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The Effect of Iodination of Particular Tyrosine Residues on the Hormonal Activity of Insulin

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Insulin dissolved in aqueous or methanolic buffer was iodinated to give preparations containing an average of between one and five iodine atoms per insulin monomer. The resultant preparations were fragmented in various ways and the ratio of tyrosine to monoiodotyrosine and di-iodotyrosine was determined in each fragment. This has allowed the distribution of iodine between the combined A-chain tyrosine residues and the individual B-chain tyrosine residues to be determined. The hormonal activity of each of these iodinated insulin preparations was measured from their effect on the production of $^{14}CO_2$ from [1-^{14}C]glucose by isolated adipose cells. The results were interpreted as meaning that the iodination of tyrosine residue A19 or B16 leads to the inactivation of insulin. Speculations are made about the nature of an interaction between insulin and a receptor site on the target tissue.

The concept that insulin interacts with a specific receptor site in its target tissue to produce an effect on metabolism is an old one (Stadie *et al.*, 1949). Garratt (1964*a*) and Garratt *et al.* (1966) have produced evidence that the association is reversible and have postulated that the observed insulin effect depends on the number of receptor sites associated with insulin and the time for which the association lasts. Measurement of the formation of this complex is complicated by the fact that insulin appears to associate with surfaces other than a specific receptor site (Newerly & Berson, 1957; Garratt *et al.*, 1966), so that the precise nature of any interaction has never been determined. However, it is possible, at least in theory, to study indirectly which amino acid residues of the insulin molecule are involved in the interaction with the insulin receptor site, by selectively modifying specific residues and measuring the effect of these modifications on the biological activity of insulin. Iodination of tyrosine residues is an attractive general modification to study, because low extents of iodination apparently have no effect on the insulin molecule whereas the complete iodination of all tyrosine residues leads to complete loss of activity (Garratt, 1964*b*; Izzo *et al.*, 1964). Iodination therefore provides a range of possible modifications, some of which will and some of which will not alter the activity of the hormone.

Because it is difficult to produce homogeneous samples of insulin in which only one tyrosine residue is iodinated, the interpretation of results from iodination studies is difficult. It is essential that the degree of iodination of each insulin sample and the distribution of iodine throughout the molecule should be known. Further, in comparing the biological activities of modified samples of insulin it is essential that the molar concentrations of the samples under test should be accurately determined and that the hormonal effect should be measured at a range of insulin concentrations. We know of no study of chemically modified insulins that satisfies all these criteria.

In this paper we present a detailed analysis of various iodinated insulins and of their hormonal activities. We speculate about the correlation between loss of activity and the iodination of particular residues and discuss a possible interaction between insulin and its receptor site in the target tissue.

Materials and Methods

Preparation of iodinated insulins

Insulin (10 times recrystallized) was a generous gift from Novo Industri A.S. (Copenhagen, Denmark). Samples (100mg) were dissolved in 10ml of aqueous or methanolic sodium borate buffer, pH9.0. Sodium borate buffers were made from a stock solution of 1M-boric acid adjusted to pH9.0 by the addition of 10M-NaOH. This stock solution was diluted with 5vol. of water or of A.R. methanol before using. The iodination procedure was slightly modified from that described by Brunfeldt *et al.* (1968). Calculated volumes of a solution of iodine in KI that contained approx. 2.5mg of available iodine/ml were added at a rate of 5ml/h to the insulin solution at room temperature, with constant stirring. The iodinated

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insulin preparations were purified by elution with aqueous borate buffer from a column of Amberlite IRA-400 anion-exchange resin. Samples of eluate containing insulin were combined and the pH was adjusted to 5.5 to precipitate the insulin. The supernatant was removed after centrifugation and the insulin was redissolved at pH9 and dialysed against three changes of water for 3h. As a control experiment two samples of insulin were treated as described above by using methanolic borate buffer without the addition of iodine.

Analysis of iodinated tyrosine residues

Samples of iodinated insulin or insulin fragments prepared as described below were hydrolysed in Teflon vials with methanolic 2M-NaOH, at 110°C for 16h. Under these conditions quantitative yields of tyrosine, monoiodotyrosine and di-iodotyrosine are obtained (Harrison & Garratt, 1970). The relative proportions of each amino acid in the hydrolysate were measured by automatic amino acid analysis as described by Harrison & Garratt (1970).

Cleavage of iodinated insulins

Samples (20mg) of iodinated insulin preparations were dissolved in 1ml of 0.35M-tris adjusted to pH7.5 with HCl. Sodium sulphite (35mg) and sodium tetrathionate (17mg) were added and the solution was left at 37°C for 3h to cleave the molecules into *S*-sulphonated A and B chains (Varandani, 1966). These preparations were acidified with acetic acid. Any precipitate that appeared was assumed to be iodinated B chain (see below) and was removed by centrifugation before applying the residue to a column (2.5cm×50cm) of Sephadex G-75. The *S*-sulphonated chains were separated by elution with 50% (v/v) acetic acid.

Although *S*-sulphonated chains from normal insulin can be completely separated by this procedure (Varandani, 1966) we found in preliminary investigations that the sum of the monoiodotyrosine and di-iodotyrosine found in the *S*-sulphonated A and B chain eluted from the column was consistently less than the monoiodotyrosine and di-iodotyrosine found in the iodinated insulin from which they were derived (Harrison, 1970). We conclude that this loss of iodinated derivatives is caused by the non-quantitative recovery of *S*-sulphonated iodinated B chain. This conclusion is based on the behaviour of pure *S*-sulphonated iodinated A and B chains. Samples of *S*-sulphonated A and B chains were obtained as described above from unmodified insulin, and amino acid analysis after hydrolysis with 6M-HCl for 21h at 110°C showed that these samples were pure: the proportion of each amino acid found was in good agreement with the theoretical value and we found no trace of threonine, proline, phenylalanine, lysine, histidine or arginine in the *S*-sulphonated A chain nor of isoleucine in the *S*-sulphonated B chain. These preparations were iodinated with an excess of iodine, dissolved in 1ml of 8M-urea, and acidified by the addition of 1ml of acetic acid. No precipitate was observed with the iodinated *S*-sulphonated A chain and the clear solution was applied to the Sephadex G-75 column and eluted with 50% acetic acid. It was eluted in a single peak in precisely the same position as un-iodinated *S*-sulphonated A chain. Amino acid analysis after alkaline hydrolysis showed that it contained di-iodotyrosine but no tyrosine or monoiodotyrosine and no phenylalanine. The preparation of iodinated *S*-sulphonated B chain precipitated on acidification. As normal *S*-sulphonated B chain is soluble under these conditions we concluded that iodination leads to decreased solubility and therefore that elution of *S*-sulphonated B chain from iodinated insulins from Sephadex G-75 with 50% acetic acid is non-quantitative.

In subsequent experiments the *S*-sulphonated iodinated insulin preparations were acidified before being applied to the column and only those fractions eluted in the position of the *S*-sulphonated A chain were collected for analysis. Amino acid analysis for tyrosine, monoiodotyrosine and di-iodotyrosine allows phenylalanine to be measured, but no trace was ever detected, and so we conclude that these preparations were not contaminated by insulin B chain.

Samples (20mg) of insulin and iodinated insulins were also hydrolysed with trypsin into a heptapeptide containing tyrosine-B26, the *C*-terminal alanine residue from the B chain and des-octapeptide-insulin containing the remaining tyrosine residues (DHA insulin; Nicol, 1960). Trypsin was purchased from Sigma Chemical Co. Ltd., London S.W.6, U.K., and treated with chloromethyl L-1-tosylamido-2-phenyl ethyl ketone to destroy chymotryptic activity (Kostka & Carpenter, 1964). The insulin samples were dissolved in 20ml of 1mM-$CaCl_2$ and the pH was adjusted to 9.5 with 0.1M-NaOH. Trypsin (1mg) was added and the solution was incubated at 37°C for 3h, during which time the pH of the digest was kept as close as possible to 9.5 by the frequent addition of 0.1M-NaOH. After 3h an additional 0.05mg of trypsin was added and the solution was left at 37°C for a further 10h.

The trypsin-treated samples were freeze-dried, redissolved in 2ml of 50% acetic acid and eluted from a column (2.5cm×50cm) of Sephadex G-50 by using 50% acetic acid as a solvent. Two peaks were obtained from samples of insulin or of iodinated insulins after tryptic cleavage. The amino acid composition of the material in each peak obtained from normal insulin was determined after acid hydrolysis. The first peak, which was eluted at about 140ml, contained no detect-

able threonine, proline or lysine and the other amino acids were present in proportions corresponding to des-octapeptide-insulin. The second peak, which was eluted at about 180ml, contained only threonine, proline, glycine, tyrosine, lysine and phenylalanine, the latter amino acid being present in double the quantity of the others. We therefore concluded that the trypsin had completely hydrolysed the arginine–glycine and the lysine–alanine bond.

In experiments with iodinated insulins only the fractions corresponding to the heptapeptide were analysed further, so that there is no direct evidence that in these samples the arginine–glycine bond was completely hydrolysed. However, the heptapeptide preparations from these samples never contained detectable amounts of alanine, so the lysine–alanine bond must have been quantitatively hydrolysed. It seems safe to conclude that the susceptibility of the arginine–glycine bond to trypsin has not been affected by the iodination of tyrosine and that heptapeptide was quantitatively recovered. Attempts were made to hydrolyse the des-octapeptide-insulin with chymotrypsin to separate the two A-chain tyrosine residues, and so these preparations were not analysed for tyrosine, monoiodotyrosine or di-iodotyrosine. These attempts were unsuccessful, and it has been shown that chymotrypsin does not hydrolyse peptide bonds adjacent to iodinated tyrosine residues (Garratt & Harrison, 1970).

Determination of the concentration of insulin solutions

Samples (6mg) of the iodinated insulin preparations were dissolved in 10ml of 10mM-HCl. The exact molarity was determined on duplicate 0.5ml samples by using the Folin reaction (Lowry *et al.*, 1951). The colour yield was compared with that from a series of accurate dilutions made from a solution of bovine insulin of accurately known concentration (Harrison & Garratt, 1969). The colour yield from the Folin reaction is not affected by the presence of iodinated tyrosine residues. The concentration of each iodinated preparation was adjusted to exactly 10μM and the solution was stored frozen for bioassay.

Bioassay of iodinated insulin preparations

Insulin preparations were bioassayed by using isolated adipose cells prepared by a slight modification of the method described by Gliemann (1965). The cells were incubated for 2h at 37°C in 0.5ml of Krebs–Ringer bicarbonate buffer, pH7.4 (Umbreit *et al.*, 1964), containing 20mg of albumin/ml, 0.1mg of glucose/ml and approx. 20nCi of [1-^{14}C]glucose (2–4mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). Insulin or iodinated insulin was present at known concentrations obtained by diluting the 10μM solutions prepared as described above. The incubation was stopped by the addition of 0.5ml of 20% (w/v) trichloroacetic acid and the $^{14}CO_2$ was trapped in Hyamine hydroxide inserted into a small polythene cup hanging from the rubber stopper of the incubation vessel. The $^{14}CO_2$ was counted for radioactivity with a liquid-scintillation counter and the amount produced was used to compare the activity of the iodinated insulins as described below.

Bioassay experiments were performed in two different ways.

In single-point-comparison experiments the activities of iodinated insulins were directly compared with the activity of bovine insulin at one concentration only. Six to nine replicate measurements of $^{14}CO_2$ production were made in the presence of 30pM-bovine insulin and iodinated insulin. Great care was taken to ensure that the molarity of each iodinated sample was equal to that of the standard bovine insulin. Also, $^{14}CO_2$ production in the presence of 2nM-insulin and in the absence of insulin was measured to show that each preparation of fat-cells showed an adequate insulin response.

In insulin-response-curve experiments the activity of an iodinated insulin sample was compared with the activity of bovine insulin over a range of concentrations. Five replicate measurements of $^{14}CO_2$ production were made in the absence of insulin and four in the presence of 2nM-bovine insulin. In addition duplicate measurements of $^{14}CO_2$ production were made at eight different insulin concentrations between 5 and 200pM and at eight different concentrations of iodinated insulin. The concentrations of iodinated insulin were varied to produce a range of effects comparable with those produced by unmodified bovine insulin.

Analysis of bioassay experiments

The single-point-comparison bioassays were analysed by calculating the mean value of $^{14}CO_2$ production obtained from the replicate samples incubated with each insulin preparation and by using Student's t test to compare standard bovine insulin with each of the treated insulins under study.

The insulin response curves were analysed assuming that insulin concentration is related to insulin effect by the relationship: $n.\log i = K + \log[e/(E-e)]$ where n and K are constants, i is the concentration of insulin, e is the observed effect of that concentration of insulin and E is the maximum observed insulin effect (Garratt *et al.*, 1970). The insulin effect was defined as the difference between the production of $^{14}CO_2$ in the absence and presence of insulin. The maximum observed insulin effect in any experiment was defined as the insulin effect in the presence of 2nM-normal bovine insulin. The least-mean-squares method was used to calculated the regression line

of log$i$ on log$[e/(E-e)]$ and to find the intercept on the log$i$ axis together with its standard error. This intercept is the logarithm of the insulin concentration required to give the half-maximum insulin effect. We have called the concentration derived from this intercept K_e and it is a measure of the insulin activity. Student's t test has been used to test whether K_e for the modified insulins varied from the K_e for normal insulin obtained in the same experiment.

The mean of the duplicate values obtained for the insulin effect observed in the presence of the highest concentration of each iodinated insulin concentration was divided by the maximum insulin effect observed in that experiment. We have called this figure the $E_{max.}$ ratio and it shows whether any modified insulin preparation is capable of producing the maximum insulin effect.

Results and Discussion

Distribution of iodine in iodinated insulins

Four samples of insulin were iodinated in aqueous borate buffer and three samples in methanolic borate buffer. The amount of iodine added was calculated to be enough to substitute between one and four atoms into each insulin molecule.

The ratio of tyrosine to monoiodotyrosine and di-iodotyrosine was measured in each sample of insulin, in the isolated *S*-sulphonated A chains, and in the isolated heptapeptides. These ratios were converted into numbers of residues by assuming that the sum of tyrosine, monoiodotyrosine and di-iodotyrosine residues in the hydrolysates of insulin, the *S*-sulphonated A chains and the heptapeptide were respectively four, two and one residues. When duplicate samples of any peptide were hydrolysed and analysed for tyrosine, monoiodotyrosine and di-iodotyrosine the results from each analysis never varied from the mean value by more than 0.03 residue/peptide.

The tyrosine, monoiodotyrosine and di-iodotyrosine content of the heptapeptide represents the iodination of tyrosine residue B26. The iodine content of tyrosine residue B16 can be calculated from the difference between the iodine content of the whole molecule and the sum of the iodine contents of the A chain and the heptapeptide. It is not possible to separate tyrosine residues A14 and A19.

Table 1 shows the total number of iodine atoms/insulin molecule as obtained by direct analysis of the unfragmented insulin molecule. This is defined as the degree of iodination. Table 1 also shows the mean values obtained as described above for the number of tyrosine, monoiodotyrosine and di-iodotyrosine residues in the A chain, in residue B16 and residue B26.

These results contrast with those published by other workers: de Zoeten & de Bruin (1961) and Massaglia *et al.* (1969) concluded that when insulin is iodinated in aqueous solution most of the iodine is substituted into the A-chain residues and that the reactivities of the two B-chain residues are similar and low. This difference from our results can be accounted for by the different analytical technique used by these earlier workers, who relied on electrophoretic separation at pH 3.2 of *S*-sulphonated A and B chains iodinated with radioactive iodine and the measurement of radioactivity in these chains. At this pH value iodinated *S*-sulphonated B chains have limited solubility even in the presence of urea (Harrison, 1970), so that they might be accidentally lost from a solution applied to electrophoresis paper and so give the impression that the B-chain residues are unreactive to iodine.

Table 1. *Distribution of iodinated tyrosine residues in iodinated insulin preparations*

Samples of insulin were iodinated in aqueous solutions (samples 1–4) or in methanolic solution (samples 5–7). The iodinated insulins were fragmented as described in the text and the proportion of tyrosine, monoiodotyrosine and di-iodotyrosine in each fragment was determined. The fraction of each residue which remained unmodified (Tyr) or which was monoiodinated (ITyr) or di-iodinated (I_2Tyr) was calculated as described in the text.

Sample	Total number of iodine atoms per monomer	Residue A14+A19			Residue B16			Residue B26		
		Tyr	ITyr	I_2Tyr	Tyr	ITyr	I_2Tyr	Tyr	ITyr	I_2Tyr
1	0.98	1.42	0.58	—	0.75	0.12	0.13	0.98	0.02	—
2	2.00	0.91	1.09	—	0.59	—	0.41	0.91	0.09	—
3	2.49	0.70	1.24	0.07	0.49	0.06	0.42	0.81	0.19	—
4	4.20	0.13	1.20	0.67	0.30	0.26	0.43	0.60	0.26	0.14
5	0.93	1.61	0.39	—	0.71	0.31	—	0.77	0.23	—
6	1.74	1.35	0.65	—	0.31	0.69	—	0.60	0.40	—
7	2.80	0.78	1.22	—	0.21	0.56	0.22	0.42	0.58	—

The iodination of tyrosine residue B16 is curious, since very little monoiodotyrosine is found, suggesting that its formation is rate-limiting in the formation of di-iodotyrosine, and in this respect it differs from the reactivity of the other residues.

Bioassay of iodinated insulins

The results of four single-point-comparison experiments are shown in Table 2. In each case $^{14}CO_2$ production was stimulated three- to four-fold by 2nM-insulin and the $^{14}CO_2$ production by the test samples was between these two values. These experiments show that treatment with methanol alone does not cause any loss in biological activity but that only one of the iodinated samples showed no loss of activity. The sample iodinated in methanol with 1.74 atoms/molecule is anomalous, as iodination to both higher and lower values causes loss of activity.

The results obtained from the insulin-response curves are shown in Table 3. The $E_{max.}$ ratio for all samples of iodinated insulin is close to 1, showing that each preparation is able to produce the maximum effect provided that the concentration is sufficiently high. The significance of this is discussed below.

Table 3 also shows that iodinated samples have a higher value of K_e (the concentration of insulin required to produce the half-maximal effect) than the value obtained for normal insulin in the same experiment. The exception to this is the same anomalous sample iodinated in methanol with 1.74 atoms/molecule. The results from the duplicate experiments on the iodinated insulins show that the ratio of the K_e of normal insulin to that of the iodinated sample is relatively consistent between experiments. In view of this we feel justified in using the best-fit value obtained for K_e compared with the value obtained from normal insulin in the same experiment as a measure of the loss of hormonal activity of the modified insulins even though the actual value obtained for K_e is subject to large experimental error.

Comparison of the iodination of specific tyrosine residues with loss of hormonal activity

In attempting to correlate loss of activity with modification of a particular residue or residues two problems arise when dealing with heterogeneous mixtures of iodinated insulins. First, it is not possible to find out whether the iodinated residues are randomly distributed throughout the insulin molecules or whether iodination of one residue leads to the preferential iodination of another in the same molecule. This means that it is not possible to estimate with certainty the proportion of, for example, completely un-iodinated insulin molecules in a preparation even when the average distribution of iodine amongst the different tyrosine residues is

Table 2. *Single-point-comparison bioassay experiments*

Isolated adipocytes were incubated with [1-^{14}C]glucose and 30pM-normal bovine insulin or treated insulins (see the text for details of treatments). Production of $^{14}CO_2$ is expressed in c.p.m. (the numbers of determinations made are shown in parentheses). In each experiment 3nM-normal bovine insulin was found to stimulate $^{14}CO_2$ production above the basal value but below the maximally stimulated value.

	Sample*	$^{14}CO_2$ production ± S.D.	*P*
Expt. 1	Normal insulin	11940 ± 1030 (9)	
	1	10080 ± 1060 (9)	<0.001
	2	8520 ± 690 (8)	<0.001
	5	9820 ± 640 (9)	<0.001
Expt. 2	Normal insulin	4460 ± 395 (9)	
	Methanol-treated insulin	4570 ± 275 (9)	>0.1
	5	3920 ± 320 (9)	<0.01
	6	4540 ± 275 (9)	>0.1
Expt. 3	Normal insulin	12250 ± 945 (6)	
	Methanol-treated insulin	12400 ± 850 (7)	>0.1
	5	8720 ± 515 (6)	<0.001
	6	11920 ± 890 (6)	>0.1
	7	9300 ± 340 (6)	<0.001
Expt. 4	Normal insulin	11100 ± 685 (9)	
	Methanol-treated insulin	11280 ± 565 (9)	>0.1
	7	9680 ± 805 (9)	<0.01

*The sample numbers are the same as in Table 1.

Table 3. *Insulin response curves*

In these experiments $^{14}CO_2$ production from [1-^{14}C]glucose by isolated adipocytes was measured in the presence of a range of concentrations of normal bovine insulin and of different iodinated insulins. The $E_{max.}$ ratio shows the ratio of the maximum stimulation of $^{14}CO_2$ production caused by insulin to the maximum stimulation caused by each sample of iodinated insulin. K_e is the concentration of normal bovine insulin (control) or of iodinated insulin (sample) required to produce half the maximum effect. The % activity of each iodinated sample is obtained by dividing K_e (control) by K_e (sample) and multiplying by 100.

		$10^{11} \times K_e \pm$ S.E.M. (M)			
Sample*	$E_{max.}$ ratio	Control	Sample	P	% activity
1	1.01	1.39±1.26	3.31±1.52	>0.1	42
1	0.92	4.71±1.39	7.89±1.48	<0.02	60
2	1.01	1.90±1.23	14.90±2.51	<0.001	13
2	1.01	3.52±1.34	18.50±1.98	<0.001	19
3	0.95	2.94±1.26	41.10±1.69	<0.001	7
3	1.09	2.19±1.30	31.40±2.39	<0.001	7
4	1.00	3.69±1.24	182.00±2.24	≪0.001	2
5	0.97	2.39±1.34	4.09±1.56	>0.1	59
5	0.94	3.98±1.26	6.46±1.35	>0.1	62
6	0.97	3.67±1.27	2.87±1.15	>0.1	128
6	0.98	1.41±1.34	0.78±1.25	>0.1	181
7	1.00	3.40±1.16	7.97±1.61	>0.1	43
7	1.01	1.69±1.17	3.48±1.35	>0.1	49

*The sample numbers are the same as in Table 1.

established. The second problem is that it is not possible to test whether partial loss of the hormonal activity results from the total loss of activity of some modified molecules or from the partial loss of activity of a larger proportion of molecules. In spite of these problems we feel justified in speculating about possible relationships between loss of activity and the iodination of particular residues, since such speculations could suggest possible fruitful lines for future study. This is especially true now that the position of each residue is known in the insulin crystal (Adams *et al.*, 1970).

Our first step in attempting these speculations was to compare the observed loss of hormonal activity (obtained from K_e; Table 3) with the proportion of molecules in each preparation iodinated in particular residues or groups of residues. In calculating these proportions we have assumed that the two A-chain residues display an identical reactivity towards iodine, although we recognize that de Zoeten & Havinga (1961) have evidence that A19 residues react faster than A14 residues. We have also assumed that the iodination of one residue does not affect the iodination of other residues in the same molecule so that the iodinated residues in any particular position are randomly distributed amongst all the insulin molecules. Thus Table 1 shows that sample 1 contains 1.42 residues of unchanged tyrosine in the A chain. We therefore assume that there are 0.71 tyrosine residues in both the A14 and the A19 position and that the fraction of insulin molecules containing no iodine in either A-chain tyrosine residue is 0.71×0.71 or 0.5. Proportions with other distributions of iodine were calculated in the same way.

Table 4 summarizes these calculations and compares the residual insulin activity with the proportion of molecules that do not have iodine in particular residues. It is important to remember that, as Table 3 shows, there is considerable error in measuring the residual insulin activity in a preparation particularly when the loss of activity is small.

Column 1 of Table 4 shows the proportion of completely un-iodinated insulin in each preparation estimated as described. It is clear that there is too little unmodified insulin to account for the activity of these preparations, especially those that were iodinated in methanol. It follows that at least some of the iodinated molecules must have at least partial activity.

Columns 2–4 show the residual activity that would be expected if there were one critical tyrosine residue whose iodination caused total loss of hormonal activity. The values shown in these columns represent the percentage of molecules containing no iodine in such a hypothetical critical residue. The observed residual activities are lower than these percentages. The loss of activity cannot therefore be equated with the iodination of a single particular residue and

Table 4. *Percentage of each insulin preparation not containing the modification listed*

These values are calculated from the distributions of iodine shown in Table 1 and by making the assumptions described in the text. The percentage of the molecules that are not iodinated in particular residues is shown; thus, where more than one residue is being considered, molecules have been excluded if they are iodinated in one or more of the positions noted. In column 9 molecules have not been excluded when residue B16 was monoiodinated: in all other cases iodination means either monoiodination or di-iodination. As stated in the text we have assumed that residues A14 and A19 contain identical amounts of iodine so that residue A14 can be substituted for A19 in any of the column headings without affecting the percentage values. The observed activities are taken from Table 3.

	Percentage of insulin molecules in which tyrosine is not iodinated in the positions indicated									
Sample no.	1 A11	2 A19	3 B16	4 B26	5 B16 or B26	6 A19 or B26	7 A19 or A14	8 A19 or B16	9 A19 or B16 (di-iodinated)	Observed activity as % of normal insulin
1	37	71	75	98	73	70	50	53	62	51
2	21	45	59	91	54	41	21	27	27	16
3	5	35	49	81	40	28	12	17	19	7
4	1	6	30	60	18	4	4	2	4	2
5	35	80	70	77	54	62	65	56	80	60*
6	8	67	31	60	19	40	44	21	67	155*
7	1	39	21	42	9	16	15	8	30	46*

* It is not possible to be sure that these values differ from 100% (see Table 3), but Table 2 shows that sample 5 was less than 100% and that samples 6 and 7 did not differ significantly from 100% in a more accurate comparison.

so the iodination of at least two different residues must lead separately to loss of activity.

Columns 5–8 in Table 4 show the residual activity that would be expected if there were two critical tyrosine residues, and that iodination of either of them independently led to complete loss of hormonal activity. Thus the results show the percentage of molecules containing no iodine in either of these positions. There is no agreement between the values in columns 5 or 6 with the observed residual activity, but substantial agreement between those in columns 7 and 8 with the residual activity. This could be interpreted as meaning that iodination of either A-chain residue (column 7) causes loss of activity or that iodination of either residue B16 or one of the A-chain residues (column 8) causes loss of activity. The two samples that are most at variance with this interpretation are samples 6 and 7. Sample 6, as was noted previously, shows no apparent loss of activity (see Table 2) and sample 7 has a higher activity than can be easily reconciled with the percentage of molecules shown in columns 7 and 8. For this reason and also because we noted that the relative distribution of monoiodotyrosine and di-iodotyrosine in residue B16 is unusual (see Table 1) we also calculated the residual activity that would be expected if insulin molecules only lose activity when they either contain iodine in one of the A-chain residues or di-iodotyrosine in residue B16. The results of this calculation are shown in column 9. In our view it is possible to reconcile the values in this column with all the observed residual activities. Our preferred interpretation of our results is thus that two different modifications can cause loss of insulin activity. One of these is the di-iodination of residue B16. The other is the iodination of one of the A-chain residues.

We have no evidence to allow us to decide whether A14 or A19 is the critical tyrosine residue in the A chain. However, Massaglia *et al.* (1969) have attributed loss of insulin activity to iodination of residue A19. Also, Blundell *et al.* (1971) point out that residue A19 is tyrosine in all insulin species of known sequence and that it lies amongst an invariant group on the surface of the insulin molecule that could be specifically involved in insulin activity. For these reasons we incline to the view that the critical A-chain tyrosine residue is A19.

Reaction of insulin with its receptor

We assume that insulin associates reversibly with a limited number of specific receptor sites in the target tissue and that the effect on the tissue is related to the proportion of receptor sites that are associated with insulin. We have used this model, together with the evidence presented above that iodination of tyrosine residue A19 or di-iodination of residue B16 causes loss of hormonal activity of insulin, to

speculate about the nature of an interaction between insulin and a receptor site in the target tissue.

A loss of biological activity of insulin could occur in two different ways. The first way requires a decrease in the association constant between iodinated insulin and its receptor site so that a higher concentration is required to reach the same degree of saturation of receptor sites. In the limiting case the modified insulin would be completely unable to associate with its receptor site. In the second possibility the association constant could be unchanged but the complex formed between the modified insulin and the receptor site would produce a decreased effect. In the limiting case the formation of the complex would have no effect at all on the target tissue.

Our results eliminate the second possibility, as the maximum insulin effect could never have been observed in any preparation containing molecules that competed effectively with normal insulin for the receptor site but nevertheless formed a complex that produced no effect. We therefore deduce that any decrease in biological activity is related to a decrease in affinity of the iodinated insulins for the insulin receptor site.

In interpreting the observation that loss of activity can be correlated with the iodination of two different residues we use the hypothesis that more than one molecule of insulin must associate with a receptor site in order to produce an effect (Garratt *et al.*, 1970). This hypothesis is based on the observation that there is a sigmoid relationship between the concentration of insulin and the effect on [^{14}C]glucose metabolism by isolated adipose cells. This relationship can be explained if n molecules of insulin (I) associate co-operatively with a single receptor site (R) and that the concentration of the resulting complex (InR) is directly proportional to the insulin effect. This can be represented schematically:

$$n\mathrm{I} + \mathrm{R} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{IR} + (n-1)\mathrm{I} \underset{k_{-n}}{\overset{k_{+n}}{\rightleftharpoons}} \mathrm{I}n\mathrm{R} \longrightarrow \text{effect}$$

If no insulin effect occurs until the complex InR is formed it follows that a change in any of the rate constants k_{+1} to k_{+n} will result in a change of the hormonal activity measured by the concentration required to produce the half-maximal effect. We suggest that different rate constants are affected by iodinating either tyrosine residue A19 or B16.

Tyrosine residue A19 is exposed on the 'surface' of the insulin monomer in a region composed of non-polar residues. This surface is not involved in any subunit interactions causing the formation of the dimer or hexamer (Adams *et al.*, 1969). We suggest that this hydrophobic surface interacts with a hydrophobic receptor site, which might involve the lipid matrix of a cell membrane. Iodination of tyrosine A19 could cause a local distortion in the insulin conformation or cause a small increase in polarity of that area of its surface, so that hydrophobic bonding between the insulin monomer and its receptor site would be decreased.

Tyrosine residue B16 is located on the 'surface' of the insulin monomer that interacts with another monomer to form the insulin dimer. We are still uncertain whether insulin exists primarily as a monomer or as a dimer at physiological pH, concentration and ionic strength (Capaldi, 1970), but whichever is the case, we suggest that the formation of a dimer is an essential step in insulin action. We suggest that the insulin dimer must be formed on the surface of the receptor site if it is not present in solution. As diiodination of tyrosine B16 is likely to change the dissociation constant of the insulin dimer it would cause loss of insulin activity.

General conclusions

Speculations about the precise nature of any interaction between insulin and its receptor site can be firmly based only when modified insulins are bioassayed over a wide range of concentrations and when these concentrations are precisely determined. The conclusions that we have drawn about the interaction of insulin with its receptor site are necessarily speculative because of the heterogeneous nature of our iodinated preparations, the imprecision of the bioassay system and uncertainty about the physiological state of insulin aggregation. Nevertheless, we believe that our general method for analysing the hormonal activity of iodinated preparations is of value for further studies of modified insulins.

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