The chemical characterization of the radioactive products derived from [^3H]Phe $^{\text{B1}}$]insulin in the circulation of the rat

J. Gwyn DAVIES* and Robin E. OFFORD

Département de Biochimie Médicale, Centre Médical Universitaire, 9 Avenue de Champel, CH-1211 Genève 4, Switzerland

1. Gel filtration of rat plasma taken 1 h after subcutaneous injection of [^3H]Phe $^{\text{B1}}$]insulin gives three peaks of radioactivity. 2. The material in these peaks was characterized by electrophoresis and chromatography. 3. We conclude that [^3H]Phe $^{\text{B1}}$]insulin is rapidly degraded to free tritiated phenylalanine. The phenylalanine is subsequently used for synthesis of plasma proteins *de novo*.

INTRODUCTION

The processes that regulate the removal of insulin from the circulation play an important role in the control of metabolism by this hormone. One method for investigating these processes is to use a valid insulin tracer and to characterize the labelled species derived from it that are found in the circulation. The principal tracer used in such studies has been insulin labelled with radioactive iodine, usually on the side chains of tyrosine residues. However, the iodine atom is rather large and very hydrophobic, and it cannot be certain that even iodinated tracers that have worked well for receptor-binding studies (Gliemann *et al.*, 1979) will behave authentically in all the reactions of metabolic processing that insulin might undergo.

Insulin labelled with tritium has suffered only a virtually imperceptible alteration to its structure. Thus phenylalanine-B1 can be substituted with tritiated phenylalanine by semisynthesis to give a tracer which behaves authentically in all systems studied so far (Halban & Offord, 1975; Halban *et al.*, 1976; Morishima *et al.*, 1985). When this tracer is injected into rats subcutaneously, radioactive species of the apparent molecular size of insulin can be detected by gel filtration of the plasma, together with products both larger and smaller than the original hormone (Berger *et al.*, 1978). The time course of appearance of these peaks has been investigated and their presence in the tissues and urine of the rat has been demonstrated, although the relative proportions of the peaks vary with the tissue extracted (Berger *et al.*, 1978; Halban *et al.*, 1978, 1979). Preliminary characterization of the product of large apparent size had shown that, although it was heterogeneous on gel filtration at acid pH, under strongly dissociating conditions (e.g. in buffer containing SDS) it did not produce radioactive material of low M_r . Immunoprecipitation failed to reveal any insulin immunoactivity (Halban *et al.*, 1978). Further investigation of this peak of material is justified by a series of experiments with radioiodinated insulin that had suggested that polymeric forms of insulin occur in the circulation (Antoniades *et al.*, 1974). In addition, careful examination of all labelled species is justified by the possibility that biologically important derivatives of insulin are released into the circulation from the sites of degradation of the hormone.

The purpose of the present study was to identify the major products of the processing of [^3H]Phe $^{\text{B1}}$]insulin *in vivo* as a first step in the understanding of the biochemistry of the processing of this insulin. Useful information can be gained about such products by electrophoretic and chromatographic analysis (Davies *et al.*, 1980). We have exploited the fact that, as a defined semisynthetic product, our tracer has the label in a known position on the molecule and it is chemically stable.

MATERIALS AND METHODS

All reagents were analytical grade unless otherwise indicated. Subtilisin Carlsberg was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Semisynthetic [^3H]Phe $^{\text{B1}}$]insulin was prepared by the procedure of Halban & Offord (1975) as modified by Halban *et al.* (1976). Male Wistar rats were injected subcutaneously with this radioactive insulin at 0.02–0.1 mCi/kg body wt. (i.e. 0.3–1.5 units/kg at 10 Ci/mmol). After 60 min rats were decapitated, and the blood was collected and the plasma prepared. Injections of tritiated phenylalanine were made with the same quantity of radioactivity as for the tritiated insulin. The results presented are the cumulative data from injections into eight rats.

Isoelectric focusing was carried out in Sephadex G-75 in a Pharmacia FBE 3000 apparatus according to the manufacturer's instructions. Ampholines (LKB, Bromma, Sweden) were used to form the pH gradient between pH 5 and pH 8. After focusing, the gel was cut into sections, which were then packed into 5 ml syringes and eluted with 2.5 ml of 1 M-acetic acid. Samples were taken for determination of radioactivity and measurement of absorption at 280 nm.

Gel filtration was carried out on Sephadex gels (Pharmacia, Uppsala, Sweden). Details of individual columns are given in the Figure legends.

For performic acid oxidation, 100 mg of the fraction to be oxidized was dissolved in 9 ml of formic acid. H_2O_2 (1 ml) was added and the mixture left for 15 min at room temperature. Then 100 ml of water was added and the solution was freeze-dried.

For digestion with subtilisin Carlsberg, the fraction to be digested was dissolved at 10 mg/ml in 1% (w/v) NH_4HCO_3 , and the enzyme was added as a solution of

* To whom correspondence should be addressed.

1 mg/ml in water to a final enzyme/substrate ratio of 1:100 by weight. After 4 h at 37 °C the reaction was stopped by freeze-drying.

Immunoaffinity columns were prepared by mixing 4 mg of anti-(pig insulin) serum (lot GP-4; Miles Laboratories, Slough, Berks., U.K.) dissolved in 0.15 ml of coupling buffer (0.1 M-NaHCO₃/0.5 M-NaCl adjusted to pH 8.3 with 6 M-HCl) with 0.3 ml of CNBr-activated Sepharose 4B that had been freshly re-swollen. After 20 h on an end-over-end mixer at 4 °C, the excess liquid was removed and the gel was washed with 1 M-ethanolamine for 2 h at room temperature. The gel was then washed alternately in coupling buffer and acetate buffer (0.1 M-sodium acetate/0.5 M-NaCl adjusted to pH 4 with 6 M-HCl). Samples were bound to and eluted from the column (0.3 cm × 1.5 cm) by the method of Akanuma *et al.* (1970).

Paper electrophoresis was carried out as described by Offord (1977) and paper chromatograms were developed in butanol/acetic acid/water/pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953). The papers were cut into strips for scintillation counting and soaked in 0.5 ml of 1 M-acetic acid for 5 min before the addition of scintillant.

Electrophoresis on cellulose acetate was based on the method of Carpenter & Hayes (1963). For pH 6.5, 0.52 M-NaH₂PO₄ was mixed with 0.52 M-Na₂HPO₄ to give pH 6.5. Then 1 vol. of this solution was mixed with 7 vol. of 8 M-urea to give a final concentration of 0.065 M-phosphate in 7 M-urea. For pH 8.9, a 1.7 M solution of aq. NH₃ was adjusted to pH 8.9 by the addition of solid CO₂. Then 1 vol. of this solution was diluted with 7 vol. of 8 M-urea to give the final buffer. After electrophoresis

Fig. 1. Elution profile on Sephadex G-50 of the plasma from a rat that had been injected with either [³H]Phe^{B1} insulin (×—×) or [³H]phenylalanine (+---+)

In addition, the elution profile of the tritiated insulin mixed with unlabelled plasma is shown (—). The column (internal diam. 2 cm, height 100 cm, volume 314 ml) was equilibrated and eluted with 1 M-acetic acid.

Fig. 2. Elution profile on Sephadex G-75 of the radioactivity in peak 1 after oxidation in performic acid

As controls, insulin was mixed with an unlabelled rat plasma fraction equivalent to peak 1 and applied to the column either before (I) or after (II) oxidation in performic acid. The column (internal diam. 1 cm, height 127 cm, volume 100 ml) was equilibrated and eluted with acetic acid/water (1:1, v/v). After gel filtration, the radioactivity in the control that had been oxidized in performic acid was loaded on to paper for electrophoresis in water/formic acid (7:3, v/v). The mobility of the major peak of radioactivity corresponds to that of the oxidized B-chain and not to that of insulin (not shown), which is well separated in this system. This indicates that oxidation of the radioactive insulin had occurred under these conditions.

the strips were dried and cut into sections, which were dissolved by heating at 100 °C in 0.5 ml of 6 M-HCl for 7 min, and the radioactivities of the samples were counted after the addition of scintillant.

A Beckman LS230 scintillation counter was used with Biofluor scintillation cocktail (New England Nuclear, Boston, MA, U.S.A.).

RESULTS

Recovery of tritium in the plasma

After 1 h the total tritium in the plasma represents 2–3 % of the injected dose, or around 5 % of the insulin absorbed from the injection site by this time (Berger *et al.*, 1979). The injected dose of insulin gave rise to circulating concentrations high in the physiological range (peak concentration 1–5 nM; Berger *et al.*, 1978).

Gel filtration of plasma samples

A typical profile on Sephadex G-50 of plasma samples from rats injected with tritiated insulin is shown in Fig. 1 (curve ×—×). The first peak corresponds to material of high *M_r* (peak 1), the second is of *M_r* similar to that of insulin (peak 2) and the third is eluted at the total column volume representing small molecules (peak 3).

Fig. 3. Electrophoresis at pH 1.9 on paper of subtilisin digests of performate-oxidized [^3H]Phe $^{\text{B1}}$ insulin (—) and of performate-oxidized peak 1 (—)

Each paper was cut into 0.5 cm strips, which were wetted with 1 M-acetic acid and then mixed with scintillation cocktail for counting of radioactivity (see the Materials and methods section). The Figure shows the profiles of the radioactivity in the two digests. The distances of the sections of the tritiated insulin digest have been adjusted for mobility on the basis of an internal standard (dansyl-arginine) for direct comparison with the peak 1 digest. The principal band in the digest of tritiated insulin is probably the *N*-terminal tetra- or tri-peptide of the B-chain. It is not well separated from phenylalanine in this system, but at pH 3.5 93 % of the radioactivity migrates with mobilities other than that of phenylalanine (results not shown).

Plasma from rats injected subcutaneously with tritiated phenylalanine gives two peaks of radioactivity on Sephadex G-50 (curve +—+ in Fig. 1); the first (peak A) emerges at the position of peak 1 and the second (peak B) emerges at the position of peak 3.

Distribution of radioactivity among the proteins in peak 1 is similar to that in peak A

Peak 1 and peak A were separated into fractions by isoelectric focusing in a flat bed with Sephadex gel as support. The radioactivity is distributed in the pH gradient in a manner similar to, although not identical with, that of the proteins as detected by absorption at 280 nm. After taking into account the differences in the shape of the pH gradients in the two cases, the distribution of radioactivity with pH for peak A is found to be similar to that for peak 1 (results not shown).

Peak 1 is not a disulphide-linked polymer of insulin chains

Oxidation of peak 1 in performic acid does not release radioactive material of lower M_r . On the other hand, when tritiated insulin was oxidized in the presence of an unlabelled fraction of high M_r obtained from rat plasma in the same way as peak 1 (Fig. 2), we observed the expected chromatographic behaviour of the oxidized insulin B-chain.

Amino acid sequence of the *N*-terminal region of the insulin B-chain is not detectable in peak 1

Had the insulin sequence been incorporated into peak 1 by covalent cross-linking, proteolytic digestion of the labelled peak 1 material would normally release the same

radioactive peptides as those liberated by action of the same proteinase on the original labelled insulin. Only if cross-linking had taken place near residue B1 would the peptides released be different, but the electrophoretic pattern of such peptides would still be expected to contain relatively few bands, with mobilities susceptible to interpretation on the basis of a single sequence.

Digestion with subtilisin Carlsberg of tritiated insulin mixed with an unlabelled plasma fraction equivalent to peak 1 gave electrophoretograms where the distribution of radioactivity had the appearance of a singly labelled homogeneous sequence (----- profile in Fig. 3). The corresponding digest of peak 1 has the appearance expected of a multiply labelled heterogeneous sequence (— profile in Fig. 3). This appearance is maintained even when the region of this latter profile in Fig. 3 that corresponds in mobility to the major products of digestion of the insulin sequence is subjected to separation in a second dimension (paper partition chromatography; see the Materials and methods section). A very similar result is obtained by the analogous series of operations carried out on peak A. Had as little as 5 % of the radioactivity in peak 1 been related to the insulin sequence, the principal band derived from it would have been seen superimposed on the much more flat heterogeneous distribution actually seen for peak 1 and peak A. (This statement was verified by visual inspection of computer-simulated traces made by combining the data for an insulin digest with that of the peak 1 digest, and with that of the peak A digest, in various proportions.) Thus the label in peak 1 can be accounted for by incorporation of free phenylalanine into plasma proteins and there is no reason to propose that it results from any other process.

Peak 2 is principally insulin

Peak 2 was further purified by immunoaffinity chromatography. Its subsequent electrophoretic behaviour was found to be closely comparable with that of an authentic sample of tritiated insulin that had been mixed with unlabelled rat plasma and subsequently prepared in the same way as peak 2 (Table 1). Both samples had apparently undergone some deamidation during the preparation. As immunochromatography columns do not give 100 % yields (recovery of authentic insulin after two cycles of immunochromatography was 92 %, that of the peak 2 material was 86 %), it is possible that partially degraded forms of insulin were lost to the analysis. In support of this is the observation that peak 2 appears to be asymmetric, containing a trail on its descending edge. Such a product, if it exists, could be of great functional importance. We believe that the best way of settling the question is to use the methods of Muir *et al.* (1986) and Davies *et al.* (1986).

Peak 3 does not contain peptides derived from insulin

Peak 3 on Sephadex G-10 chromatography gives a major peak eluted in the position of a phenylalanine standard (just after the salt; cf. Bretthauer & Golichowski, 1967), representing between 75 and 90 % in different samples. In addition to this peak, up to 20 % of the radioactivity was eluted with the salt and 4–6 % after the phenylalanine. Electrophoresis at pH 1.9 and 3.5 and paper chromatography of the major peak each gave a single peak of radioactivity having the mobility or R_f of phenylalanine. Peak B (^3H]phenylalanine injection) on

Table 1. Electrophoresis on cellulose acetate of purified peak 2 from rat plasma

Peak 2 from Sephadex G-50 chromatography was further purified by immunoaffinity chromatography and then analysed by electrophoresis on cellulose acetate either at pH 6.5 or at pH 8.9. The cellulose acetate was cut into strips and dissolved in acid for determination of the radioactivity, as described in the Materials and methods section. As a control, tritiated insulin was mixed with unlabelled rat plasma and treated as peak 2. The results are given for the regions identified from the radioactivity profiles (not shown) and are expressed as percentages of the total radioactivity recovered from the strips.

Sample	pH 6.5	
	[³ H]Insulin*	Peak 2
Radioactivity associated with serum albumin	6 %	10 %
Radioactivity associated with unlabelled insulin	67 %	60 %
Radioactivity of mobility higher than that of insulin	27 %	31 %

Sample	pH 8.9	
	[³ H]Insulin*	Peak 2
Radioactivity associated with serum albumin	7 %	5 %
Radioactivity associated with unlabelled insulin	72 %	74 %
Radioactivity of mobility higher than that of insulin	21 %	21 %

* Mixed with plasma (see the text) and runs as expected of a mixture of insulin and desamido-insulin.

Sephadex G-10 chromatography gave 64% of the radioactivity in the position of phenylalanine, 20% being eluted with the salt and a further 16% being eluted after phenylalanine. The minor components observed on gel filtration of peak 3 are thus also seen in similar quantities with peak B, and there is once again no reason to propose that these minor peaks have any special structural relationship with the insulin molecule.

DISCUSSION

Characterization of the new products found in the circulation of the rat after injection of tritiated insulin, and a check on the nature of what was apparently unchanged insulin, seemed to be an important first step in understanding the biochemistry of insulin processing *in vivo*. The results suggest that the major metabolic processing of this tracer consists of rapid degradation to tritiated phenylalanine, presumably at the tissues, which can join the metabolic pool of unlabelled amino acids and constitutes a labelled precursor for protein biosynthesis. As such it is recycled into newly synthesized plasma proteins. There remains the possibility that the small percentage lost during purification of peak 2 represented partially degraded insulin molecules, but this

requires further investigation. In addition, it should be noted that the loss of a few residues from the *N*-terminus of the A-chain would not alter significantly the mass or charge of the insulin molecule and might not have been detected if the molecule remained immunologically active. However, pharmacokinetic experiments with [³H]Gly^{A1}insulin (Davies & Offord, 1985; Davies *et al.*, 1985) have shown that it is cleared from the circulation at a similar rate to [³H]Phe^{B1}insulin. This suggests that the *N*-terminal region of the A-chain is not separated rapidly from that of the B-chain.

This processing could involve the action of aminopeptidases cleaving the labelled amino acid from the molecule. Such aminopeptidases would have to cleave both phenylalanine and glycine at the same rate given the similar metabolic clearance rates for A1- and B1-labelled insulins (Davies *et al.*, 1985). This question could be resolved by comparison of the clearance of *N*-terminally labelled insulins with that of [³H]Ala^{B30}insulin (Davies *et al.*, 1987). Preliminary studies suggest no major differences in clearance of these tracers in the rat (Davies *et al.*, 1983).

No evidence was found for a disulphide-linked polymer of insulin chains as suggested by Antoniadou *et al.* (1974). There are several differences between the present work and that study: the type of tracer, the position of the label on the molecule and the route of injection. However, the present study provides at least one instance where a labelled insulin that behaves authentically gives rise to a product of high *M_r*, but in which the product does not have the structure proposed by Antoniadou *et al.* (1974).

We thank the Fonds National Suisse de la Recherche Scientifique, the Medical Research Council of the United Kingdom and the Stanley Thomas Johnson Foundation for financial support. We thank Dr. P. Halban and Dr. M. Berger for useful discussions and Mr. C. Bradshaw and Mlle. D. Carmignac for expert technical assistance.

REFERENCES

- Akanuma, Y., Kuzuya, T., Hayashi, M., Ide, T. & Kuzuya, N. (1970) *Biochem. Biophys. Res. Commun.* **38**, 947–953
- Antoniades, H. N., Stathakos, D. & Simon, J. D. (1974) *Endocrinology* (Baltimore) **95**, 1543–1553
- Berger, M., Halban, P. A., Muller, W. A., Offord, R. E., Renold, A. E. & Vranic, M. (1978) *Diabetologia* **15**, 133–140
- Berger, M., Halban, P. A., Girardier, L., Seydoux, J., Offord, R. E. & Renold, A. E. (1979) *Diabetologia* **17**, 97–99
- Bretthauer, R. K. & Golichowski, A. M. (1967) *Biochim. Biophys. Acta* **155**, 549–557
- Carpenter, F. H. & Hayes, S. L. (1963) *Biochemistry* **2**, 1272–1277
- Davies, J. G. & Offord, R. E. (1985) *Biochem. J.* **231**, 389–392
- Davies, J. G., Offord, R. E., Halban, P. A. & Berger, M. (1980) in *Insulin: Chemistry, Structure and Function of Insulin and Related Hormones* (Brandenburg, D. & Wollmer, A., eds.), pp. 517–523, Walter de Gruyter, Berlin and New York
- Davies, J. G., Carmignac, D., Bradshaw, C. G. & Offord, R. E. (1983) *Diabetologia* **25**, 149
- Davies, J. G., Watarai, T., Pye, S. & Radzuik, J. (1985) *Diabetes Res. Clin. Pract. Suppl.* **1**, S123
- Davies, J. G., Muir, A. V. & Offord, R. E. (1986) *Biochem. J.* **240**, 609–612
- Davies, J. G., Rose, K., Bradshaw, C. G. & Offord, R. E. (1987) *Protein Eng.* **1**, 407–411

- Gliemann, J., Sonne, O., Linde, S. & Hansen, B. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1183–1190
- Halban, P. A. & Offord, R. E. (1975) *Biochem. J.* **151**, 219–225
- Halban, P. A., Karakash, C., Davies, J. G. & Offord, R. E. (1976) *Biochem. J.* **160**, 409–412
- Halban, P. A., Berger, J., Gjinovci, A., Renold, A. E., Vranic, M. & Offord, R. E. (1978) in *Semisynthetic Peptides and Proteins* (Offord, R. E. & DiBello, C., eds.), pp. 237–247, Academic Press, London and New York
- Halban, P. A., Berger, M. & Offord, R. E. (1979) *Metab. Clin. Exp.* **28**, 1097–1104
- Morishima, T., Bradshaw, C. G. & Radziuk, J. (1985) *Am. J. Physiol.* **248**, E203–E208
- Muir, A. V., Offord, R. E. & Davies, J. G. (1986) *Biochem. J.* **237**, 631–637
- Offord, R. E. (1977) *Methods Enzymol.* **47**, 51–59
- Waley, S. G. & Watson, J. (1953) *Biochem. J.* **55**, 328–337

Received 25 June 1987/27 November 1987; accepted 10 December 1987