatography. The crude insulin hydrochloride obtained from rat pancreas, as described in the preceding paper, was dissolved in 0.5 ml. of ethanol-0.8N-HCl (3:1, v/v), and applied to Whatman no. 1 paper as a streak about 15 cm. long. This was chromatographed overnight in butan-2-ol-aq. 1% acetic acid (1:1, v/v), as described by Fenton (1959). Crystalline ox insulin was run alongside as a marker. After staining with bromocresol green, a large amount of material was visible at the origin, and in addition a band was obtained with approximately the same R_f as ox insulin. Alternatively, guide strips were cut from the edges of the chromatogram and stained to locate the insulin. Chemical and biological tests carried out on the main part of the band demonstrated that insulin was present in this zone (Taylor, Smith & Gardner, 1962).