Biological Properties of Chemically Modified Insulins.

I. Biological Activity of Proinsulin and Insulin Modified at A₁-glycine and B₂₉-lysine*

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Summary. Beef insulin, pork proinsulin and four derivatives of beef insulin modified at the A₁-B₂₉ site on the molecular surface have been studied. Three derivatives had a synthetic crosslink between the A and B chains. Previous studies with these materials [2, 3 and 5] had demonstrated in vivo bioactivities which were much higher than those displayed in vitro. This paper reports experiments which explain this discrepancy. The analogues were administered at equimolar rates to anaesthetised greyhounds by a priming-dose constant infusion technique and the plasma concentrations achieved were estimated by radioimmunoassay. Proinsulin and the modified insulins were metabolised more slowly than insulin. Biopotency values, which related fall in plasma glucose concentration to the total administered dose of analogue, agreed broadly with published results of conventional in vivo bioassays. On the other hand, calculation of potency in relation to the serum concentration of analogue actually achieved, yielded results which agreed more closely with in vitro assay data. We conclude that for these analogues, reported discrepancies between in vitro and in vivo biopotencies can be largely explained by the different rates at which these materials are metabolised.

Key words: Insulin structure-function, chemically modified insulin, proinsulin, bioactivity of insulin analogues.

With the increasing availability of specifically chemically modified insulins it is now becoming possible to study in detail structure-function relationships of the

molecule. By studying the way in which a change in structure at a known site of the molecule alters its bioactivity, attempts are made to define those characteristics of the structure of insulin which are essential for its activity. Ideally it is hoped to identify an active site on the molecular surface; a site which is involved in binding to the insulin receptor on the target cell.

One group of analogues on which there is already a considerable body of information comprises those with a chemical modification of the N terminal glycine of the insulin A chain and of the ϵ amino group of B₂₉ lysine, two groups which lie in close spatial proximity, (Blundell et al. [1]).

Freychet et al. [2], have examined some of these materials for their capacity to displace radio-iodinated insulin from isolated liver cell membranes and expressed this as a percentage of that of insulin. Gliemann and Gammeltoft [3] and Gliemann and Sørensen [4] have derived similar results by examining the ability of the analogues to displace labelled insulin from isolated fat cells, and also by studying lipogenesis in the same preparation. Brandenburg, Puls et al. [5] examined the biological activity of these materials by following the fall in plasma glucose concentration after bolus injection in the rabbit, and Lazarus et al. [6] studied proinsulin in a similar way in the rat. Thus Freychet and Gliemann have expressed potency in relation to the concentration of the analogue in the medium surrounding the biological preparation while Brandenburg et al. and Lazarus et al. expressed their results in relation to the total dose administered to the intact animal. The former methods have all given results which are significantly lower than the latter for all the materials we have studied and this is also generally true for other modifications at the A₁B₂₉ site in the insulin molecule (Blundell et al. [7]).

A possible explanation for this observation is that modifications at that site impair not only the biological

* Dedicated to Professor H. Zahn on the occasion of his 60th birthday

Fig. 1. Diagrammatic representation of insulin, proinsulin, and the chemically modified materials studied. The crosslinked analogues have an aliphatic link between the free amino groups of A₁ glycine and B₂₉ lysine. A₁, B₂₉ diacetyl insulin has no synthetic crosslink, but the free amino groups are chemically modified in a comparable fashion

effectiveness of insulin but also reduce its susceptibility to degradation. If this were the case administration of these materials to an animal would result in higher concentrations in the plasma and at the receptor site than would be achieved with equimolar doses of insulin.

The experiments reported here allow us to test this hypothesis. The fall in plasma glucose concentration during an *in vivo* experiment can be related to either the dose used or to the serum concentration achieved. We have infused the materials under study at a constant rate, thus achieving a steady state, and have related their hypoglycaemic activity to the concentrations actually present in the plasma. In this way we have simulated the conditions which apply in an *in vitro* assay. We have also related the fall in plasma glucose concentration to the total dose of material administered during the experiment, a calculation which allows comparison with published conventional *in vivo* assay data.

The possibility of an *in vivo* conversion of a relatively inactive analogue to a more active form or to insulin itself also requires consideration, as this would give rise to apparently higher activity in the whole animal than that demonstrable *in vitro*.

For one of the materials we have studied it was possible to select an insulin antiserum which could distinguish between insulin-like immunoreactivity and analogue-like immunoreactivity in the plasma. We have thereby been able to test this suggestion.

Materials and Methods

Bovine insulin, pork proinsulin and four semisynthetic analogues of beef insulin were studied. These were A₁, B₂₉ diacetyl insulin and three crosslinked materials of the form, A₁ Gly-NH-CO-(CH₂)_x-CO-NH-B₂₉ Lys. ie. A₁-B₂₉ oxalyl insulin $x = 0$; A₁-B₂₉ suberoyl insulin $x = 6$; A₁-B₂₉ dodecyl insulin $x = 10$. These materials were prepared in Aachen from crystalline beef insulin (Brunnengraber Lübeck 24 U/mg) as described by Brandenburg [5, 8, 9] and shown to be homogeneous by electrophoresis and other techniques. The pork proinsulin was obtained from Dr. Schlichtkrull, Novo Research Laboratories, Copenhagen.

Experimental Protocol

Experiments consisted of the administration of each material to intact anaesthetised greyhounds by a priming-dose constant infusion technique similar to that previously used in man (Sönksen et al. [11]). The animals were studied after an overnight fast. Anaesthesia was induced by intravenous Pentobarbitone (30 mg/kg body weight) and the animals maintained on intermittent positive pressure ventilation with air to achieve an end-expiratory CO₂ concentration between 4–5% as measured by an infra-red CO₂ analyser.

Blood samples were obtained from a plastic cannula in the external jugular vein. The material under study, dissolved in isotonic saline with 1% human serum albumin was infused into a hind-limb vein, the rate of infusion being accurately controlled by a slow infusion pump. 1 to 1½ L of isotonic saline were also infused during each experiment to maintain an adequate diuresis, and urine was collected via an indwelling urethral catheter. Each experiment lasted 4½ h and was divided into five periods of 30 min and a final period of 90 min. The first period [1] was used for baseline sampling. During the following four periods (II–V) graded infusions of insulin or analogue were administered, the rate being doubled every 30 min. The infusion was stopped at the beginning of the final period (VI). The priming-doses given at the beginning of each infusion period were calculated assuming a distribution space of approximately 10% of body weight. Dilutions of the insulin, insulin analogues, and proinsulin were adjusted to give equimolar infusion rates per kg body weight. Blood samples were taken at 10 min intervals throughout the experiment except during period VI when samples were taken every minute for the first ten minutes, every five minutes for the next twenty minutes, every ten minutes for the next thirty minutes and every fifteen minutes for the final

$1/2$ h. Urine from each period was collected separately and aliquots taken. The experimental protocol is illustrated in Figure 2.

Nine experiments were conducted with beef insulin, six with pork proinsulin, seven with A_1 , B_{29} diacetyl insulin and with A_1 - B_{29} dodecoyl insulin, five with A_1 - B_{29} oxalyl insulin, and three with A_1 - B_{29} suberoyl insulin.

Assay Methods

Plasma glucose was estimated with a Beckman glucose analyser using a glucose oxidase method.

Immuno-reactive serum insulin-like material was assayed by the double antibody method described by Morgan and Lazarow 1963 [10] as modified by Sönksen et al. [11], except that charcoal-treated dog serum was used in place of human serum. Human monocomponent insulin (Novo) was iodinated with ^{125}I by a chloramine T method (Greenwood, Hunter and Glover [12]) and purified by gel filtration on a G50 sephadex column.

23 antisera to porcine or human insulin and one to porcine proinsulin were initially tested to define the cross-reactivity of the analogues with insulin. Two antisera were selected for use in the assays. The first, (GP5) showed no apparent difference in affinity for insulin, proinsulin or the analogues and gave suitable standard dilution curves for the assay of any of these materials. The second (Ab1) showed higher specificity such that inhibition of I^{125} insulin binding was less marked with the cross-linked analogues than with insulin, so that non-parallel dilution curves were obtained. GP5 was used to assay serum immunoreactive material in all the experiments, the concentration being read from a standard curve of the relevant analogue. Each assay also included dilutions of the infusion mixture. Ab1 was used to assay serial dilutions of six serum samples from experiments with A_1 - B_{29} dodecoyl insulin, three from the end of the period of most rapid infusion (V 30) and three collected 60 min after stopping the infusion (VI 60). In these assays standard curves of both A_1 - B_{29} dodecoyl insulin and beef insulin were included.

Fat Cell Experiments

The ability of the analogues to stimulate lipogenesis in a preparation of isolated fat cells was compared to that of insulin. Epididymal fat cell suspensions from 100–120 G male Wistar rats were prepared by the method described by Gliemann [12] and their incorporation of ^3H from ^3H -3-glucose into lipids measured as described by Moody et al. [14].

Fig. 2. Experimental design. (1) Each experiment was divided into six periods I–V of 30 min each, and VI of 90 min. (2) Insulin or analogue was administered by continuous infusion during periods II–V. The infusion rate was doubled at the end of each period. (3) Serum samples were estimated for insulin or analogue by radioimmunoassay. The diagram demonstrates the steady-state concentrations achieved during the infusion periods II to V and the decay after the infusion was stopped. The reduced rate of metabolism of A_1 - B_{29} dodecoyl insulin is clearly demonstrated by the higher concentrations achieved and longer decay time. (4) Plasma glucose was measured throughout the experiments. The reduced hypoglycaemic activity of A_1 - B_{29} dodecoyl insulin can be seen.

Data Handling

Estimates of the potency of proinsulin and analogues in relation to insulin were calculated in two ways. By the first method, hypoglycaemic activity was related to the total dose of material administered to the animal, and by the second, to the plasma concentration actually achieved.

Method 1

The fall from mean baseline plasma glucose concentration to the lowest value reached was taken as the measure of activity in each experiment and this was related to the total dose of material administered to the animal (Fig. 3).

Fig. 3. Representation of dose-related method of bioassay calculation. The total dose of the hormone administered during the course of the experiment was related to the fall in glucose concentration from baseline to the lowest value achieved

Fig. 4. Representation of concentration-related method of bioassay calculation. Plasma steady-state hormone concentration in each period was related to the fall in plasma glucose concentration from baseline to the value at the end of the relevant infusion period (Δ plasma glucose)

Hypoglycaemic activity of the compound =
overall fall in plasma glucose mmol/l

dose of compound administered pmol/kg.

For each analogue the mean value obtained for this index has been expressed as a percentage of that of insulin. The unpaired *t* test was used to test the significance of the difference of each analogue from insulin.

Method 2

For each experiment the plasma glucose concentration values were plotted on a semilog scale against time. A 'best fit' line was drawn through the points by

eye. For each infusion period, the magnitude of the fall in glucose concentration from the mean baseline value, to the value at the end of the infusion was read from the graph, Figure 4. Thus each experiment provided four observations of activity which could be related to the four steady-state insulin or analogue concentrations. In some experiments the lower concentrations of insulin or analogue resulted in no measured fall in plasma glucose concentration, and, as is conventional in bioassays, such data points were excluded from the subsequent analysis.

These results were subjected to a parallel line bioassay analysis as described by Finney [15]. This technique tests the validity of the data as a bioassay by constructing dose-response curves and testing them for linearity, nonparallelism and heterogeneity of variance. If the required conditions are met, the potency of the analogue relative to insulin is calculated using a pooled slope, and from the variance of the data, 95% fiducial limits derived. As an example, the relationship between log dose and response is shown for insulin and A₁, B₂₉ diacetyl insulin in Figure 5.

This method of quantitating activity differs in one respect from more usual bioassay experiments in which each observation is entirely independent of all the others. As has been described above, from each experiment we derived four values for activity, one for each infusion period. These observations are to some extent mutually dependent. For example a steep fall during period II, the first infusion period, will not only give rise to a large value for bioactivity for that period but will also contribute a large value to the overall fall from baseline recorded for the subsequent infusion periods. In this way it could be argued that an inevitable and perhaps spurious linearity might be introduced into the data. In spite of this reservation, this method of approach to *in vivo* bioassay represents a considerable advance on the much cruder experiments previously performed.

Data from the fat cell experiments were processed by the same bioassay procedure (Finney [15]). All the analogues produced highly significant linear regression lines of response on log-dose with no evidence of nonparallelism. Analogue potencies relative to insulin were derived from the horizontal distance between the regression lines fitted using a pooled slope.

Results

The results of assessing activity in relation to administered dose are shown in Table 1. A₁, B₂₉ diacetyl insulin and two of the crosslinked analogues, A₁-B₂₉ suberoyl and A₁-B₂₉ dodecoyl insulins were not significantly different from insulin. A₁-B₂₉ oxalyl insulin

Fig. 5. Log dose-response relationship for insulin ●, and for A₁-B₂₉ diacetyl insulin ○ by the concentration-related method of calculation. Fitted lines for insulin — and A₁-B₂₉ diacetyl insulin ----- as derived by the bioassay procedure described in the text are shown

Table 1. Results of potency estimations in relation to administered dose. Significance of difference from insulin estimated by the unpaired t test. Results of Brandenburg et al. [5] and Lazarus et al. [6] are shown for comparison. N. S. = not significant

Dose related assays

	Potency %	p	Brandenburg et al. Potency %
Insulin	100		100
A ₁ -B ₂₉ Diacetylinsulin	97	N. S.	100
A ₁ -B ₂₉ Oxalyinsulin	29	<.01	52
A ₁ -B ₂₉ Suberoylinsulin	63	N. S.	100
A ₁ -B ₂₉ Dodecoylinsulin	86	N. S.	46
Proinsulin	50	<.01	24 ^a

^a Lazarus et al.

Table 2. Results of potency estimations in relation to analogue concentration. In vivo results are presented as mean potency with 95% fiducial limits, and fat cell results as mean and total range of observations. For the published results of Gliemann et al. [3, 4] and Freychet et al. [2, 24] shown for comparison, ranges where given represent 95% confidence limits, except * = range obtained with three preparations

Concentration related assays

	In vivo potency %	Fat cells, potency %	Gliemann et al. fat cells, potency %	Freychet et al. liver membrane binding affinity %
Insulin	100	100	100	100
A ₁ -B ₂₉ Oxalyinsulin	5.2 (2.7-10.3)	4.5 (2.4-6.7)	3.7 (2.6-4.8)	1.1 (0.8-1.4)
A ₁ -B ₂₉ Suberoylinsulin	6.9 (3.4-14.0)	5.6 (3.9-8.5)	(1.9-7.1)*	(1.2-1.8)
A ₁ -B ₂₉ Dodecoylinsulin	16.1 (10.0-25.8)	7.2 (4.6-7.9)	11.5 (10-13)	2.3 (1.9-2.7)
Proinsulin	5.0 (2.74-9.22)	2.3 (2.1-2.4)	2	3.5-4
A ₁ -B ₂₉ Diacetylinsulin	30.4 (21-44)	30.2 (28-38)	28 (26-32)	25 (20-31)

had an activity of 29% that of insulin and proinsulin 50% that of insulin. For comparison, the reported in-vivo results for the semisynthetic materials (Brandenburg et al. [5]) and for proinsulin (Lazarus et al. [6]) are also shown in Table 1.

The potencies of the analogues as related to plasma concentrations are shown in Table 2. They are compared with previously published data of Gliemann and Gammeltoft [3] and Gliemann and Sorensen [4] derived from an in vitro bioassay with isolated fat cells, and with results obtained in our own laboratory

with a similar preparation. In addition, their relative binding affinities for liver cell membranes are also shown (Freychet [2]).

For A₁-B₂₉ diacetyl insulin and the two cross-linked materials A₁-B₂₉ oxalyl and suberoyl insulins there is close agreement in the assays of biological activity. This is the first in vivo estimation that achieves this agreement and is the first that relates activity to prevailing plasma analogue concentration rather than to the administered dose. With proinsulin and A₁-B₂₉ dodecoyl insulin there is still some discre-

Fig. 6. Radioimmunoassay results with Ab₁ of serial dilutions of serum from 3 dogs undergoing experiments with A₁-B₂₉ dodecyl insulin. Inhibition of ¹²⁵I insulin binding by increasing concentrations of bovine insulin -----, and of standard A₁-B₂₉ dodecyl insulin — and of serial dilutions of the following sera: Dog 1 at the end of infusion period V ○; Dog 2 at the end of infusion period V △; Dog 3 at the end of infusion period V □; Dog 1 60 min after stopping infusion ●; Dog 2 60 min after stopping infusion ▲; Dog 3 60 min after stopping infusion ■. The immunoreactive material in the sera is shown to resemble A₁-B₂₉ dodecyl insulin and not bovine insulin

pancy in that the potencies in our *in vivo* experiments are slightly higher than those achieved with the fat cells.

Results of experiments using the discriminating antiserum Ab₁ to assay immunoreactive material from dogs infused with A₁-B₂₉ dodecyl insulin are shown in Figure 6.

Each sample in serial dilution is plotted on the graph in the appropriate position as indicated by assay with the non-discriminating antiserum GP5. It is clear that both the samples taken at the end of the infusion of the analogue, and 1 h after the infusion had ceased, behave immunoreactively in a way identical to standards of the original A₁-B₂₉ dodecyl insulin, and not like standards of insulin. Therefore the suggestion that this analogue may be converted *in vivo* seems unlikely.

Discussion

There are a number of possible explanations for the observation that some insulin analogues are apparently more active *in vivo* than *in vitro*.

Lower rates of metabolism of these materials will result in a prolonged survival time and higher concentrations in plasma. In addition there is likely to be an effect on tissue sensitivity to a given plasma concentration. This is particularly true for tissues such as the liver which are not only responsive to insulin but also known to have a high capacity to degrade the hormone (Ansorge [16]).

In such tissues the concentration of the hormone at the receptor site will be determined not only by the concentration in plasma, but also by the rate at which the tissue is itself degrading it. For example, if insulin is very rapidly irreversibly removed by the degradative mechanisms, they will lower the hormone's con-

centration at the cell surface to significantly below that in the plasma. On the other hand, with any given plasma concentration, an analogue that is more slowly metabolised will be present at a relatively higher concentration at the tissue receptors. This will remain true even if binding to the receptor sites is an essential prerequisite of degradation, as recently suggested by Terris and Steiner [17, 18].

For this effect to occur, an important assumption is that the vascular endothelium is sufficiently impermeable to insulin for an appreciable gradient to exist between the plasma and the interstitial fluid. Expressed alternatively, the apparent distribution space of insulin *in vivo* must be less than the extracellular volume. This assumption is supported by the observations of Sönksen et al. [11] who found both insulin and proinsulin to be distributed in a volume greater than that of the plasma, but smaller than the extracellular space.

Our second method of calculating bioactivity allows for the effect of metabolism on the plasma concentration, but does not correct for this latter effect on concentration at the cell surface. We have already shown in glucose turnover studies with these analogues that when compared to insulin, the crosslinked materials, especially proinsulin and A₁-B₂₉ dodecyl insulin, have a proportionately greater effect in reducing glucose appearance from the liver than in stimulating peripheral glucose uptake in muscle and adipose tissue (C. V. Tompkins et al. [19]). Freychet's results [2], see Table 2, show that the binding affinity of these analogues for liver membranes is, if anything, less avid than would be expected from their bioactivity on the fat cell. Reduced local degradation in the liver, and proportionally higher concentrations at liver receptors *in vivo* would therefore satisfy Freychet's observations and our own on glucose turnover. It would also explain our observation

that A₁-B₂₉ dodecyl insulin and proinsulin have higher activities in our *in vivo* experiments than in isolated fat cells even after allowing for differences in plasma concentration.

The alternative suggestion that conversion of these materials to insulin takes place *in vivo* is effectively ruled out, at least for A₁-B₂₉ dodecyl insulin, by the results presented in Figure 6.

With A₁-B₂₉ oxalyl, A₁-B₂₉ suberoyl, and A₁, B₂₉ diacetyl insulins the activities *in vivo* by the concentration related method of calculation compare very well with those of Gliemann [3, 4] and Freychet [2] and with our own data with isolated fat cells. We suggest therefore that quantitative differences in the metabolism of these materials compared to insulin explain adequately the discrepancies found in previous estimates of potency.

Clearly an experimental protocol such as ours, with a stepped infusion, does not allow a direct comparison with other *in vivo* data using a bolus technique, and perhaps this is reflected in the differences we find from Brandenburg, Puls et al. [5] (Table 1) who were also using a different species of animal.

It is of interest that the three semisynthetic crosslinked derivatives studied have markedly different biological activities. It is known that both A₁ glycine and B₂₉ lysine lie on the surface of the molecule and it has been suggested that this part of its structure is intimately concerned with binding to the receptor site (Blundell et al. [7]). Alternatively, Brandenburg et al. [5] have shown by circular dichroism studies that the addition of the A₁-B₂₉ crosslink does cause small conformational changes in the molecular structure in solution and it is possible that the reduced activity is caused by distortion of a binding site on the molecular surface which does not immediately involve the A₁-B₂₉ residues themselves. For example the Shanghai insulin research group [20] have presented evidence that the B₂₃ and B₂₄ amino acids are essential for activity and Wood et al. [21] and Pullen et al. [22] have also suggested that the area involving these residues may well constitute the active site, with the polar residues A₁, A₄, A₁₉, and A₂₁ at its periphery. They also have evidence that this area of the molecule is somewhat altered in conformation in A₁ modified derivatives (personal communication).

By whatever mechanism the crosslink between A₁ and B₂₉ interferes with activity it is perhaps not surprising that there should be an optimum distance between these two groups, so that crosslinks of different lengths will vary in the degree to which they impair function.

Glieman, Freychet and ourselves find A₁-B₂₉ dodecyl insulin, with a chain length of 12 carbon atoms, has more activity than those with a shorter

aliphatic bridge [2, 3, 4]. Brandenburg finds A₁-B₂₉ suberoyl insulin, with 8 carbon atoms in the crosslink, to be more active *in vivo* than either A₁-B₂₉ dodecyl insulin or the more closely linked A₁-B₂₉ derivatives [5]. The distance between the A₁α and B₂₉ε amino groups in the crystal is 1 nm [1], corresponding to the chain length of the suberoyl group [2]. However, in solution the side-arm of the B₂₉ lysine is probably free to move relative to the rest of the molecule (Dodson, personal communication) and the crystallographic distance between these groups need not accurately indicate the distance between them when insulin is bound to the receptor.

With proinsulin, the position adopted by the C-peptide and the basic amino-acids which attach it to the A₁ glycine and B₃₀ alanine groups has recently been studied by Snell and Smyth [23]. They suggest a conformation in which the C-peptide overlies and interacts with several of the surface structures in insulin which may well be involved in the active site. It is of interest that this natural crosslink between the A and B chains reduces biological activity to about 5% of that of insulin in the greyhound and to 2% in the rat fat cell. These results compare well with previous estimates of the activity of pork proinsulin in man, 5% Sönksen et al. [11] and with fat cells, 2% Gliemann [4] and liver cell membranes, 3.5–4% Freychet [24].

A₁, B₂₉ diacetyl insulin shows a concentration related biological activity intermediate between that of the crosslinked materials, both natural (proinsulin) and semisynthetic, and that of insulin. This too is in support of the findings of Gliemann and Freychet.

Work with specifically chemically modified insulins is beginning to improve our understanding of the relationships between the biological properties of the insulin molecule and its structure. It is clear that there are highly specific structural requirements that have to be met not only for receptor binding and activation, but also if the molecule is to be susceptible to degradation. To understand the behaviour of an insulin analogue in the whole animal it is clearly essential to study both the interactions with the receptor and the characteristics of its metabolism.

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