Diabetologia 15, 403–410 (1978)

Diabetologia

In Vitro Bioactivity of Insulin Analogues: Lipogenic and Anti-Lipolytic Potency and Their Interaction with the Effect of Native Insulin

M. J. Ellis[1], S. C. Darby, R. H. Jones, and P. H. Sönksen

Department of Medicine, St. Thomas' Hospital Medical School, London, England

Summary. This paper presents a survey of the biological potencies of a variety of naturally-occurring and semi-synthetic insulin analogues and a study of the joint biological action of some of these materials with native insulin. Biological activity was tested on isolated rat fat cells using lipogenesis from glucose as the metabolic index. A brief comparison using inhibition of fat cell lipolysis was included. The results indicated: 1. Analogue potencies varied considerably (0.4–100% insulin activity). Values obtained were mainly confirmatory but included two further B1-modified materials and a tricarbamylated insulin. The results supported previous indications on the relative roles of the A1, B1, and B_{29} residues of insulin for hormone activity. 2. Analogue bioactivities, whether assessed by stimulation of lipogenesis or inhibition of lipolysis, were similar for the four materials tested in both systems. The response of fat cells with respect to both metabolic indices occurred over a comparable range of insulin concentrations, with half maximal effects at 30–35 pmol l^{-1} insulin. 3. The presence of modified insulins appeared to alter the biological action of native insulin *in vitro*. Small effects of both potentiation and antagonism were identified.

Key words: Insulin analogues, isolated fat cells, biological potency, lipogenesis, inhibition of lipolysis, combined biological action, potentiation, antagonism.

Analogues of hormones provide a tool for investigating many aspects of the mechanisms of hormone action. For example, information has been derived from extensive studies using a group of insulins modified at the A1, B1, and B_{29} positions of the molecule in terms of their bioactivity [1–5], receptor binding affinity [5, 6], conformation [4, 7, 8] and metabolic clearance [9].

The initial step in this *in vitro* study on the action of insulin analogues was to establish the biological potencies of 31 available materials from their ability to stimulate lipogenesis in isolated rat fat cells, most of these data being an independent confirmation of results published previously. The abilities of four of the analogues to inhibit adrenaline-stimulated lipolysis in fat cells were also investigated and compared with their lipogenic potencies.

Insulin appears to inhibit lipolysis in adipose tissue by a mechanism independent of glucose uptake [10, 11] and it is not clear whether the insulin receptor as well as the effector mechanism for the two processes is different. The anti-lipolytic action of insulin has been reported to occur at lower hormone concentrations [12, 13] than effects on glucose uptake. The experiments described here enabled us to compare the concentrations of insulin causing half-maximal effects on these two metabolic effects in the same preparation of fat cells.

Finally, the established biological potencies of four A1, B_{29} modified insulins and proinsulin were used to design experiments investigating the effect of these analogues on the metabolic response of fat cells to native insulin. The availability of materials with the ability to modify insulin action at the tissue level could lead to a new approach to the treatment of diabetes mellitus. An analogy would be the successful use of analogues of acetylcholine to treat myasthenia gravis [14]. This treatment is based on the ability of compounds structurally related to acetylcholine to potentiate or increase the action of the neurotransmitter at the motor end-plate.

[1] Present Address: The Medical Unit, The Princess Margaret Hospital, Christchurch, New Zealand

0012-186X/78/0015/0403/$01.60

Materials and Methods

Materials

[3-^{3}H] glucose was obtained from the Radiochemical Centre, Amersham. Crude collagenase (138–211 units mg^{-1}), was purchased from the Worthington Biochemical Corporation, New Jersey, and human serum albumin was a generous gift from the Blood Products Laboratory, Lister Institute, Hertfordshire. Adrenaline ("Injection", 5.46 mmol l^{-1}) was obtained from the St. Thomas' Hospital Pharmacy.

Repurified bovine insulin [15] used as reference standard, and the following semi-synthetic insulins [14, 15] were donated by D. Brandenburg, Wollforschungsinstitut, Aachen:

A_1 acetyl insulin
B_{29} acetyl insulin
A_1, B_{29} diacetyl insulin
A_1-B_{29} desphe.^{B1} suberoyl insulin
A_1-B_{29} diamino suberoyl insulin
A_1-B_{29} dodecoyl insulin
A_1-B_{29} cystinyl insulin
A_1-B_{29} (butyl oxycarbonyl)$_2$ cystinyl insulin
A_1-B_{29} (Z.lysyl alanyl lysyl) insulin
A_1-B_1 diamino suberoyl insulin
A_1-B_1 (Z. lysyl alanyl lysyl) insulin

The following materials were gifts from:

D. G. Lindsay, University of Sussex, Brighton [2, 3, 16].
A_1 carbamyl insulin
A_1 acetoacetyl insulin
A_1 succinyl insulin
A_1 thiazolidyl insulin
B_1 carbamyl insulin
B_1 acetoacetyl insulin
B_{29} succinyl insulin
A_1, B_1, B_{29} tricarbamyl insulin
A_1-B_{29} succinyl insulin
A_1-B_{29} adipoyl insulin

R. Geiger, Farbwerke Hoechst A.G., Frankfurt [17, 18]:
[D-ala.]^{A1} insulin
B_1-3,5-diiodotyr. insulin

J. C. Sodoyez, University of Liege, Belgium [19]:
[^{127}I] monoiodoinsulin

S. Emdin, Umea School of Medicine, Sweden [20]:
hagfish insulin

The Novo Industri A/S, Copenhagen:
porcine proinsulin
guinea pig insulin

Materials were disolved in hydrochloric acid (1 mmol l^{-1}) and diluted in normal saline (150 mmol l^{-1}) containing human serum albumin (10 g l^{-1}) to concentrations of 1g l^{-1} or 4g l^{-1} and stored frozen.

Isolated Fat Cell Assays

Isolated fat cells were prepared as described by Gliemann [21] from the epididymal fat pads of male Wistar rats weighing between 100 and 120 g and which had been fed *ad libitum*. The medium for cell isolation and incubation was Krebs-Ringer bicarbonate [22] in a gas phase of 95:5 (oxygen:carbon dioxide) or Krebs-Ringer HEPES [N-2-hydroxyethyl piperazine -N^1-2 ethonesulphonic acid, 25 mmol l^{-1}] buffer, both containing albumin (10g l^{-1}). Cell numbers were determined using a haemocytometer and incubations performed in 20 ml polyethylene vials.

Lipogenesis from glucose was determined by the method of Moody et al. [23] after fat cells ($\leq 0.25 \times 10^5$ cells ml^{-1}) had been incubated with shaking at 37° for 90 minutes in 1 ml medium containing D-glucose (0.55 mmol l^{-1}), [3-^{3}H] glucose (0.1 μCi) and insulin (0–872 pmol l^{-1}) or analogue. Results were expressed as glucose (nmol) converted to toluene-extractable lipid in 90 minutes by 10^5 cells.

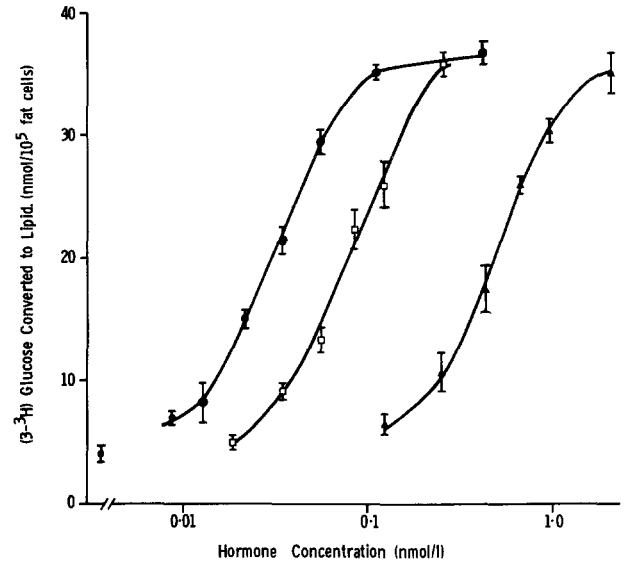

Fig. 1. Rat fat cell lipogenesis from [3-^{3}H] glucose. Results are the mean ± SEM of triplicate determinations of response in the presence of bovine insulin (●), A_1, B_{29} diacetyl insulin (□) and A_1-B_{29} oxalyl insulin (▲)

Fat cell lipolysis was determined by measuring glycerol release following simulation by adrenaline (0.68μmol l^{-1}). Incubations were performed in 2 ml Krebs-Ringer HEPES buffer containing albumin (30 gl^{-1}), fat cells ($\leq 1 \times 10^5$ cells ml^{-1}), glucose (0 or 0.55 mmol l^{-1}) and hormone for 30 minutes at 37° C. Aliquots of the medium were removed and added to perchloracetic acid (1.41 mol l^{-1}). The extract was neutralised using potassium hydroxide and glycerol concentration determined enzymatically [24].

Biological potencies were determined by including bovine insulin (0–872 pmol l^{-1}) and appropriate dilutions of insulin analogues in an assay. Data in the linear portion of the log dose-response curves were analysed using parallel-line bioassay techniques [25, 26]. The potency of an analogue relative to insulin was derived from the horizontal distance between fitted linear regression lines of response on log-dose for the materials. The results of analysis of variance and estimates of assay precision indicated assay validity.

The combined action of insulin and an analogue was investigated by comparing in one assay the abilities of insulin, an analogue and mixtures of the two materials to stimulate fat cell lipogenesis. Five concentrations of insulin and four concentrations of analogue were included as reference standards. Mixtures of insulin and analogue standard solutions were prepared in varying proportions and in initial experiments two dilutions of each mixture included in an assay. Subsequently one dilution of a mixture was assayed to enable investigation of a wider range of molar ratios of insulin: analogue. Fat cell responses were analysed using a likelihood ratio test [27] designed to detect any deviation from simple addition of the biological effects of two materials which have similar actions. The likelihood ratio test statistic was referred to the F-distribution for significance. The results were also plotted as isobols [28] i. e. plots of equi-effective doses of materials, to indicate visually whether any synergistic effect could be classified as antagonistic or potentiating.

Table 1. Biological potencies of analogues relative to insulin

Analogue	Potency (percent) relative to insulin (mean)	Range of estimates in 'n' assays	No. of assays 'n'
a) *Substitutions*			
A_1 acetyl insulin	40	35– 46	4
A_1 carbamyl	34	32– 35	2
A_1 acetoacetyl	27	24– 31	3
A_1 succinyl	17	13– 19	4
A_1 thiazolidyl	18	17– 20	4
B_1 carbamyl	116	114–118	2
B_1 acetoacetyl	86	85– 87	2
B_{29} acetyl	87		1
B_{29} succinyl	80	73– 84	3
A_1, B_{29} diacetyl	29	23– 38	13
A_1, B_1 B_{29} tricarbamyl	10	10– 10	2
b) *Replacements*			
A_1-D-ala	66	61– 72	2
B_1-3,5-diiodotyr	109	107–114	3

Results are the mean and range of estimates of potency obtained in 'n' assays

Table 2. Biological potencies of analogues relative to insulin

Analogue	Potency (percent) relative to insulin (mean)	Range of estimates in 'n' assays	No. of assays 'n'
a) *Cross-linked insulins*			
A_1-B_{29} oxalyl insulin	5.1	4.4– 6.6	3
A_1-B_{29} succinyl	8.1	7.6– 8.7	2
A_1-B_{29} adipoyl	4.2	3.8– 4.6	3
A_1-B_{29} suberoyl	5.4	4.4– 7.2	12
A_1-B_{29} desphe.^{B1}suberoyl	6.3	6.3– 6.3	2
A_1-B_{29} diaminosuberoyl	6.1	5.2– 7.4	3
A_1-B_{29} dodecoyl	6.8	5.5– 9.4	11
A_1-B_{29} cystinyl	3.1	3.0– 3.2	2
A_1-B_{29} (B.o.c.)$_2$ cystinyl	2.2	1.7– 2.6	2
A_1-B_{29}Z. (L.A.L.)	3.7	3.3– 4.1	2
A_1-B_1 diamino suberoyl	2.3	2.2– 2.4	2
A_1-B_1 Z. (L.A.L.)	0.4	0.3– 8.5	2
b) *Tracer preparations*			
Monoiodo ^{127}insulin	89.2	81.0–103.1	3
B_1-3,5-diiodo^{127}tyr. insulin	109.0	107.0–114.5	3
c) *Naturally-occurring*			
Porcine proinsulin	3.4	2.2– 4.4	10
Guinea pig insulin	1.2	1.1– 1.3	2
Hagfish insulin	3.3		1

Results are the mean and range of estimates of potency obtained in 'n' assays. B.o.c. = butyloxycarbonyl. Z.(L.A.L.) = Z. lysyl alanyl lysyl

Results

Figure 1 illustrates the parallel log dose-response curves for the effects of insulin and two semi-synthetic derivatives on fat cell lipogenesis from glucose. The analogues studied appeared to retain the intrinsic activity of native insulin, i. e. achieved the same maximum response but varied in their potency relative to insulin as shown in Tables 1 and 2. Analogues substituted at the B1 position resembled native insulin in potency. B_1-carbamyl insulin showed slightly more bioactivity than insulin on isolated fat cells in keeping with full-activity reported *in vivo* [16]. The potency of B_1 acetoacetyl insulin was 85 per cent relative to insulin, comparable to reported values for B_1 succinyl and B_1 acetyl insulins [5]. Replacement of the B_1 residue with diiodotyrosine did not reduce bioactivity as we have reported elsewhere [29]. A_1,

Table 3. Potencies of analogues relative to insulin estimated from their effects on lipogenesis and inhibition of adrenaline-stimulated lipolysis

Analogue	Potency relative to insulin (percent) (mean and 95 percent fiducial limits[a] or range[b] of estimates)	
	Lipogenesis	Anti-lipolysis
Hagfish insulin	3.3 (2.9– 3.8)[a]	3.7 (3.3– 4.1)[a]
A_1, B_{29} diacetyl insulin	28.5 (23.1–37.5)[b]	24.1 (17.4–30.8)[b]
A_1-B_{29} suberoyl insulin	5.4 (4.4– 7.2)[b]	3.6 (3.3– 3.9)[b]
A_1-B_{29} dodecoyl insulin	6.8 (5.5– 9.4)[b]	4.6 (4.1– 5.0)[b]

[a] 95 percent fiducial limits in one assay
[b] Range obtained in two or more assays
Results with hagfish insulin were obtained using the same preparation of fat cells for the lipogenic and anti-lipolytic assays (n=1). The anti-lipolytic effects of A_1, B_{29} modified insulins were estimated in separate assays (n≥2) from their lipogenic potencies (n>2, Tables 1, 2)

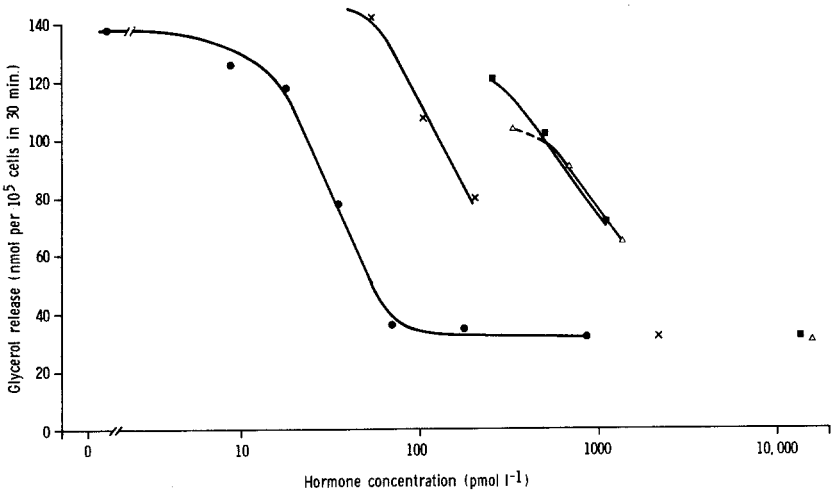

Fig. 2. Inhibition of adrenaline-stimulated glycerol release in isolated fat cells. Values are single determinations of glycerol release (nmol per 10^5 cells in 30 minutes) in the presence of bovine insulin (●); A_1, B_{29} diacetyl insulin (×); A_1-B_{29} dodecoyl insulin (■)

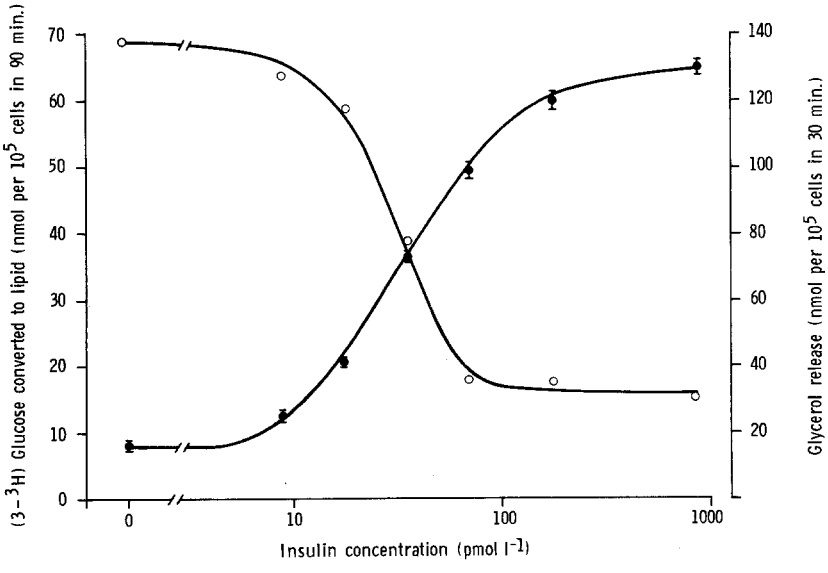

Fig. 3. Comparison of insulin effects on lipogenesis and inhibition of lipolysis. Isolated fat cells were incubated in medium containing albumin (30 g l^{-1}), glucose (0.55 mmol l^{-1}) and insulin (0872 pmol l^{-1}). Lipo genesis from [3-^{3}H] glucose and inhibition of adrenaline-stimulated lipolysis were estimated as described in the text. Results are the mean ± SEM of triplicate determinations of lipogenesis (●) and single determination of glycerol release (○) by aliquots of the same preparation of cells

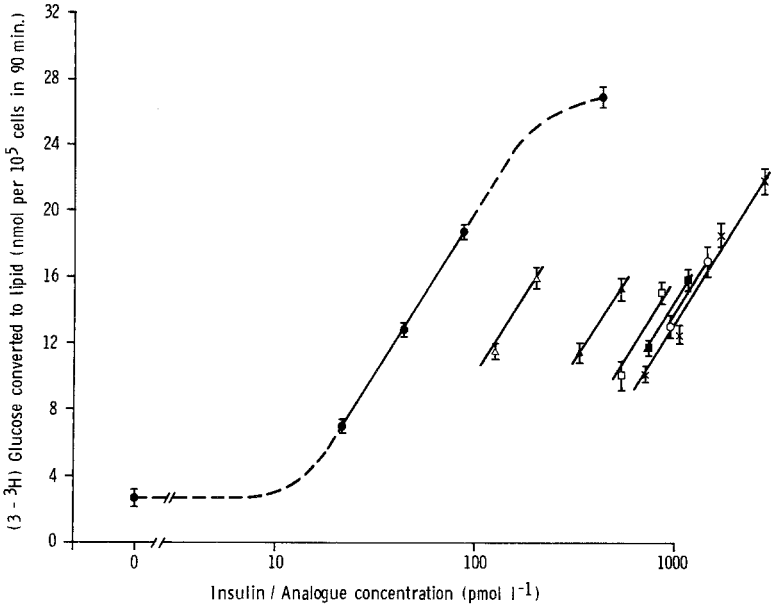

Fig. 4. Isolated fat cellresponses to bovine insulin (●), porcine proinsulin (×) and mixtures of the two hormones in molar ratios of 1:450 (○); 1:117 (■); 1:50 (□); 1:21.4 (▲) and 1:5.6 (△). Values are the mean ± SEM of triplicate determinations of lipogenesis from [3-^{3}H] glucose

B_1, B_{29} tricarbamyl insulin exhibited a markedly reduced potency (10 per cent relative to insulin). This value is similar to that reported for trisuccinyl insulin but lower than for triacetyl insulin [5]. The potencies of the remaining analogues shown in Tables 1 and 2 were consistent with previously reported values [4, 5, 15, 17, 20, 30–34].

Table 3 lists the biological potencies of hagfish insulin and 3 A_1, B_{29} modified insulins estimated from both their lipogenic and anti-lipolytic effects relative to insulin. Similar values were obtained using these two metabolic variables. The analogues appeared to be full agonists i. e. achieved the same maximum antilipolytic effect as insulin as shown in Figure 2. The action of insulin in stimulating lipogenesis and inhibiting lipolysis in aliquots of the same preparation of fat cells is shown in Figure 3. Half-maximal effects on both processes occurred at similar insulin concentrations (30–35 pmol l^{-1}) in two experiments under the conditions described.

Figure 4 illustrates the independent and joint action of insulin and proinsulin on isolated fat cell lipogenesis. Responses produced by two dilutions of insulin/proinsulin mixtures were linear and parallel to the log dose-response curves of the reference preparations of the two hormones. This observation was true for all analogues studied. Analysis of responses by the likelihood ratio test gave the results shown in Table 4 and summarised in Table 5. In seven control assays with insulin, no significant deviation from simple addition of biological effects was observed. In most assays (33–71 per cent) with analogues, however, their combined effect with insulin could not adequately be described by the additive model. A significant result was not observed on every occasion; for example A_1, B_{29} diacetyl insulin appeared to modify insulin action significantly ($p < 0.05$) in four out of seven assays. A visual indication of the type of modifying action of this analogue is shown in Figure 5. This "isobol" plot suggests a potentiating role for diacetyl insulin since the defined response was produced by lower concentrations of hormone when acting in combination with insulin than by either hormone acting alone. This can be contrasted to the pattern observed in control assays with insulin (Figure 5b) which indicates simple addition of biological effects. The isobol patterns (not shown) for the remaining analogues were consistent with a predominantly antagonistic action for A_1-B_{29} suberoyl insulin but did not indicate a consistent effect for A_1-B_{29} dodecoyl insulin or proinsulin.

Discussion

Estimates of the biological activity of hormone analogues have enabled elucidation of areas of the molecule involved in maintaining structure and activity. The results presented here confirm previous observations of the importance of A_1 glycine and relative unimportance of the B_1 and B_{29} groups for the structure and activity of insulin. Substitution of A_1 glycine with groups of increasing size caused a progressive decrease in activity. Formation of an intramolecular cross-link between A_1 and either B_1 or B_{29} reduced potency to very low values. The negligible activity of A_1-B_1 Z (LAL) insulin (0.4 per

Table 4. Analysis of assay responses for evidence of deviation from simple additivity

Insulin/ Analogue	Likelihood ratio test statistic	F-distribution level		Insulin/ Analogue	Test statistic	F-distribution level	
		Degrees of freedom	Significance p			Degrees of freedom	Significance p
Insulin	1.03	8,37	0.432	A_1-B_{29}	3.85	5,48	0.005
	0.45	5,40	0.811	suberoyl	2.02	6,39	0.086
				insulin			
	1.58	8,37	0.164		6.29	6,39	0.001
	0.78	8,37	0.623		1.37	5,40	0.256
	1.64	10,38	0.132		32.30	8,37	<0.001
	0.53	8,37	0.826		2.21	10,32	0.044
	0.91	10,35	0.535		1.33	10,35	0.253
					3.59	10,36	0.002
A_1, B_{29}	2.12	5,48	0.079		2.07	10,41	0.050
Diacetyl	8.69	3,27	<0.001				
Insulin	1.94	4,35	0.126	A_1-B_{29}	2.68	5,48	0.032
	0.37	4,32	0.828	dodecoyl	0.83	9,35	0.594
	3.22	8,25	0.011	insulin	2.41	10,35	0.027
	1.71	5,31	0.161		2.62	10,35	0.017
	4.15	10,41	0.001		3.24	10,32	0.005
	1.45	9,39	0.200		1.33	9,33	0.260
	2.93	9,39	0.010		1.45	10,35	0.200
Porcine	4.93	5,43	0.001	A_1 acetyl	0.38	5,31	0.851
proinsulin	20.60	5,43	<0.001	insulin	2.00	9,39	0.065
	6.29	10,35	<0.001		4.39	10,41	0.001
	0.16	9,35	0.997				
	8.38	10,35	<0.001				
	1.11	9,35	0.383				
	2.93	9,39	0.009				

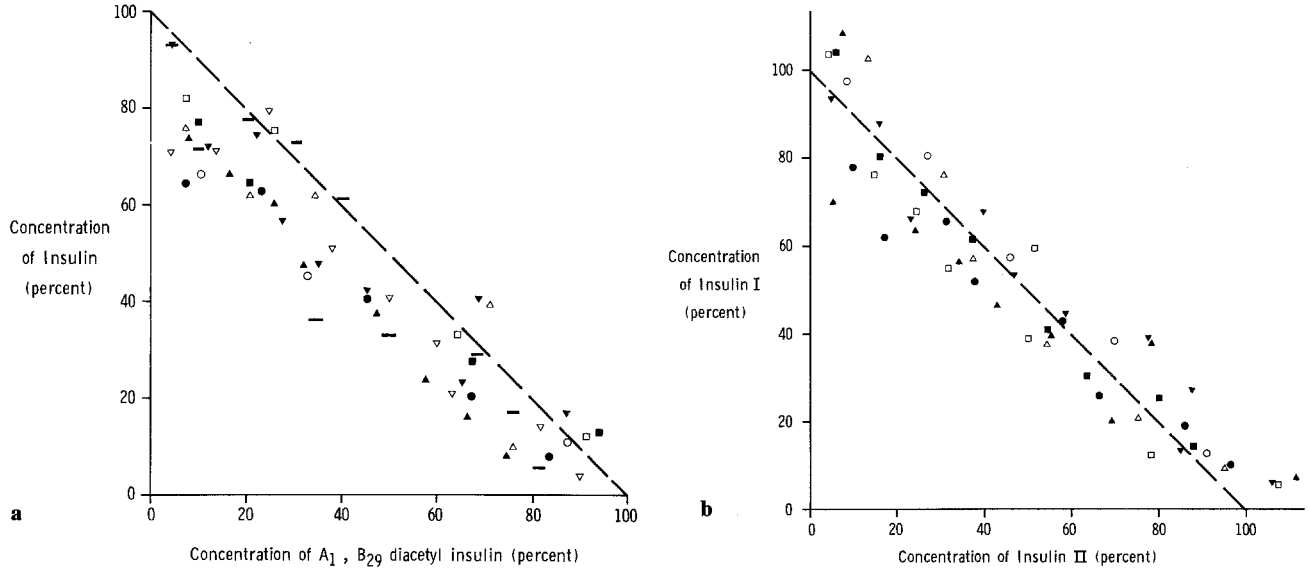

Fig. 5. Combined isobols from: **b** 7 control experiments with insulin. **a** 9 assays with A_1, B_{29} diacetyl insulin. The isobol points were derived as explained in the text. The doses (D_o) of insulin/analogue which alone gave the defined response value (Y=O) in each assay have been normalised to 100 per cent and the mixture doses presented as a percentage of D_o Symbols represent individual assays

cent) indicated the purity of this analogue i. e. any contamination with insulin must be $\leq$ 0.4 per cent.

Potency estimates of modified insulins appear to be relatively independent of the tissue or metabolic effect of insulin used in their estimation. For example, the *in vitro* bioactivity of proinsulin has been reported as 2–4 per cent relative to insulin when assessed from various metabolic effects. These include stimulation of glucose uptake by rat diaphragm [33]; glucose oxidation or lipogenesis in iso-

Table 5. Summary of number of assays with evidence for significant interaction by the likelihood ratio test

Insulin/Analogue	No. of assays	Assays with significant ($p \leq 0.05$) deviation from additivity %
Insulin	7	0
A_1, B_{29} diacetyl insulin	9	44.4%
A_1-B_{29} suberoyl insulin	9	66.6%
A_1-B_{29} dodecoyl insulin	7	55.5%
A_1 acetyl insulin	3	33.3%
Proinsulin	7	71.4%

lated fat cells [31, 33]; amino acid uptake by cultured human fibroblasts [35] and inhibition of rat fat cell lipolysis at low concentrations or stimulation at high concentrations of hormone [36]. The *in vivo* hypoglycaemic activity of proinsulin, when corrected for the slower metabolism of this hormone, was similar in man [37] and the dog [9] to the bioactivity determined *in vitro*. It was observed in this study that the potencies of hagfish insulin and three of the A_1, B_{29} modified insulins were similar whether estimated from stimulatory effects on lipogenesis from glucose or inhibition of adrenaline stimulated lipolysis in isolated rat fat cells. Rudman et al. [38] had reported a difference in potencies relative to insulin of desoctapeptide insulin and desala. ^{B30}, des asn. ^{A21} insulin when determined from their effects on glucose oxidation and inhibition of lipolysis. In their study, however, different species (rat and hamster) were used as tissue source. Furthermore the analogues were reported to be partial agonists whereas later investigations [5, 6] have shown these materials to behave as full agonists of insulin.

The *in vitro* biological activities of many analogues relative to insulin have been shown to agree with the relative binding affinities of the materials determined on isolated fat cells [5] and liver plasma membranes [6]. The altered biological activity of modified insulins therefore seems to reflect a change in their affinity for cell receptors, with the possible exception of hagfish insulin [20]. The observation that the relative bioactivities of some insulin derivatives are comparable whether estimated from effects on lipogenesis or lipolysis, despite the different "effector" mechanisms for these two processes would be consistent with the same receptor being involved in both actions.

Studies on the bioactivity of modified hormones allow not only investigation of structure-function relationships but also prediction of materials potentially useful for therapeutic or research purposes. For example, replacement of B_1 phe. with a radioactive residue should produce a fully active derivative and hence a valid tracer for studies on insulin action and metabolism, as shown for B_1-3,5-diiodotyr. Insulin [29]. Similarly, the observations [9] that the greater activity of A_1, B_{29} modified insulins *in vivo* than *in vitro* could be explained by their reduced susceptibility to degradation might suggest a therapeutic use for the materials, either as longer-acting forms of the hormone or possibly as potentiators of insulin action through an ability to inhibit degradation of the native hormone. It therefore seemed desirable to investigate whether the biological activity of insulin could be potentiated or antagonised by analogues of the hormone. We have presented results suggesting that materials with this ability can be identified in an *in vitro* isolated cell system.

The combined effects of insulin and an analogue on fat cell lipogenesis were analysed by a likelihood ratio test and shown to deviate significantly from simple additivity. By comparison, simple addition of responses was observed in control experiments with insulin. The modifying action of the analogues was not always reproducible, being significant in 33–71 per cent of assays; this lack of reproducibility possibly being due to collagenase effects on the cell membrane. The type of action, i. e. potentiation or antagonism, was indicated by isobol patterns for A_1, B_{29} diacetyl insulin (potentiation) and A_1-B_{29} suberoyl insulin (predominantly an antagonist). No clear effects of proinsulin or A_1-B_{29} dodecoyl insulin were observed by this method. These overall results suggest that the materials studied were not strong synergists. This may explain the discrepancy between these results and those of Gliemann and Gammeltoft [5] who suggested that A_1-B_{29} dodecoyl insulin had purely additive effects with insulin, as did various A_1 modified insulins [8] and hagfish insulin [20]. Their approach (addition of sub-maximally stimulating concentrations of insulin and analogue) would not allow sufficiently rigorous statistical analysis to determine whether the net response was simply additive or consistent with significant potentiation or antagonism. As pointed out by these workers [5, 8], a model in which two drugs act on the same receptor as full agonists, although possibly differing in affinity, would require that the materials have additive effects. This model, however, assumes that no interaction occurs between adjacent receptor sites. The observations presented here suggest that the basic assumption of independent receptor sites may not be applicable to insulin's action on fat cells.

Acknowledgements. We wish to thank Professor D. J. Finney, Professor P. Armitage, Mr. D. I. Dron and R. J. Corkhill for assistance with statistical analysis. M. J. Ellis and S. C. Darby were supported by Medical Research Council and Commonwealth Postgraduate Scholarships respectively. The work was supported by a project grant from the British Diabetic Association.

References

1. Brandenburg, D.: Preparation of $N^{\alpha A1}$, $N^{\varepsilon B29}$ adipoyl insulin. An intramolecular cross-linked derivative of beef insulin. Hoppe Seylers Z. Physiol. Chem. **353**, 869–873 (1972)
2. Lindsay, D. G., Shall, S.: The acetylation of insulin. Biochem. J. **121**, 737–745 (1971)
3. Lindsay, D. G.: Intramolecular cross-linked insulin. FEBS Lett. **21**, 105–108 (1972)
4. Brandenburg, D., Busse, W.-D., Gattner, H-G., Zahn, H., Wollmer, A., Gliemann, J., Puls, W.: Structure-function studies with chemically modified insulins. Proceedings of the Twelfth European Peptide Symposium, p. 270–284. Amsterdam: North Holland Publishing Co. 1973
5. Gliemann, J., Gammeltoft, S.: The biological activity and binding affinity of modified insulins determined on isolated rat fat cells. Diabetologia **10**, 105–113 (1974)
6. Freychet, P., Roth, J., Neville, D. M.: Insulin receptors in the liver: specific binding of ^{125}I insulin to the plasma membrane and its relation to insulin bioactivity. Proc. Natl. Acad. Sci. USA **68**, 1833–1837 (1971)
7. Blundell, T. L., Dodson, C. G., Hodgkin, D. C., Mercola, D.: Insulin: The structure in the crystal and its reflection in chemical and biological properties. Adv. Protein Chem. **26**, 279–402 (1972)
8. Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J., Gammeltoft, S.: Receptor-binding region of insulin. Nature **259**, 369–373 (1976)
9. Jones, R. H., Dron, D. I., Ellis, M. J., Sönksen, P. H., Brandenburg, D.: Biological properties of chemically modified insulins. 1. Biological activity of proinsulin and insulin modified at A_1-glycine and B_{29}-lysine. Diabetologia **12**, 601–608 (1976)
10. Perry, W. F., Bowen, H. P.: Factors affecting the *in vitro* production of non-esterified fatty acid from adipose tissue. Canad. J. Biochem. Physiol. **40**, 749–755 (1962)
11. Jungas, R. L., Ball, E. G.: The effects of insulin and epinephrine on free fatty acids and glycerol production in the presence and absence of glucose. Biochemistry **2**, 383–388 (1963)
12. Fain, J. N., Kovacev, V. P., Scow, R. D.: Anti-lipolytic effect of insulin in isolated fat cells of the rat. Endocrinology **78**, 773–778 (1966)
13. Renner, R., Hepp, K. D.: Evidence for different membrane receptors at the fat cell mediating glucose transport and antilipolysis. Diabetologia **10**, 384 (1975)
14. Koelle, G. B.: The pharmacological basis of therapeutics, 4th ed., pp. 442–465. New York: Macmillan Company 1970
15. Brandenburg, D., Gattner, H.-G., Wollmer, A.: Darstellung und Eigenschaften von Acetylderivaten des Rinderinsulins. I. Hoppe Seylers Z. Physiol. Chem. **353**, 599–617 (1972)
16. Lindsay, D. G., Loge, P., Losert, W., Shall, S.: Carbamyl and methylthiocarbamyl insulins. Biochim. Biophys. Acta **263**, 658–665 (1972)
17. Geiger, R., Geisen, K., Summ, H.-D., Langer, D.: [A 1-D-Alanin] insulin. Hoppe Seylers Z. Physiol. Chem. **356**, 1635–1649 (1975)
18. Teetz, V., Eckert, H. G., Geiger, R.: [B1-3,5-diiodo-L-tyrosine] insulin. Proc. 14th European Peptide Symposium Belgium, 1976. Editions de l'Universite de Bruxelles (in press)
19. Sodeyez, J. C., Sodoyez-Goffaux, F., Goff, M. M., Zimmerman, A. E., Arquilla, E. R.: [^{127}I] or carrier-free [^{125}I] monoiodoinsulin: Preparation, physical, immunological and biological properties and susceptibility to "insulinase" degradation. J. Biol. Chem. **250**, 4268–4277 (1975)
20. Emdin, S. O., Gammeltoft, S., Gliemann, J.: The biological activity on rat fat cells of insulin from the Atlantic hagfish. Diabetologia **11**, 340 (1975)
21. Gliemann, J.: Assay of insulin-like activity by the isolated fat cell method. I. Factors affecting the crystalline response to insulin. Diabetologia **3**, 382–388 (1967)
22. Cohen, P. P.: Monometric techniques, 3rd ed., p. 149. Minneapolis: Burgess Publ. Co. 1957
23. Moody, A. J., Stan, M. A., Stan, M., Gliemann, J.: A simple free fat cell bioassay for insulin-like activity. Horm. Metab. Res. **6**, 12–16 (1975)
24. Eggstein, M., Kreutz, F. H.: Eine neue Bestimmung der Neutralsette in Blutserum und Gewebe. Klin. Wochenschr. **44**, 262–267 (1966)
25. Finney, D. J.: Statistical methods in biological assay. 2nd ed. London: Charles Griffin & Co. Ltd. 1964
26. McArthur, J. W., Ulfelder, H., Finney, D. J.: A flexible computer programme for the composite analysis of symmetrical and asymmetrical biologic assays of parallel-line type. J. Pharmacol. Exp. Ther. **153**, 573–580 (1966)
27. Darby, S. C., Ellis, M. J.: A test for synergism between two drugs. Appl. Statist. **25**, 296–299 (1976)
28. Loewe, S.: Antagonisms and antagonists. Pharmacol. Rev. **9**, 237–242 (1957)
29. Ellis, M. J., Jones, R. H., Thomas, J. H., Geiger, R., Teetz, V., Sönksen, P. H.: B_1-3,5-diiodotyrosine insulin: A valid tracer for insulin. Diabetologia **13**, 257–261 (1977)
30. Henrichs, H. R., Buche, Th., Hofbauer, Kerp, L.: In vitro studies on receptor binding and biological activity of porcine insulin with chemical modifications in position A1. Diabetologia **12**, 397 (1976)
31. Gliemann, J., Sørensen, H. H.: Assay of insulin-like activity by the isolated fat cell method: IV. The biological activity of proinsulin. Diabetologia **6**, 499–504 (1970)
32. Zimmerman, A. E., Kells, D. I. C., Yip, C. C.: Physical and biological properties of guinea-pig insulin. Biochem. Biophys. Res. Commun. **46**, 2127–2133 (1972)
33. Stoll, R. W., Ensinck, J. W., Williams, R. H.: Immunologic and biologic activities of the heterogeneous components of insulin. Diabetes **18**, 392–396 (1969)
34. Weitzel, G. Stratling, W.-M., Hahn, J., Martini, O.: Insulin vom Schleimfisch. Hoppe Seylers Z. Physiol. Chem. **348**, 525–532 (1967)
35. Hollenberg, M. D.: Action of insulin analogues on cultured human fibroblasts reflects biological potency. Life Sci. **18**, 521–528 (1976)
36. Lavis, V. R., Williams, R. H.: Comparison of the lipolytic effects of insulin and proinsulin on isolated fat cells. Diabetes **24**, 238–239 (1975)
37. Sönksen, P. H., Tompkins, C. V. T., Srivastava, M. C., Nabarro, J. D. N.: A comparative study on the metabolism of human insulin and porcine proinsulin in man. Clin. Sci. Mol. Med. **45**, 633–654 (1973)
38. Rudman, D., Garcia, A. L., Del Rio, A., Akgun, S.: Effects on mammalian adipose tissue of fragments of bovine insulin and of certain synthetic peptides. Biochemistry **7**, 1864–1874 (1968)

Received: July 4, 1977,
and in revised form: June 30, 1978

Dr. P. H. Sönksen
Department of Medicine
St. Thomas's Hospital Medical School
London, SE1 7EH
England