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The Acetylation of Insulin

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The acetylation of the free amino groups of insulin was studied by reaction of the hormone with *N*-hydroxysuccinimide acetate at pH 6.9 and 8.5. The products formed were separated by chromatography on DEAE-Sephadex and were characterized by isoelectric focusing, by end-group analysis, by the incorporation of [^{3}H]acetyl groups in the molecule, and by treatment with trypsin that had been treated with 1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one ('tosylphenylalanyl chloromethyl ketone'). Three monosubstituted products, two disubstituted products and one trisubstituted derivative were prepared. The α-amino groups of the terminal residues and the ϵ-amino group of the lysine-B29 were the sites of reaction. Acetylation of any of the free amino groups did not affect the biological activity of insulin. It was demonstrated, however, that substitution at the glycine-A1 amino group by the larger residues, acetoacetyl or thiazolidinecarbonyl, produced a decrease in biological activity. Modification of the lysine-B29 or phenylalanine-B1 amino groups with these larger reagents did not affect the biological activity. Modification of the phenylalanine-B1 amino group by any of the three substituents resulted in a large decrease in the affinity of insulin for anti-insulin antibodies raised in the guinea pig. Modification of the other two amino groups did not affect the reaction with antibody. These observations are correlated with the tertiary structure of insulin.

Numerous derivatives of insulin have been prepared in which the N^{α}-amino groups of phenylalanine-B1 and of glycine-A1 and the N^{ϵ}-amino group of lysine-B29 are modified. In every case, except acetylation, a significant decrease in the biological activity of insulin occurred when more than one of the amino groups was modified (Mills, 1953; Anderson, 1956; Bromer, Sheehan, Berns & Arquilla, 1967; Levy & Carpenter, 1967; Lindsay & Shall, 1970; Africa & Carpenter, 1970).

The observation by Fraenkel-Conrat & Fraenkel-Conrat (1950) that the amino groups of insulin could be acetylated without any appreciable loss in biological activity appears to contradict the above results, and it was decided to reinvestigate the acetylation of insulin and to characterize the acetyl-insulins in the manner previously reported for the acetoacetyl- and thiazolidinecarbonyl-insulins (Lindsay & Shall, 1969, 1970). By isolation of the products of reaction with each one of the amino groups in turn, it was hoped that further information would be obtained that would rationalize the contradiction.

The effectiveness of hydroxysuccinimide esters for the acylation of amino groups in insulin (Lindsay & Shall, 1970) led us to synthesize *N*-hydroxysuccinimide acetate and to examine the products of its reaction with insulin.

This work, in conjunction with our previous results (Lindsay & Shall, 1969, 1970), permit some general conclusions to be drawn relating the tertiary structure of insulin to its biological and immunological activity.

MATERIALS

Crystalline porcine insulin (ten-times recrystallized) was a product of Novo Industri A/S, Copenhagen, Denmark, and was used without further purification. The ox insulin also used in this work was a product (Batch no. 26918) of British Drug Houses Ltd., Poole, Dorset, U.K.

N-Hydroxysuccinimide was prepared by the method of Anderson, Zimmerman & Callahan (1964) and was recrystallized from ethyl acetate. It had m.p. 97–100°C (literature m.p. 99–100°C).

NN-Dicyclohexylcarbodi-imide was the product of Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

[^{3}H]Acetic acid (480 mCi/mmol) was a product of The Radiochemical Centre, Amersham, Bucks., U.K.

Ampholytes were the product of LKB Instruments Ltd., South Croydon, Surrey, U.K.

Trypsin that had been treated with 1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one ('tosylphenylalanyl chloromethyl ketone') was the product of Worthington Biochemicals Corp., Freehold, N.J., U.S.A.

Urea (A.R. grade) was de-ionized by passing a 7M solution through a mixed-bed Amberlite MB 3 column and used immediately after preparation.

METHODS

Melting points. These are uncorrected and were measured on a Kofler block.

Conductance. This was measured with an LKB model 5300B Conductolyser fitted with a dip-cell.

U.v.-absorption spectra. These were taken in a Cary model 15 recording spectrophotometer.

N.m.r. spectra. These were taken on a Varian A60 machine, with tetramethylsilane being used as an internal standard.

N-*Hydroxysuccinimide acetate.* Acetic acid (3.0g, 50 mmol) was dissolved in dichloromethane (30ml), and a solution of *N*-hydroxysuccinimide (5.76g, 50mmol) in anhydrous dioxan (10ml) was added. *NN'*-Dicyclohexylcarbodi-imide (10.32g, 50mmol) was added and the reaction mixture was stirred overnight at 4°C and for a further 2h at room temperature. The dicyclohexylurea formed was filtered off and the solvent concentrated on a rotary evaporator under reduced pressure. Light petroleum (b.p. 60–80°C) was added to the supernatant until the solution became turbid. Filtration of the crystalline material and recrystallization from ethyl acetate gave colourless needles of *N*-hydroxysuccinimide acetate (7.5g, 95.5% yield), m.p. 131–134°C [literature m.p. 131°C (Hurd, Buess & Bauer, 1954)] (Found C, 46.0; H, 4.8; N, 8.8. Calc. for $C_6H_7NO_4$: C, 45.9; H, 4.5; N, 8.9%), n.m.r. spectrum ($CDCl_3$): 7.18τ, a singlet, weight 4, assigned to the succinimide protons, and 7.67τ, a singlet, weight 3, assigned to the acetyl protons.

Reaction of insulin with N-*hydroxysuccinimide acetate.* Zinc–insulin (200mg, 38μmol) was dissolved in 0.1M-HCl (40ml) and the solution adjusted with NaOH to pH6.9 in the titration vessel of a Radiometer type TTT1 pH-stat. *N*-Hydroxysuccinimide acetate (6mg, 38μmol) was dissolved in dioxan (100μl) and added to the above solution in 10μl portions, the pH being maintained at 6.9 by the addition of alkali every 30min.

The reaction mixture was left overnight, and was then dialysed against distilled water and freeze-dried to yield 185mg of the modified protein.

In a second experiment zinc–insulin (500mg, 85μmol) was treated with *N*-hydroxysuccinimide acetate (15.2mg, 85μmol) at pH8.5 exactly as described above, portions of the activated ester being added every 5min.

In a further experiment zinc–insulin (500mg, 85μmol) was treated with the activated ester (40mg, 255μmol) at pH8.5 as described above.

Chromatographic separation of the acetyl-insulins. The acetyl-insulins were separated on a column (2.5cm×40cm) of DEAE-Sephadex A-25 by a modification of the method described by Bromer & Chance (1967). This column was equilibrated with buffer containing 10mM-tris and 50mM-NaCl in de-ionized 7M-urea adjusted to pH7.30 with M-HCl. The acetyl-insulins were dissolved in this buffer (50mg/ml) and the column was developed at a flow rate of 54.0ml/h, 9.7ml fractions being collected. A 97ml volume of eluent was collected before a linear gradient was applied. For insulin treated with a molar equivalent of *N*-hydroxysuccinimide acetate a linear gradient obtained by running 10mM-tris and 100mM-NaCl in de-ionized 7M-urea (1litre) at pH7.30 into the stirred reservoir of the starting buffer (1litre) was applied.

For insulin treated with a threefold molar excess of *N*-hydroxysuccinimide acetate, the concentration of NaCl in the final buffer was increased to 150mM.

The protein concentration was determined from the extinction of the solution at 277nm.

The contents of tubes around the centre of each peak from the chromatography were pooled and separately rechromatographed under the same conditions. This process was repeated until each peak appeared homogeneous by isoelectric focusing. The material was freeze-dried and the residue de-ionized on a column (1.5cm×60 cm) of Sephadex G-10 equilibrated with 5% (v/v) acetic acid. The fractions were pooled and the protein was freeze-dried.

Radioactivity. The radioactivities of 25μl portions of the fractions isolated from the chromatographic separation of insulin treated with a sample of the ^{3}H-labelled *N*-hydroxysuccinimide acetate (0.57mCi/mmol) were measured in a Beckman model 233 liquid-scintillation counter; 0.2ml of a solution of Hyamine hydroxide and 10ml of 0.5% (w/v) 2,5-diphenyloxazole in toluene was added to each pot.

Dansylation. Samples containing 10nmol of the isolated derivatives were dansylated by the method of Gray (1967). The residue after acid hydrolysis was dissolved in 15μl of acetone–acetic acid (3:2, v/v) and the extracted DNS-amino acids were separated by paper electrophoresis at pH4.38.

Isoelectric focusing of acetyl derivatives in polyacrylamide gel. The 7.5% polyacrylamide gels, 4M in urea and containing 0.04% of the pH4–6 range LKB Ampholine, were photopolymerized by using riboflavin and loaded with 100μg samples of each of the [^{3}H]acetyl-labelled insulin derivatives. The gels were subjected to electrophoresis for 2h at 150V according to the method of Wrigley (1968). The protein bands were detected by immersion of the gels in 5% (w/v) trichloroacetic acid for 30min. The gels were also cut into 0.2cm slices and the radioactivity in each slice was determined in a liquid-scintillation counter by the method of Young & Fulhorst (1965).

Treatment with 1-*chloro*-4-*phenyl*-3-*toluene*-p-*sulphonamidobutan*-2-*one-treated trypsin.* Acetyl-insulins (3mg) were dissolved in Na_2CO_3 buffer, pH9.2, and treated with a solution of 1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one-treated trypsin (125μl; 1.2mg/ml) in 1mM-$CaCl_2$ by the method of Levy & Carpenter (1967). A 0.1μmol portion of the above solution was analysed for free alanine on the amino acid analyser.

Biological assay. The insulin derivatives were separated from trace amounts of urea and salt by elution from a column (2.5cm×60cm) of Sephadex G-10 that had been equilibrated with aq. 5% (v/v) acetic acid and the protein, after being freeze-dried, was dried over P_2O_5 at room temperature for 2 days. The derivatives were assayed against a neutral insulin solution as standard by the mouse-convulsion method (*British Pharmacopoeia*, 1968).

All injections were of solutions in sodium acetate buffer, pH 8.0.

Radioimmunoassay was performed according to the method of Hales & Randle (1963). Both types of assay were undertaken by Dr G. A. Stewart at the Wellcome Foundation Ltd., Dartford, Kent, U.K.

RESULTS AND DISCUSSION

Treatment of insulin with an equimolar amount of *N*-hydroxysuccinimide acetate gives an 80% yield of reaction products both at pH 6.9 and at 8.5, and effectively demonstrates the usefulness of *N*-hydroxysuccinimide acetate for the acetylation of the α- and ε-amino groups of insulin.

Acid anhydrides undergo rapid hydrolysis at neutral pH and have to be used in large excess to give an appreciable yield of the acylated protein. In part this could account for their lack of selectivity towards amino groups (Cohen, 1968). Highly reactive acylating agents, such as hydroxysuccinimide esters, used at a relatively low pH are often more selective than weaker acylating agents. The selectivity of hydroxysuccinimide esters towards amino groups has also been demonstrated by de Groot, Lapidot, Panet & Wolman (1966). Treatment of aminoacyl-tRNA with *N*-hydroxysuccinimide acetate was more specific for the amino group present than was acetic anhydride. *N*-Hydroxysuccinimide acetate should also prove a useful reagent for the incorporation of radioactive acetyl groups into proteins.

At low reagent concentrations and near neutral pH, the predominant products of the reaction are the two monosubstituted acetyl-insulins in which the two terminal α-amino groups are modified (Fig. 1). However, if the pH is raised the amount of the Phe^{B1}-acetyl-insulin isolated is decreased and Lys^{B29}-acetyl-insulin is also produced (Fig. 2).

The chromatographic separation of the products of the reaction of insulin with a threefold excess

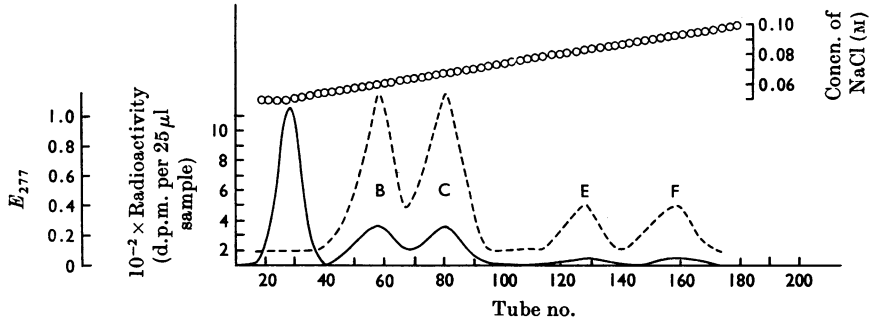

Fig. 1. Chromatographic separation of the reaction products of insulin (200 mg), treated with an equimolar amount of *N*-hydroxysuccinimide acetate at pH 6.9, on a column (40 cm × 2.5 cm) of DEAE-Sephadex A-25 at pH 7.30. ———, E_{277}; ----, radioactivity; ○——○, NaCl gradient.

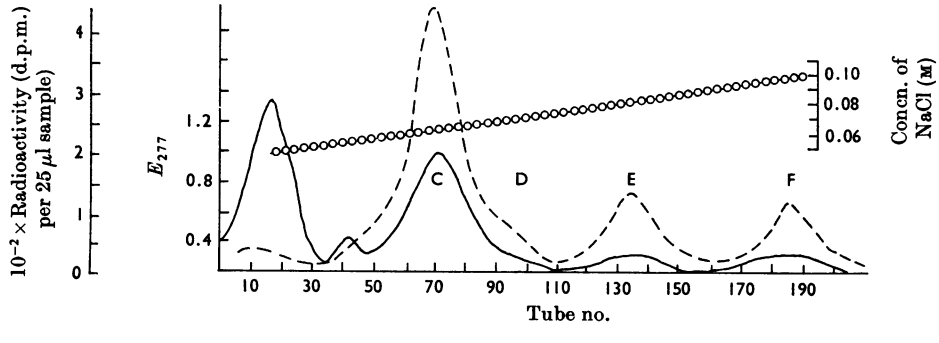

Fig. 2. Chromatographic separation of the reaction products of insulin (500 mg), treated with an equimolar amount of *N*-hydroxysuccinimide acetate at pH 8.5, on a column (40 cm × 2.5 cm) of DEAE-Sephadex A-25 at pH 7.30. ———, E_{277}; ----, radioactivity; ○——○, NaCl gradient.

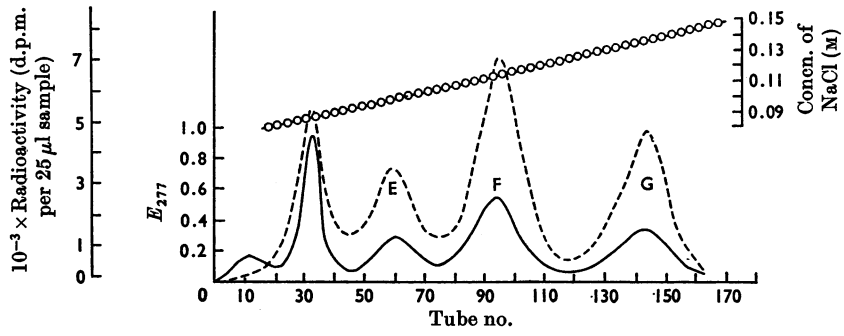

Fig. 3. Chromatographic separation of the reaction products of insulin (500mg), treated with a threefold molar amount of *N*-hydroxysuccinimide acetate at pH8.5, on a column (40cm×2.5cm) of DEAE-Sephadex A-25 at pH7.30. ———, E_{277}; ----, radioactivity; ○——○, NaCl gradient.

Table 1. *Characterization of the acetyl-insulin derivatives*

Material	Reaction conditions	Fraction	Overall yield* (%)	Relative yield† (%)	Purity‡ (%)	Incorporation of acetyl groups	Major end groups (dansyl method)	Alanine released by trypsin (mol)
Bovine insulin	pH6.9; 1 equiv. of activated ester	B	80	25	92	0.8	Gly; Lys (ε)	0.8
		C		30	—	0.9	Phe; Lys (ε)	
Bovine insulin	pH8.5; 1 equiv. of activated ester	C	80	44	99	0.9	Phe; Lys (ε)	1.1
		D		10	90	1.0	Phe; Gly	0.2
		E		7	—	2.3	Lys (ε)	
		F		7	—	1.8	Phe	
Porcine insulin	pH8.5; 3 equiv. of activated ester	E	60	16	96	2.3	Lys (ε)	0.9
		F		27	98	2.3	Phe	0.1
		G		15	95	2.7	—	0.1

* Based on total incorporation of radioactivity into protein.
† Based on the amount of product formed relative to total amount of protein isolated.
‡ Based on contamination by products that are separated by isoelectric focusing.

of the activated ester at pH8.5 is shown in Fig. 3. Characterization of the two disubstituted acetyl-insulins formed (Table 1) shows that they are Phe^{B1},Gly^{A1}-diacetyl-insulin and Gly^{A1},Lys^{B29}-diacetyl-insulin respectively. Under the present chromatographic conditions it was not possible to separate Phe^{B1},Lys^{B29}-diacetyl-insulin, and we were not able to find any evidence for the production of this material. This evidence suggests that the order of reactivity of the amino groups of insulin under these conditions is glycine>phenylalanine≃lysine. This is in contrast with the situation at pH6.9, when the order of reactivity is glycine≃phenylalanine≫lysine. From the order of elution of the various derivatives on DEAE-Sephadex chromatography, one can conclude that the pK_a values of the amino groups in insulin are glycine< phenylalanine<lysine. This is consistent within the limits of experimental error with the order of reactivity of these groups at pH6.9 but not at 8.5. A local change in conformation or charge affecting the reactivity of the amino group of phenylalanine-B1 might explain the dependence of the order of reactivity of the α-amino groups on the reaction pH. The variation in reactivity could also depend on the nature of the modifying reagent and the solvent in which the reaction occurs. Previous work on the chemical modification of the amino groups in insulin has suggested a variation in the order of the reactivity of the α-amino groups under different conditions (Mills, 1953; Anderson, 1956; Bromer *et al.* 1967; Africa & Carpenter, 1970; Lindsay & Shall, 1970).

Isoelectric focusing on polyacrylamide gel of each of the derivatives (Fig. 4) showed them to be homogeneous and to contain less than 10% of any material that could be separated by this technique. It was not possible to distinguish between the three

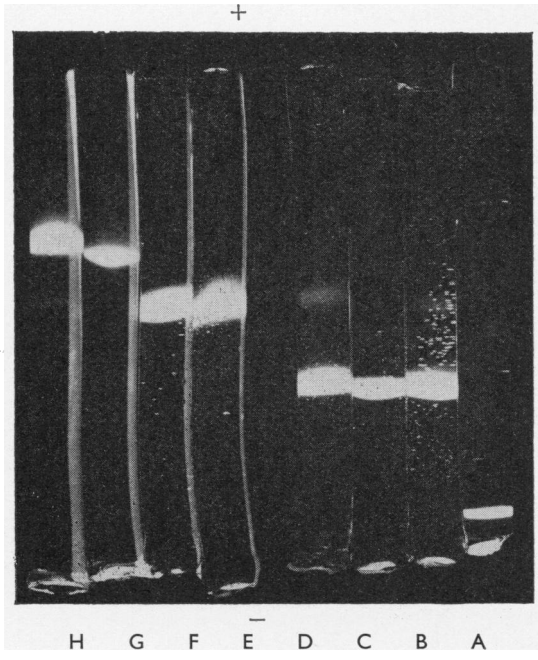

Fig. 4. Separation of the purified acetyl-insulin derivatives by isoelectric focusing in polyacrylamide gels containing 0.04% of pH range 4–6 Ampholine. A, Native insulin; B, Phe^{B1}-acetyl-insulin; C, Gly^{A1}-acetyl-insulin; D, Lys^{B29}-acetyl-insulin; E, Phe^{B1},Gly^{A1}-diacetyl-insulin; F, Gly^{A1},Lys^{B29}-diacetyl-insulin; G, Phe^{B1},Gly^{A1},Lys^{B29}-triacetyl-insulin; H, Phe^{B1},Gly^{A1},Lys^{B29}-triacetoacetyl-insulin.

monoacetyl-insulin isomers or the two diacetyl-insulin isomers by using this method.

Biological activity. The mouse-convulsion method for the assay of the acetyl-insulins confirms the results of Fraenkel-Conrat & Fraenkel-Conrat (1950) and suggests that the α- and ϵ-amino groups of insulin are not required for its hormonal activity.

The acetoacetyl-insulins, prepared as previously described (Lindsay & Shall, 1969), were retested under the same conditions as the acetyl-insulins. The results (Table 2) show a small, but significant, decrease in the mouse-convulsion activity of the glycine-modified monoacetoacetyl-insulin. An increase in the size of the modifying group on glycine as in the thiazolidine-modified insulins (Lindsay & Shall, 1970) decreases the activity of this derivative to 50% of that of insulin. Several trisubstituted aminoacyl-insulins with a substituent of similar size to the thiazolidine group show a similar decreased activity (Levy & Carpenter, 1967).

This evidence suggests that, although the glycine N^{α}-amino function in insulin is not involved in its hormonal function, it is close to the region of the molecule that is biologically important.

We now discuss in general terms some features of the structure that appear relevant to the results described in this paper.

The glycine-A1 residue of insulin is situated close to a region of the molecule that is bounded by the twofold axis of symmetry that relates the two monomers of insulin in the insulin dimer (Adams *et al.* 1969), which is shown in projection in Fig. 5. An examination of the tertiary structure of rhombohedral 2Zn–insulin in relation to the known sequences of insulins from various species shows that a species-invariant region of the molecule occurs near to the β-pleated sheet that joins the two terminal parts of the B-chains in the insulin dimer (T. L. Blundell, G. G. Dodson, D. C. Hodgkin & M. Vijayan, unpublished work). Many of the invariant residues such as B7-9, B11-12, B15-16, B23-26, A1-2 and A21 appear to be on the outside of a common surface, which points towards the twofold axis of symmetry OP that relates the two insulin monomers in the insulin dimer. This surface probably extends along the whole length of the insulin monomer from the A21 residue to the B9 residue and after a sharp turn involving residues B6–B8 finally ends at the histidine-B5 residue.

The residues that comprise this invariant region are also predominantly hydrophobic in character. If the invariant residues are important for the highly specific interaction of insulin with its receptor, then the mechanism of interaction would appear to involve a complementary fit of hydrophobic residues on the surface of the receptor site with the appropriate residues in the insulin molecule.

The hydrophobic residues that form the interface between the monomers will become accessible only when the dimer dissociates into the monomer. Roth, Gordon & Pastan (1968) have shown that immunoreactive plasma insulin is eluted on Sephadex G-50 as a molecule of mol. wt. 6000. This evidence is indicative of the 6000 monomer unit of insulin as being a biologically active unit.

Modification of the α-amino group of glycine-A1 by groups larger than an acetyl group causes a loss in activity that in our opinion could be due to the shielding of important interactions with the receptor site or by a distortion of configuration of the invariant residues.

The work of Africa & Carpenter (1970) has shown that the biological activity of des-Phe^{B1}-des-Gly^{A1}-insulin is 10% of that of the activity of insulin. However, des-Phe^{B1}-insulin has full biological activity (Brandenburg, 1969). Glycine-A1 must therefore exert some stabilizing effect in the region of the 'active site' of insulin. As our work shows that the free α-amino group of glycine-A1 is not essential for this stabilization, the forces stabilizing the active site probably involve hydrogen-bond interactions between glycine and adjacent atoms.

Table 2. *Activity of insulin derivatives*

	Mouse-convulsion assay		Radioimmunoassay	
	Mean potency (units/mg)	Fiducial limits ($P=0.95$)	Immunoreactivity (units/mg)	Fiducial limits ($P=0.95$)
Insulin chromatographed in 7 M-urea	23.3	(21.1–25.8)	20.8	(18.0–24.0)
Crystalline bovine insulin	22.4	(20.6–24.3)	24.4	(23.6–25.4)
Phe^{B1}-substituted insulins				
(*a*) Acetyl (bovine)	22.4	(20.2–24.9)	9.3†	(8.9– 9.7)
(*b*) Acetoacetyl (bovine)	24.5	(22.2–26.9)	2.1	(2.0– 2.15)
(*c*) Thiazolidine* (bovine)	23.2	(21.1–25.6)	6.63	(6.37–6.91)
Gly^{A1}-substituted insulins				
(*a*) Acetyl (bovine)	23.6	(21.4–25.9)	17.3	(16.5–18.1)
(*b*) Acetoacetyl (bovine)	19.8	(19.1–20.6)	21.2	(20.4–21.9)
(*c*) Thiazolidine* (bovine)	12.8	(11.8–14.0)	19.8	(19.1–20.6)
Lys^{B29}-substituted insulins				
(*a*) Acetyl (bovine)	25.7	(23.4–28.3)	20.2	(19.3–21.3)
(*b*) Thiazolidine* (bovine)	22.8	(20.9–25.0)	20.6	(17.3–24.6)
Phe^{B1},Gly^{A1}-disubstituted insulins				
(*a*) Acetyl (porcine)	20.2	(18.4–22.1)	6.2	(5.8– 6.6)
(*b*) Acetoacetyl (bovine)	17.0	(15.5–18.7)	3.3	(3.1– 3.6)
Gly^{A1},Lys^{B29}-disubstituted insulin				
(*a*) Acetyl (porcine)	21.0	(19.0–23.3)	16.3	(15.5–17.0)
Phe^{B1},Gly^{A1},Lys^{B29}-trisubstituted insulins				
(*a*) Acetyl (porcine)	21.6	(19.6–23.9)	2.5	(2.4– 2.7)
(*b*) Acetoacetyl (bovine)	17.4	(15.7–19.2)	4.2	(4.0– 4.5)

* Data from Lindsay & Shall (1970).

† The immunoreactivity of this sample appeared to vary on standing. The value quoted was that obtained after storage of the solution in acetate buffer at pH 8.0 and 5°C for between 8 and 15 days. After 1 and 2 days storage the potency of the immunoreactive material was 5.5 units/mg and 7.3 units/mg respectively.

Modification of the α-amino group of glycine-A1 by reasonably large residues would be expected to alter these secondary forces by distortion and this distortion could be transmitted through a large part of the invariant region. A similar explanation could account for the large loss in activity on removal of the *C*-terminal asparagine residue on the A-chain (Slobin & Carpenter, 1963).

Thus the mechanism of interaction of insulin with its receptor may not involve reactive amino acid side chains but may depend on a mechanism of 'exact fit' only.

A three-dimensional electron-density difference 'map' between insulin and the crystalline and isomorphous glycine-modified thiazolidinecarbonyl-insulin (Lindsay & Shall, 1970) would test the validity of the above hypothesis. This difference 'map' should indicate whether the loss of activity in this derivative is due to a transmission effect throughout the invariant region of insulin by modification of the α-amino group on glycine-A1, or whether the loss of activity is due solely to the shielding of certain of these residues from interaction with the receptor site because of the presence of the thiazolidine ring.

Immunological activity. Table 2 shows that independent of the size of the modifying group, modification of the phenylalanine-B1 amino group causes a marked decrease in affinity for anti-insulin antibodies raised as is customary in normal guinea pigs, whereas both the glycine-A1 and lysine-B29 amino groups are unimportant in the reaction between insulin and anti-insulin antibodies.

The weakness with which the phenylalanine-modified insulin derivatives bind to guinea-pig anti-insulin antibodies suggests that the area around this part of the insulin molecule forms the antibody-combining site. The sequence B1–B4, A9–A10 and A12–A15 are all in the same region of the molecule (Fig. 6) and are on the surface of the molecule rather than buried in the interior. Residues A8–10, A13, B2–4, B27 and B30 show three or more non-conservative alternatives in amino acid composition among the various insulins whose sequences have been determined, and can be considered to be highly species-variable.

Our previous results (Lindsay & Shall, 1969, 1970) have shown that the phenylalanine-modified insulin derivatives cannot be crystallized in the rhombohedral space group. The packing of dimers

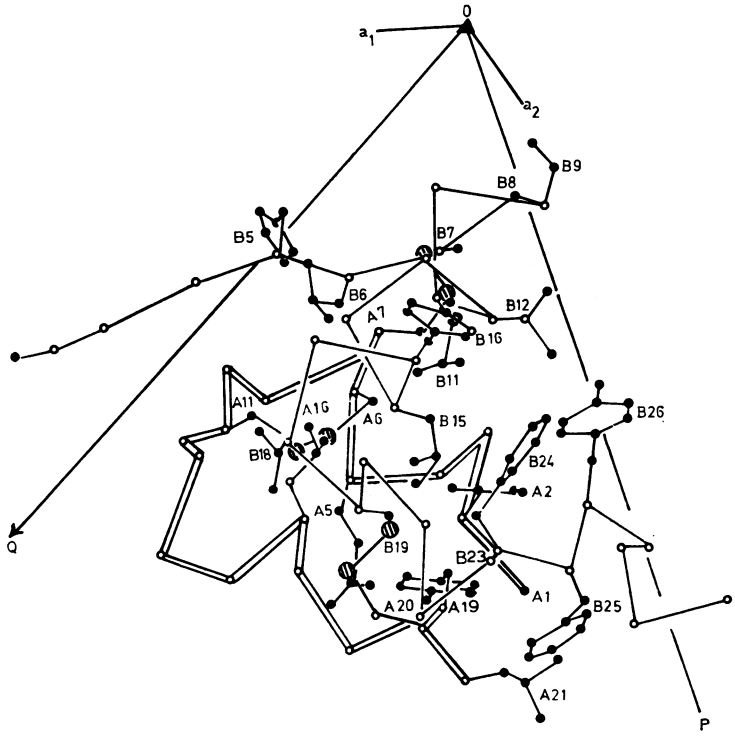

Fig. 5. View down the threefold axis of the species-invariant residues in the insulin monomer that are common to all the mammalian, fish and bird insulins whose sequences have been published [data taken from the *Atlas of Protein Sequence and Structure*, vol. 4, published by the National Biomedical Research Foundation, Silver Spring, Md., U.S.A. 1969)]. Conservative alternatives are excluded from this figure.

in the rhombohedral crystal involves close contacts between the phenylalanine-B1 residues and other nearby groups of adjacent molecules. Because of these close contacts modification of phenylalanine-B1 could produce a conformational change that may affect binding to antibody.

The inability of the phenylalanine derivatives to bind to antibody may also be due to the substituent preventing close approach of the antibody to its binding site. Des-Phe^{B1}-insulin does react with guinea-pig anti-insulin antibodies (Brandenburg, 1969), so neither the amino group nor the phenyl ring is an essential element in the binding site.

The importance of the species-variable region around phenylalanine-B1 as an immunogenic site also offers an explanation for the high immunological potency of proinsulin towards insulin antisera (Steiner, Hallund, Rubenstein, Cho & Bayliss, 1968). The connecting peptide that links the alanine-B30 and glycine-A1 residues would be unlikely to mask totally the species-variable region of the molecule, leaving it free to react with anti-insulin antibody. Evidence has been presented by Frank & Veros (1968, 1970) that proinsulin exhibits self-association behaviour in solution that is closely similar to that of insulin, and that proinsulin interacts with zinc to form a zinc–proinsulin complex corresponding to a hexamer of proinsulin. It is probable that proinsulin also has the insulin antigenic determinant in addition to those sites specific to proinsulin.

A correlation between the antigenicity and immunogenicity of insulin may be relevant to the clinical treatment of diabetes.

Wilson, Aprile & Sasaki (1967), using synthetic insulin peptides that were able to provoke cutaneous anaphylaxis in normal guinea pigs previously injected with insulin, suggested that antibodies were directed mainly towards antigenic loci in the regions A10–A21 and B1–B8 of the insulin molecule.

However, Arquilla & Finn (1963) have shown that genetic factors in the guinea pig can direct antibody production towards different determinants in the insulin molecule. It is possible that several

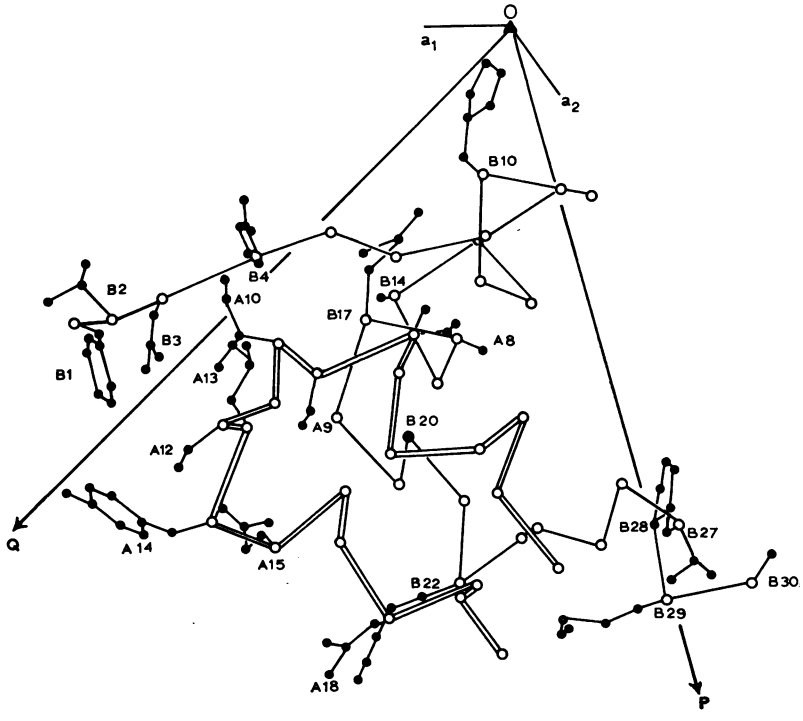

Fig. 6. View down the threefold axis of the species-variable residues in the insulin monomer. The figure includes all those residues for which there are two or more non-conservative alternatives in sequence for all the mammalian, fish and bird insulins whose sequences have been published [data taken from the *Atlas of Protein Sequence and Structure*, vol. 4, published by the National Biomedical Research Foundation, Silver Spring, Md., U.S.A. (1969)].

different regions of the insulin molecule may be antigenic in a total population of individuals. The work of Deckert & Grundahl (1970) has suggested that the antigenicity of pig insulin towards humans may be more dependent on the method of insulin administration than on the sequence difference between human and pig insulin.

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REFERENCES

Adams, M. J., Blundell, T. L., Baker, E. N., Dodson, E. J., Dodson, G. G., Harding, M. M., Hodgkin, D. C., Rimmer, B., Sheat, S. & Vijayan, M. (1969). *Nature, Lond.*, **224**, 491.

Africa, B. & Carpenter, F. H. (1970). *Biochemistry, Easton*, **9**, 1962.

Anderson, G. W., Zimmerman, J. E. & Callahan, F. M. (1964). *J. Am. chem. Soc.* **86**, 1839.

Anderson, W. (1956). *C. r. Trav. Lab. Carlsberg*, **30**, 104.

Arquilla, E. R. & Finn, J. (1963). *Science, N.Y.*, **142**, 400.

Brandenburg, D. (1969). *Hoppe-Seyler's Z. physiol. Chem.* **350**, 741.

Bromer, W. W. & Chance, R. E. (1967). *Biochim. biophys. Acta*, **133**, 219.

Bromer, W. W., Sheehan, S. K., Berns, A. W. & Arquilla, E. R. (1967). *Biochemistry, Easton*, **6**, 2378.

Cohen, L. A. (1968). *A. Rev. Biochem.* **37**, 695.

Deckert, T. & Grundahl, E. (1970). *Diabetologia*, **6**, 15.

de Groot, N., Lapidot, Y., Panet, A. & Wolman, Y. (1966). *Biochem. biophys. Res. Commun.* **25**, 17.

Fraenkel-Conrat, J. & Fraenkel-Conrat, H. (1950). *Biochim. biophys. Acta*, **5**, 89.

Frank, B. H. & Veros, A. J. (1968). *Biochem. biophys. Res. Commun.* **32**, 155.

Frank, B. H. & Veros, A. J. (1970). *Biochem. biophys. Res. Commun.* **38**, 284.

Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 139. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Hales, C. N. & Randle P. J. (1963). *Biochem. J.* **88**, 137.
Hurd, C. D., Buess, C. M. & Bauer, L. (1954). *J. org. Chem.* **19**, 1140.
Levy, D. & Carpenter, F. H. (1967). *Biochemistry, Easton*, **6**, 3559.
Lindsay, D. G. & Shall, S. (1969). *Biochem. J.* **115**, 587.
Lindsay, D. G. & Shall, S. (1970). *Eur. J. Biochem.* **15**, 547.
Mills, G. L. (1953). *Biochem. J.* **53**, 37.
Roth, J., Gordon, P. & Pastan, I. (1968). *Proc. natn. Acad. Sci. U.S.A.* **61**, 138.
Slobin, L. I. & Carpenter, F. H. (1963). *Biochemistry, Easton*, **2**, 22.
Steiner, D. F., Hallund, O., Rubenstein, A., Cho, S. & Bayliss, C. (1968). *Diabetes*, **18**, 725.
Wilson, S., Aprile, M. A. & Sasaki, L. (1967). *Can. J. Biochem.* **45**, 1135.
Wrigley, C. (1968). *Sci. Tools*, **15**, 17.
Young, R. W. & Fulhorst, H. W. (1965). *Analyt. Biochem.* **11**, 389.