PREPARATION OF [^{14}C]ACETAMIDINO-INSULIN

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SUMMARY

[^{14}C]Acetamidino-insulin was synthesized by acetamidination of insulin with methyl [^{14}C]acetimidate hydrochloride. The acetamidino-insulin contains two main components, which can be separated by DEAE-cellulose column chromatography. They correspond to glycine^{A-1}-phenylalanine^{B-1}-N$^{\varepsilon}$-lysine^{B-29}-[^{14}C]acetamidino-insulin and glycine^{A-1}-N$^{\varepsilon}$-lysine^{B-29}-[^{14}C]acetamidino-insulin. These immunological and biological activity was compared with that of native insulin.

Key Words: Insulin, Acetamidination, Methyl acetimidate, Carbon-14, DEAE-cellulose column chromatography

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INTRODUCTION

Several methods for preparation of radioactive insulin for use in studying its biochemical and physiological action have been investigated (1-5).

Repke and Zull reported on preparation of tritiated acetamidino-insulin, and stated that the insulin is biologically active (4). However, it was not fully purified and was still heterogeneous.

We attempted to isolate and to characterize each component in the insulin preparation. It was revealed that the crude acetamidino-insulin is mainly composed of glycine^{A-1}-phenylalanine^{B-1}-N^{ε}-lysine^{B-29}-acetamidino-insulin and glycine^{A-1}-N^{ε}-lysine^{B-29}-acetamidino-insulin.

EXPERIMENTAL AND RESULTS

Preparation of [^{14}C]acetamidino-insulin

[^{14}C]Acetamidino-insulin was prepared from insulin (bovine pancreas; Sigma Chemical Company, St. Louis, MO, U.S.A.) and methyl [1-^{14}C]acetimidate hydrochloride according to the method of Repke and Zull (4).

Methyl [1-^{14}C]acetimidate hydrochloride: The hydrochloride was obtained by a modification of an ordinary method for preparation of ethyl acetimidate hydrochloride (6). Dry hydrogen chloride (about 90 mg) was added to the mixture of [1-^{14}C]acetonitrile (73 mg, 0.2 mCi/mmol; The Radiochemical Centre, Amersham, U.K.) and absolute methanol (57 mg) at ice-NaCl temperature. The reaction mixture was left for 1.5 hr in an ice bath. After warming to room temperature, it was outgassed under vacuum for 2 hr in a desiccator containing NaOH and P_2O_5. The

yield of methyl [1-^{14}C]acetimidate hydrochloride is 151.6 mg (77.7 %).

[^{14}C]Acetamidino-insulin: A solution of methyl [1-^{14}C]-acetimidate hydrochloride (151.6 mg; in 9 ml of 0.4 M borate buffer and 2.3 ml of 1 N NaOH at pH 8.5) was added to 9 ml of 0.01 N HCl containing insulin (71.4 mg) at 0 °C. The mixture was adjusted to pH 8.9, and stirred for 2 hr at 4 °C. The reaction was stopped by lowering the pH to 2.8 with 1 N HCl. The mixture was desalted by the use of a membrane filter (Diaflo Membrane UM-2; Amicon Corporation, Lexington, MA, U.S.A.), and lyophylized. The yield of crude [^{14}C]acetamidino-insulin is 66.6 mg.

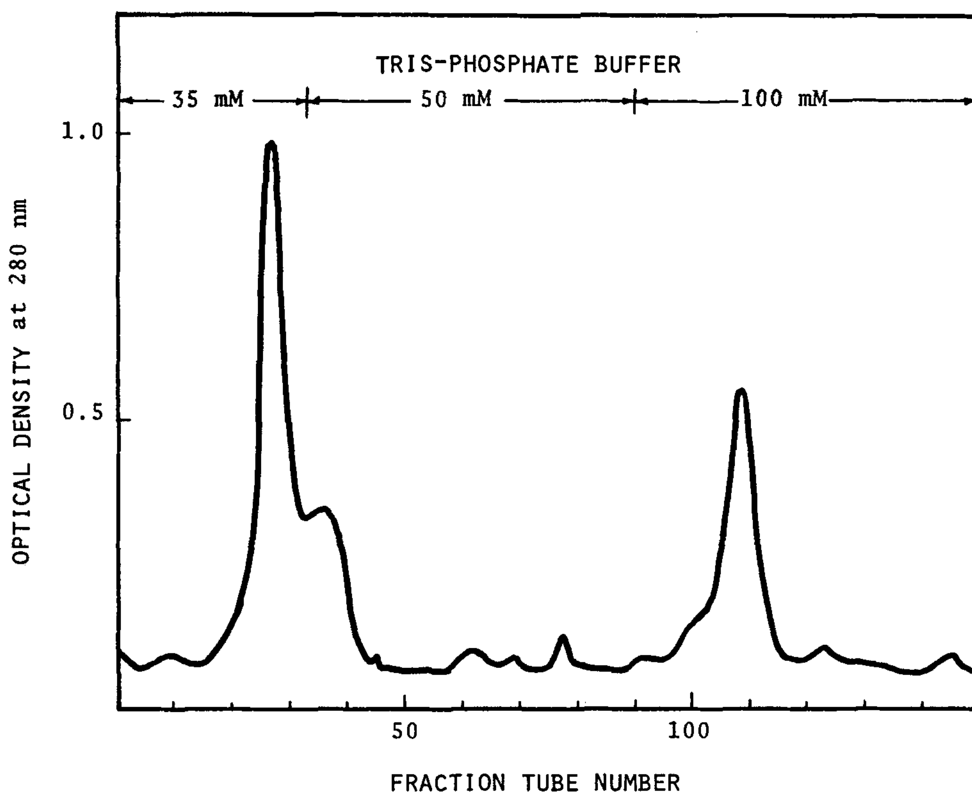

Figure 1. DEAE-cellulose column chromatography of an acetamidination reaction mixture; Column size wet DEAE cellulose 16 ml, fraction volume 3 ml.

Purification of [^{14}C]acetamidino-insulin

The crude acetamidino-insulin was purified by DEAE-cellulose column chromatography. The crude material (66.6 mg) was charged on a column of DEAE-cellulose (Whatman, Clifton, NJ, U.S.A.) equilibrated at pH 7.5 with 10 mM tris-phosphate buffer containing 4 M urea, and eluted at pH 8.5 with the flow rate of 5 ml/hr with 35, 50 or 100 mM tris-phosphate buffer containing 4 M urea. Its elution pattern is shown in Figure 1. The two main elution bands (band' A & B) were separately collected. Band A and B were desalted by passage through a column of Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.1 M formic acid, and lyophylized. The purity of each acetamidino-insulin obtained was checked by polyacrylamide disc gel electrophoresis according to the method of Repke and Zull (4) (Figure 2). Their yields based on an insulin molecular weight of 5740 (6) are below; [^{14}C]Acetamidino-insulin A (band A) 24.4 mg (33.0 %), [^{14}C]acetamidino-insulin B (band B) 13.1 mg (17.9 %)

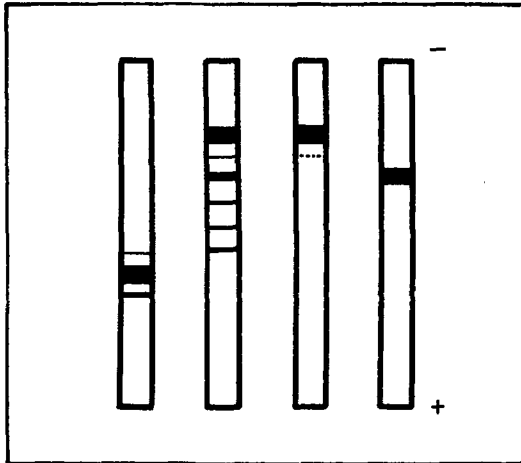

Figure 2. Polyacrylamide disc gel electrophoresis of, from left to right, native insulin, an acetamidination reaction mixture, acetamidino-insulin A and B at pH 8.9.

Structure determination of the acetamidino-insulins

Insulin or acetamidino-insulin was converted into the corresponding dinitrophenyl (abbr. DNP) derivative with 2,4-dinitrofluorobenzene (7). The 6 N HCl solution of each DNP-insulin was heated at 105 °C for 16 hr. After cooling, it was extracted with ethyl acetate. The extract was analysed by thin layer chromatography (7). The chromatogram of the DNP-insulin hydrolyzates is shown in Figure 3.

DNP-native insulin gave rise to the DNP-derivatives of glycine, lysine, and phenylalanine. No DNP-amino acid was obtained from acetamidino-insulin A treated with 2,4-dinitrofluorobenzene, and DNP-phenylalanine from DNP-acetamidino-insulin B. This shows that acetamidino-insulin A and B correspond to glycine^{A-1}-phenylalanine^{B-1}-N$^{\varepsilon}$-lysine^{B-29}- acetamidino-insulin and glycine^{A-1}-N$^{\varepsilon}$-lysine^{B-29}-acetamidino-insulin, respectively.

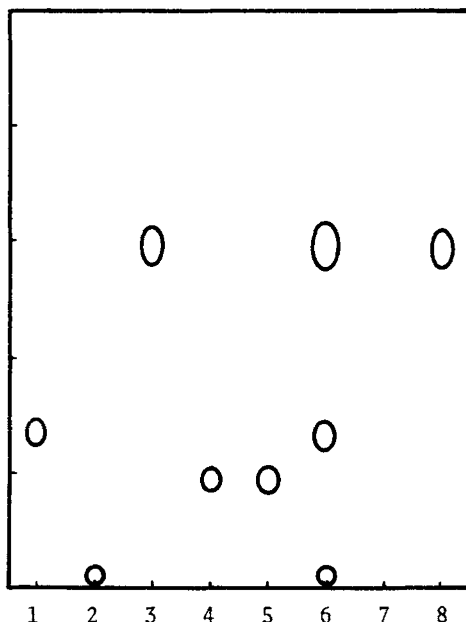

Figure 3. Thin layer chromatography of DNP-derivatives; Glycine (1), lysine (2), phenylalanine (3), urea (4), tris(hydroxymethyl)-aminomethane (5), native insulin (hydrolyzate) (6), acetamidino-insulin A (hydrolyzate) (7) and acetamidino-insulin B (hydrolyzate) (8), on Silica Gel G with chloroform/benzylalcohol/acetic acid 70:30:3.

Immunological and biological activity of acetamidino-insulin A and B

In order to check the immunological activity of acetamidino-insulin A and B, crossreactivity between acetamidino-insulin and [^{125}I]insulin was examined (8). By the use of a commercial kit for insulin quantitation (Insulin-RIA kit; Dainabot, Matsudo, Japan), the concentrations of the insulins at which the binding of 50 per cent of [^{125}I]insulin was inhibited in a [^{125}I]insulin anti-insulin system were determined (Figure 4). The relative crossreactivities of acetamidino-insulin A and B to native insulin are 0.6 and 12.5 %, respectively.

The fat cell glucose metabolism assay was utilized for the examination of the biological activity of the insulins. For the assay method, the procedure of Rodbell was slightly modified (9). Table 1 indicates that acetamidino-insulin B is more active, but acetamidino-insulin A is less active in the fat cell assay system than native insulin.

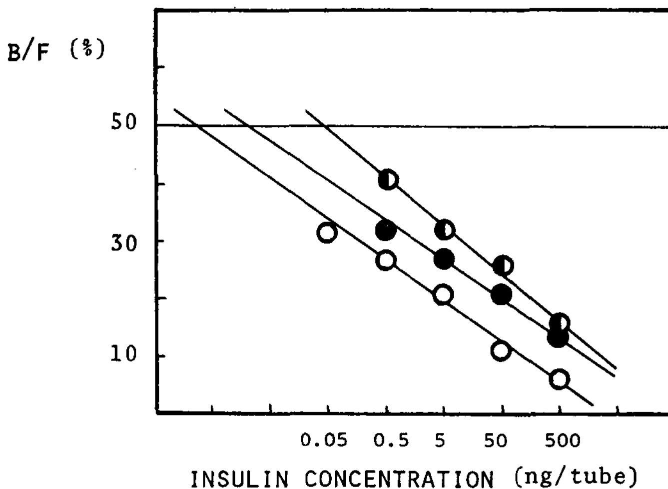

Figure 4. Antigenicity of native insulin and acetamidino-insulin; Immunoassays were performed according to Insulin-RIA kit mannual (Dainabot). o···native insulin, ◐···acetamidino-insulin A, ●···acetamidino-insulin B.

Table 1. Isolated fat cell assay[a] of native insulin and acetamidino-insulin.

Insulin	Dose (μU)[b]	CO_2 production[c] (10^4 cpm)
none	–	0.35
native insulin	40	1.56
	200	1.77
acetamidino-insulin A	100	1.56
acetamidino-insulin B	100	1.76

[a] Incubation time 2.5 hr.

[b] Based on an activity of 24 I.U. per mg for native insulin.

[c] $^{14}CO_2$ production from [1-^{14}C]glucose in fat cells isolated from rat epididymal adipose tissue; Mean of triplicated determinations.

DISCUSSION

Acetamidination of insulin gave glycine^{A-1}-phenylalanine^{B-1}-N^{ε}-lysine^{B-29}-acetamidino-insulin and glycine^{A-1}-N^{ε}-lysine^{B-29}-acetamidino-insulin. They are immunologically and biologically potent; The latter is more active than the former. Hunter and Ludwig (5) observed that the N-terminal phenylalanyl amino group of insulin is less reactive towards methyl acetimidate than the other amino group. This accounts for the two acetamidination products which we have characterized.

Our data prove the conjecture of Repke and Zull (4) that the slow moving major band on a polyacrylamide gel at pH 8.9 might be the fully modified insulin, while the fast moving one might be the acetamidino-insulin whose phenylalanyl amino group was unmodified.

It is desirable to use purified protein in biochemical studies, since the presence of impurities could cause unanticipated results. Therefore, the procedures presented here are important for the preparation of acetamidino-insulin. In addition, since a method for preparing methyl [^{3}H]acetimidate of high specific activity has been described (10), pure [^{3}H]acetamidino-insulin with high specific activity is available and should prove useful in biological and physiological experiments.

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