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A New Insulin Derivative for the Preparation of Insulin–Enzyme Conjugates: An Application in the Enzyme Immunoassay of Insulin

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Abstract

A new insulin derivative, mercaptobutyrimidyl pyridine disulfide insulin, was prepared under conditions which directed the reaction preferentially through the terminal α-amino group of the A-chain. The purified monosubstituted insulin exhibited only a marginal decrease in antigenicity as measured by radioimmunoassay; there was, however, a significant reduction in biological activity in the mouse convulsion assay. Conjugation of the insulin derivative to human placental alkaline phosphatase was carried out via a thiol interchange reaction and the resulting conjugate was active in a double-antibody, solid-phase enzyme immunoassay for insulin.

Introduction

The use of enzyme-labelled antigens and antibodies in immunological assays and histochemistry has recently gained wide acceptance (Schuurs and Van Weeman 1977). In contrast to radioisotopic labels, enzyme labels offer the advantages of long shelf life, simplicity in detection and avoid the problems associated with handling radioactive material (Landon 1977).

Numerous reagents have been used for enzyme–antigen (or antibody) conjugation (for a review of the use of these reagents see Schuurs and Van Weeman 1977). The most widely used conjugation reagent is glutaraldehyde (Avrameas 1969), a bifunctional reagent which reacts with the α- and ε-amino groups of protein (Wold 1972; Pesce *et al.* 1976). During the conjugation of two proteins with glutaraldehyde, reaction occurs in an indiscriminate manner and both large homo- and heteropolymers are formed (Pesce *et al.* 1976; Sachs and Winn 1970). In cases where enzyme–antigen conjugates have been formed there are often large losses in both enzyme (Clyne *et al.* 1973) and immunological activity (Schuurs and Van Weeman 1977).

Specific and controlled reactions for protein conjugation have been investigated, particularly for the formation of biologically active hormone–protein conjugates used in receptor studies (Hofman *et al.* 1977; May *et al.* 1978; Schechter *et al.* 1978). The synthesis routes for these conjugates are unsuitable for enzyme immunoassays since they require either reaction conditions not compatible with the maintenance of enzyme activity or are chemically complex with low overall yields. Insulin–enzyme conjugates, suitable for enzyme immunoassays, have been formed in reactions more controlled than the glutaraldehyde reaction by the use of certain heterobifunctional reagents. In particular maleimidobenzoyl *N*-hydroxysuccinimide ester (Kitagawa and Aikawa 1976) and *S*-acetylmercaptosuccinic anhydride, used in conjunction with

N,N'o-phenylenedimaleimide (Kato *et al.* 1975*a*) have been used to provide immunologically and enzymically active insulin–enzyme conjugates. These conjugates have not, however, been isolated or chemically characterized.

In this paper we describe the synthesis and isolation of a protected thiol monosubstituted derivative of bovine insulin, butyrimidyl pyridine disulfide insulin. Conditions used in the synthesis directed the reaction preferentially through the non-antigenic α-amino group of the insulin A-chain (glycine) resulting in a derivative possessing almost the full antigenic reactivity of native insulin. Conjugation of the insulin derivative to alkaline phosphatase was carried out via a thiol interchange reaction following reaction of the enzyme with methyl 4-mercaptobutyrimidate. The resulting conjugate was used to develop an enzyme immunoassay for bovine insulin.

Materials and Methods

Crystalline bovine insulin was obtained from the Production Division of these Laboratories and desalted by gel filtration on a Sephadex G25 column (2·5 by 50 cm) in 1·0 M acetic acid. The zinc-free insulin was further purified by chromatography on DEAE-cellulose columns (2·5 by 50 cm, Whatman DE-52) in 0·01 M Tris-HCl, pH 8·0, containing 7 M urea (freshly deionized) and developed with a 2-litre linear NaCl gradient (0–0·1 M). The purified insulin was desalted, as above, and lyophilized.

For investigation of the level of derivatization as a function of reagent concentration, methyl 4-mercaptobutyrimidate hydrochloride (MMBI, Pierce Chemical Co., Illinois, U.S.A.), 1–4 mg, was dissolved in methanol (250 μl) containing 2,2'-pyridine disulfide* (2PDS, Aldrich Chemical Co., Wisconsin, U.S.A.) (1:4 mole/mole) at room temperature. After 20 min, the solution was added to 2·0 ml of a 5·0 mg/ml solution of purified insulin in methanol–0·1 M borate buffer, pH 9·0 (1:1 by volume). Following a 15-min incubation at room temperature 1·0 ml of dilute acetic acid (10 ml glacial acetic acid per litre water) was added and the solution gel-filtered on a Sephadex G25 column (2·5 by 50 cm), equilibrated and eluted with dilute acetic acid. For the preparative synthesis of the 1:1 derivative, MMBI (7·5 mg) and 2PDS (30 mg) were incubated in methanol (250 μl) and then added to purified insulin (25 mg) in the methanol–borate buffer (5·0 ml) solution. After desalting by gel filtration and lyophilization, the 1:1 insulin derivative was purified by the above ion-exchange chromatographic procedure using a DEAE-cellulose column (1 by 26 cm) developed with a 300-ml linear NaCl gradient.

Human placental alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) was isolated from fresh placenta and purified by an affinity chromatography procedure (Murdoch *et al.* 1980).

Analytical Procedures

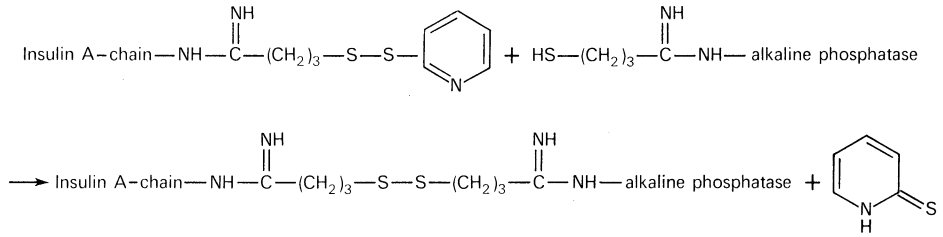
The degree of derivatization was determined by a chromatographic procedure. 1 mg derivative was dissolved in 1·0 ml borate buffer (25 mM, pH 9·0) containing 0·2 mM 2-mercaptoethanol and incubated at room temperature. After 20 min the solution was chromatographed on a Sephadex G25 column (1·5 by 5·5 cm) equilibrated and eluted with borate buffer and 1·0-ml fractions collected. The separated mercaptobutyrimidated insulin and free 2-thiopyridone concentrations were calculated from the absorbance of the fractions at both 276 and 343 nm. The protein concentration was calculated using an E_M^{1cm} of 5950 (Arquilla *et al.* 1976) and the 2-thiopyridone using an E_M^{1cm} of 8080 (Stuchbury *et al.* 1975).

Radioimmunoassay of insulin and the derivative was carried out by a modified ethanol precipitation procedure (Heding 1972) using ^{125}I-labelled insulin. The purity of the insulin derivative was assessed by disc gel electrophoresis as described by Davis (1964). The site of MMBI modification of the insulin was determined by deamination of the derivative with nitrous acid (Levy 1973), hydrolysis *in vacuo* with 6 M HCl for 18 h at 110°C and amino acid analysis on a Beckman 121 M amino acid analyser. Biological activity determinations were made using a mouse convulsion assay as described in the British Pharmacopoeia (1973 edn, p. A109).

*2,2'-Dithiobispyridine.

Conjugation

Alkaline phosphatase (sp. act. 244 U/mg), 184 units in 0·5 ml 25 mM borate buffer, pH 9·0, containing EDTA (1 mM) was reacted for 1 h with MMBI (25 μl of a 2·0 mg/ml solution in borate–EDTA buffer) under nitrogen. The thiolated enzyme was desalted on a Sephadex G25 column (1·5 by 5·5 cm) equilibrated in 25 mM borate buffer, pH 9·0, and 0·5-ml fractions collected. The two fractions containing the peak of the enzyme activity were pooled and added to 200 μg ^{125}I-trace-labelled butyrimidyl pyridine disulfide insulin. Conjugation of mercaptobutyrimidated insulin to the alkaline phosphatase occurred according to the following reaction scheme:



After 10 min at room temperature the solution was passed down a Sephacryl S200 column (1 by 27 cm) equilibrated and eluted with borate buffer. The fractions (2 ml) were screened for ^{125}I-labelled insulin and alkaline phosphatase activity. The fractions containing both activities were pooled and stored at 4°C.

Solid-phase Second Antibody

Sheep anti-guinea pig IgG serum was coupled to activated CH-Sepharose 4B (Pharmacia, South Seas, Sydney), in the ratio 0·1 ml serum to 0·5 g Sepharose, according to the manufacturer's instructions. The solid-phase antiserum was stored, at 4°C, as a 10% (v/v) suspension in phosphate-buffered saline (PBS) containing 0·02% (w/v) sodium azide, 0·5% (v/v) Tween 20 and 0·5% (w/v) bovine serum albumin (BSA). The suspension was diluted 1:4 in the same buffer for routine use in the assay.

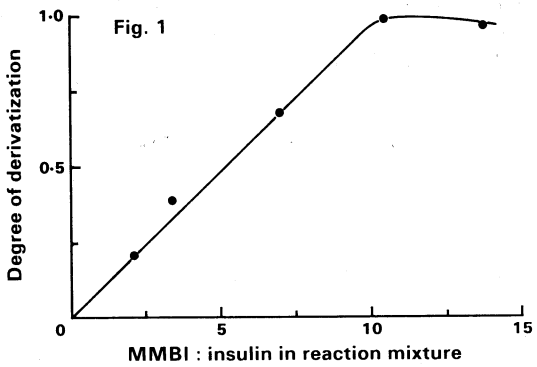
Double Antibody Solid-phase (DASP) Immunoassay

The assay was carried out in 9 by 73-mm disposable polystyrene tubes. Appropriately diluted guinea pig anti-insulin, 100 μl, was added to polystyrene tubes containing 100 μl of the alkaline phosphatase–insulin conjugate [diluted in 0·1 M Tris-HCl buffer, pH 8·0, containing 0·02% (w/v) sodium azide, 5% (w/v) BSA and $MgCl_2$ (1 mM)], and 100 μl of an insulin solution made up in PBS–Tween 20. After incubation for 1 h at 37°C, 100 μl of a 2·5% (v/v) suspension of Sepharose–anti-guinea pig IgG serum was added using a SMI glass micropipette (Calbiochem, Sydney). The tubes were placed in a rack attached to a rotary shaker and shaken at 180 rev/min for 16 h at room temperature. At the end of the incubation period 2·0 ml PBS–Tween 20 was added to each tube by automatic syringe. The beads were sedimented by centrifugation at 1400 *g* for 5 min and the supernatant aspirated. After a second, similar wash, two further washings were carried out with 10 mM Tris-HCl, pH 8·5, containing 0·15 M NaCl. Following the final centrifugation and aspiration all the liquid above the beads was carefully removed. Alkaline phosphatase activity associated with the washed beads was determined by incubation for 1 h at 37°C with 0·5 ml 15 mM *p*-nitrophenyl phosphate dissolved in 125 mM sodium carbonate buffer, pH 10·2, containing mannitol (10 mM) and $MgCl_2$ (9 mM). The tubes were intermittently shaken by hand. The reaction was stopped by the addition of 0·6 ml 1·0 M NaOH and the beads and a slight flocculant precipitant, which formed on the addition of NaOH, removed by centrifugation. The absorbance of the supernatants in the tubes was measured spectrophotometrically at 405 nm.

Results

The molar ratio of insulin to MMBI in the reaction mixture was varied between 1:3·5 and 1:14 (at a constant MMBI to 2PDS ratio of 1:4) to determine the optimal level required to give an incorporation of 1 mole butyrimidyl pyridine disulfide per

mole insulin over a 15-min incubation period. The results shown in Fig. 1 indicate that the desired 1:1 degree of derivatization was achieved at an insulin to MMBI ratio of 1:10·5. With greater amounts of MMBI precipitation occurred.



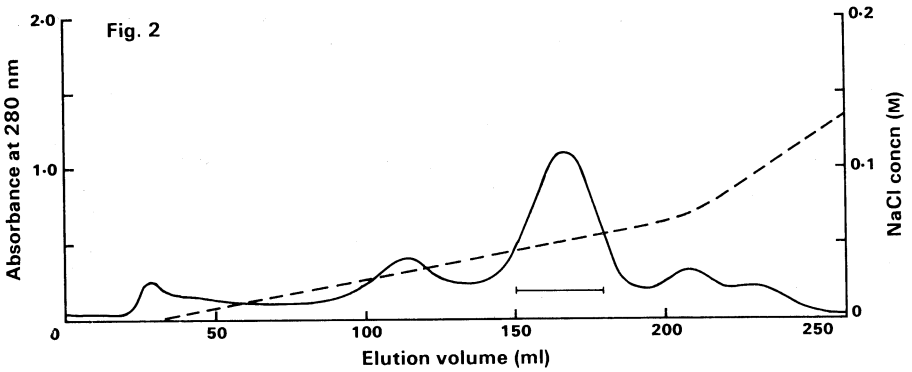


Fig. 1. Effect of varying the ratio of MMBI to insulin in the reaction mixture on the number of moles of butyrimidyl pyridine disulfide incorporated per mole of insulin plotted against degree of derivatization.

Fig. 2. DEAE-cellulose chromatography of the derivative formed in a reaction mixture containing MMBI and insulin in the ratio of 10·5:1 and incubated for 15 min at room temperature (——). The sample (26 mg) was loaded onto the column (1 by 26 cm) in 0·01 M Tris-HCl buffer (pH 8·0) containing 7 M urea. A NaCl gradient (– – –) was used to develop the column at a flow rate of 1·0 ml/min and 2·0-ml fractions were collected. Fractions indicated by the bar were pooled, desalted and lyophilized.

Purification of the desired 1:1 derivatized product was achieved by chromatography on a DEAE-cellulose column. The elution profile (Fig. 2) indicated that there was one major product (73%) and four minor products of the derivatization reaction. The minor products were not characterized. The major peak of the elution profile shown in Fig. 2, pooled as indicated, contained insulin which released 1 mole 2-thiopyridine per mole insulin on treatment with 2-mercaptoethanol. Electrophoresis in polyacrylamide gel indicated that the chromatographically purified derivative contained only a minor amount (less than 5%) of underivatized insulin as a contaminant.

Amino acid analyses of deaminated insulin derivative, deaminated native insulin and native insulin, shown in Table 1, were carried out in order to determine the site(s) of incorporation of the butyrimidyl pyridine disulfide group. The α-amidino group is completely hydrolysed by treatment with 6M HCl at 110°C for 24 h (Hunter and Ludwig 1972), hence, any protection afforded either of the α-amino groups of the amino terminal amino acids during deamination was determined by an increase in these residues in the deaminated derivative over those found in the deaminated native insulin. In contrast, the ε-amidino group is relatively resistant to acid hydrolysis, having a half-life of 46 h at 110°C in 6 M HCl (Reynolds 1968). This means that under the conditions employed in this study, ε-amidination will be seen as a small, but significant, increase in the lysine content of the deaminated native insulin. As shown in Table 1, the major product of the reaction of methyl butyrimidyl pyridine disulfide with insulin, under the conditions outlined, is the 1:1 derivative modified selectively at the α-amino group of glycine at position A 1. Of the three possible amino acids at which reaction could occur (italicized in Table 1) — glycine (at A 1), phenylalanine (at B 1) and lysine (at B 29)—only glycine was protected during deamination. Radioimmunoassay curves for both the purified derivative and native insulin are shown in Fig. 3. The points for the derivative are consistently above those for native insulin; however, most equivalent points fall within ± one standard deviation (±5%).

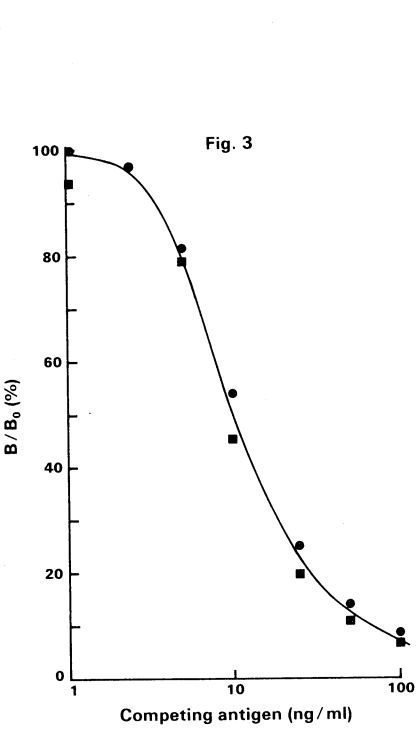
Table 1. Amino acid analyses

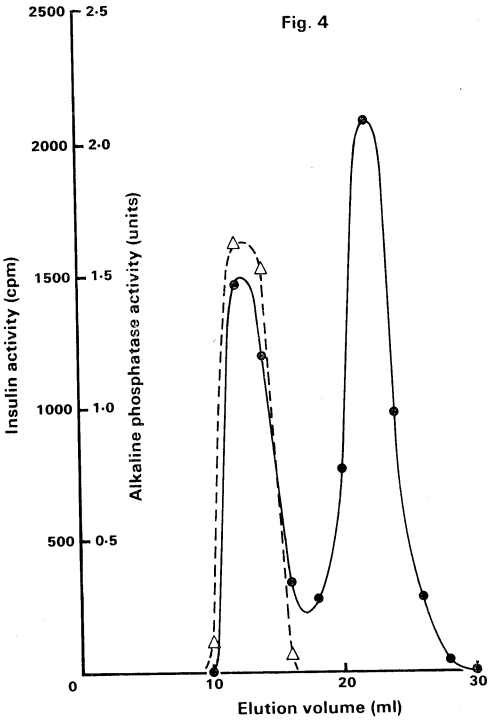
Amino acid content expressed as residues per molecule. Residues unstable to nitrous acid (tyrosine, serine, threonine) are not listed

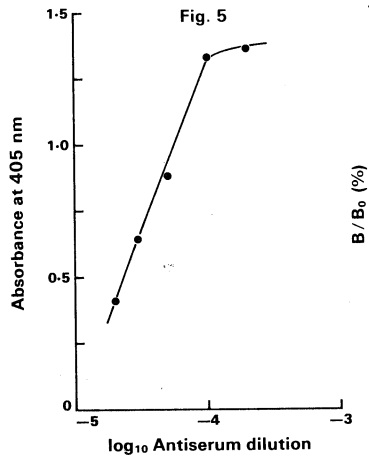
Amino acid	Deaminated insulin derivative[A]	Deaminated native insulin[A]	Native insulin[B]
Aspartic acid	2·84	2·91	2·91 (3)
Glutamic acid	7·00	6·84	7·12 (7)
Proline	1·24	1·16	1·10 (1)
Glycine	*3·60*	*2·84*	*4·08* (4)
Alanine	3·09	2·97	3·08 (3)
Valine[C]	4·26	4·56	4·41 (5)
Isoleucine[C]	0·53	0·46	0·50 (1)
Leucine	5·56	5·51	5·51 (6)
Phenylalanine	*1·74*	*1·83*	*3·09* (3)
Lysine	*0·28*	*0·28*	*1·08* (1)
Histidine	2·09	2·10	1·96 (2)
Arginine	1·18	1·09	0·92 (1)

[A]Deamination of insulin and the chromatographically purified 1:1 derivative was carried out by reaction with nitrous acid (Levy 1973).
[B]Theoretical residues per molecule are given in parentheses.
[C]Valine and isoleucine are not completely released during 18-h period of hydrolysis.

The introduction of a butyrimidyl pyridine disulfide group at position A 1 caused a marked decrease in the biological activity of insulin. The 1:1 insulin derivative had a biological potency of 9·1 international units per milligram (95% fiducial limits 7·6–11·1) compared with 25·2 international units per milligram (95% fiducial limits 22·2–29·0) measured with native insulin.







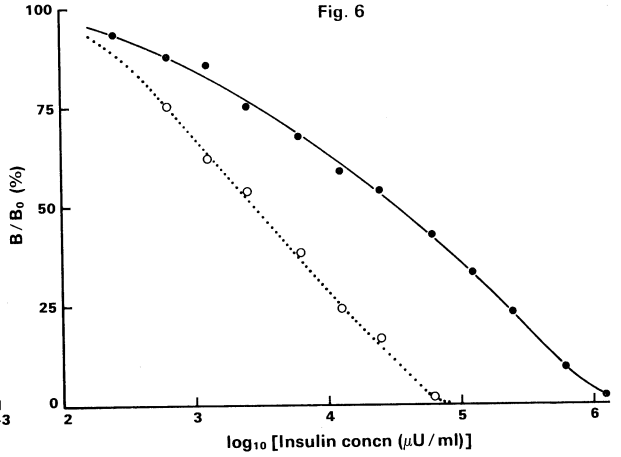


Fig. 3. Radioimmunoassay curves for native insulin (■) and the chromatographically purified 1:1 derivative (●). Guinea pig anti-insulin antiserum I was used at a 1:20 000 dilution and separation of bound from free tracer was by precipitation with 78% (v/v) ethanol. One standard deviation was within ±5% for each point. B/B_0 refers to the ratio of radioactivity (cpm) bound in the presence (B) and absence (B_0) of competing, unlabelled antigen.

Fig. 4. Sephacryl S200 gel filtration profile for the separation of unconjugated insulin–butyrimidyl pyridine sulfide from the insulin–alkaline phosphatase conjugate. The column (1 by 27 cm) was equilibrated and eluted with 25 mM borate buffer, pH 9·0. The solid line indicates the insulin radioactivity eluted and the broken line indicates the alkaline phosphatase activity (units—A_{405} per minute per 20 μl). A pool made of fractions containing enzyme activity contained $5{\cdot}0 \times 10^{-9}$ moles of alkaline phosphatase and $9{\cdot}6 \times 10^{-9}$ moles of insulin as measured by incorporation of radioactivity.

The reaction of MMBI with alkaline phosphatase did not result in a decrease in enzyme activity even after incubation for 2 h at room temperature. A rapid diminution in activity was observed on incubation with reducing agents such as mercaptoethanol and dithiothreitol. Similarly, activity was lost on incubation of MMBI-treated alkaline phosphatase with monobutyrimidyl pyridine disulfide insulin. However, this loss could be reduced to approximately 15% by restricting the reaction time to 10 min before gel filtration of the mixture on a Sephacryl S200 column. A typical elution profile for a 10-min conjugation reaction is seen in Fig. 4. The recoveries of enzyme activity and insulin in the first peak (conjugate) were: alkaline phosphatase 49·2% and insulin 27·5% of which 50% retained the immunological activity of native insulin.

Using a 1:200 dilution of the conjugate, dilutions of between 1:10 000 and 1:40 000 were satisfactory for use in the enzyme immunoassay for a series of guinea pig anti-insulin sera. A typical titration curve, for antiserum I, is seen in Fig. 5, in which the satisfactory operating dilution was determined as 1:20 000. The two major criteria for a satisfactory dilution are that antibody must be limiting and an optical absorbance of approximately 1·0 at 405 nm be attained in the absence of competing unlabelled insulin in the assay procedure.

Standard curves obtained with two antiserum pools are seen in Fig. 6. The working ranges of the two curves are antiserum I, 150–30 000 μU/ml, and antiserum II, 250–250 000 μU/ml.

Discussion

Methyl mercaptobutyrimidate was first introduced as a reversible cross-linking reagent for ribosomal proteins (Traut *et al.* 1973). The applicability of the reagent was extended by the protection of the reactive thiol group in a disulfide linkage with pyridine (King *et al.* 1978), which enabled derivatives of proteins to be formed which were stable unless exposed to a thiol group. Conjugation of these proteins to thiol-containing proteins occurred via thiol interchange and the reaction could be followed spectrophotometrically by the release of thiopyridone.

The reaction of insulin with methyl butyrimidyl pyridine disulfide, in a ratio of 1:10·5 for 15 min, gave as the major product a 1:1 derivative. Higher molar ratios, or extending the reaction time, produced an insoluble derivative presumably due to the incorporation of two or more of the large hydrophobic butyrimidyl pyridine disulfide groups into the insulin molecule. This probably accounts for the apparent low derivatization level at a 14:1 MMBI to insulin ratio seen in Fig. 1. Due to this insolubility, centrifugation removes the higher derivatives from the reaction solution and provides a partial purification of the 1:1 derivative.

The reaction of imidoesters with proteins occurs through available amino groups (Hunter and Ludwig 1962, 1972). Using model compounds, Hunter and Ludwig (1962) showed that reaction of methyl alkyl imidates occurs rapidly with α-amino groups at

Fig. 5. Titration of antiserum I using the assay procedure described in order to determine the dilution at which antibody was limiting. The insulin–alkaline phosphatase conjugate dilution was 1:200. The optimum dilution of this antiserum for use in routine assays was 1:20 000. Similar titrations were carried out for all anti-insulin sera used in routine assays.

Fig. 6. Insulin–enzyme immunoassay standard curves for two different antisera pools, antiserum I (○) and antiserum II (●). Antiserum I was used at a 1:20 000 dilution and antiserum II at 1:10 000. The insulin–alkaline phosphatase conjugate dilution was 1:200. B/B_0 as defined in Fig. 3.

pH 9·0. The site of imidination of proteins with methyl alkyl imidates does depend, however, on the nature of the alkyl group, the pH of the reaction and the pK_a of the amino groups available for reaction (Hunter and Ludwig 1962; DiMarchi *et al* 1978). Amino acid analyses of the chromatographically purified butyrimidyl pyridine disulfide insulin (Table 1) indicate that the major product of the amidination of insulin with methyl butrymidyl pyridine disulfide under the conditions described was the monosubstituted derivative modified at glycine A 1. The analyses provide no evidence of a monosubstituted derivative modified at phenylalanine B 1 in the major product chromatographically isolated from the reaction mixture (Fig. 2). The derivative modified at glycine A 1 accounted for 73% of the total insulin recovered from the DEAE-cellulose column during chromatographic fractionation of the amidination mixture (Fig. 2). Selective reaction of both the A 1 and B 1 α-amino groups has been reported by other authors (see Blundell *et al.* 1972, for a review). The relative reactivity of these two groups depends on the nature of the reagent, the solvent and the pH. *N*-Hydroxysuccinimide acetate (Lindsay and Shall 1971) and phenylisothiocyanate (Anderson 1954) have both been shown to react preferentially with the glycine A 1 α-amino group at pH 8·5–9·0. As insulin exists largely as a monomer at pH 9·0 in the absence of zinc (Fredericq 1953), and the hexamer and dimer dissociation is further favoured by the presence of an organic solvent (e.g. methanol), the most probable explanation for the selectivity of the amidination is a difference in the pK_a of the two amino terminal groups.

Selective modification of the glycine A 1 has been shown by Lindsay and Shall (1972) to cause little, if any, change in the antigenicity of insulin. This is in contrast to modification at phenylalanine B 1 which causes a large reduction in the antigenicity, the magnitude of which is dependent on the size of the modifying reagent. The results shown in Fig. 3 are consistent with those of Lindsay and Shall (1972) in that no significant decrease in antigenicity was observed in selective derivatization at glycine A 1. Further, the observation that glycine A 1 butyrimidyl pyridine disulfide insulin has a decreased biological potency is also consistent with the changes observed by Lindsay and Shall on selective glycine A 1 modification with groups larger than acetyl. Conjugation of the glycine A 1 modified insulin to alkaline phosphatase which has been pre-activated by reaction with MMBI under reducing conditions leads to the formation of a product which retains most of the enzyme activity and half of the insulin immunoreactivity. The recovery of enzyme and insulin activities in the thiol interchange reaction described is higher than those generally observed in glutaraldehyde-mediated conjugations. The isolation and characterization of the stable intermediate, butyrimidyl pyridine disulfide insulin, in the scheme for coupling insulin to alkaline phosphatase has provided the means by which the conjugation can be carried out in a controlled manner. Most probably the selective incorporation of the thiol group into the non-antigenic A-chain of the insulin molecule is responsible for the relatively high recovery (50%) of insulin antigenicity in the conjugate.

The finding that the introduction of a thiol group, via MMBI under mild reducing conditions, into alkaline phosphatase does not affect the enzyme activity suggests that this reaction will provide a mild, convenient general method of introducing accessible thiol groups into alkaline phosphatase. This opens up the possibility of using alkaline phosphatase in conjunction with strategies employing maleimides (Kato *et al.* 1975*a*, 1975*b*, 1976; Kitagawa and Aikawa 1976), in addition to the thiol interchange strategy.

The application of the insulin–alkaline phosphatase conjugate in an enzyme immunoassay (Fig. 6) indicates that, like the radioimmunoassay, both the range and the sensitivity of the enzyme immunoassay are dependent on the antiserum pool employed. The enzyme immunoassay standard curve generated with antiserum I, at a 1:20 000 dilution, provides a working range of 150–30 000 μU/ml. This compares favourably with the range of 125–1900 μU/ml provided by the radioimmunoassay (Fig. 3). The small difference between the assay methods in the lower limits of the working range is not significant for most applications. The wider range provided by the enzyme immunoassays is advantageous in many industrial situations since sample dilutions are kept to a minimum. Certainly, the replacement of radioimmunoassays with enzyme immunoassays will remove the expense and difficulty involved in the disposal of radioactive wastes as well as eliminating the health and environmental hazards of radioisotopes. The strategy of using controlled, characterized reactions for antigen–enzyme conjugation, should expedite the acceptance of enzyme immunoassays in routine immunoassay procedures.

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