Biochem. J. (1969) **115**, 11
Printed in Great Britain

Iodination of Insulin in Aqueous and Organic Solvents

BY A. MASSAGLIA, U. ROSA, G. RIALDI AND C. A. ROSSI
Nuclear Research Centre, SORIN, Saluggia (Vc), C.N.R. Institute for Clinical Physiology (Pisa), Institute of Industrial Chemistry, University of Genoa, and Institute of Biological Chemistry, University of Pisa, Italy

(*Received* 5 *March* 1969)

1. The iodination of insulin was studied under various experimental conditions in aqueous media and in some organic solvents, by measuring separately the uptake of iodine by the four tyrosyl groups and the relative amounts of monoiodotyrosine and di-iodotyrosine that are formed. In aqueous media from pH 1 to pH 9 the iodination occurs predominantly on the tyrosyl groups of the A chain. Some organic solvents increase the iodine uptake of the B-chain tyrosyl groups. Their efficacy in promoting iodination of Tyr-B-16 and Tyr-B-26 is in the order: ethylene glycol and propylene glycol $\simeq$ methanol and ethanol > dioxan > 8M-urea. 2. It is suggested that each of the four tyrosyl groups in insulin has a different environment: Tyr-A-14 is fully exposed to the solvent; Tyr-A-19 is sterically influenced by the environmental structure, possibly by the vicinity of a disulphide interchain bond; Tyr-B-16 is embedded into a non-polar area whose stability is virtually independent of the molecular conformation; Tyr-B-26 is probably in a situation similar to Tyr-B-16 with the difference that its non-polar environment depends on the preservation of the native structure.

Studies of the iodination of insulin (De Zoeten, De Bruin & Everse, 1961*a*; De Zoeten, Havinga & Everse, 1961*b*; Springell, 1962) have shown that the four tyrosyl residues display different reactivities.

Iodine reacts at the two *ortho* positions of the phenolate ion; consequently the reactivity of the tyrosyl residues in insulin will reflect differences in their ionization behaviour or in the environment of their aromatic rings or both.

Rosa, Massaglia, Pennisi, Cozzani & Rossi (1967) have found that iodination of insulin has a marked effect on the reactivity of the interchain disulphide bonds. Specifically, iodination results in the formation of an iodoinsulin species in which one of the interchain disulphide bonds does not react with sulphite, thiols and GSH–insulin transhydrogenase (Rosa, Rossi & Donato, 1968).

This suggests that, through the structural environment of the tyrosyl groups, iodination might induce disturbing effects extending to the whole molecule or to a part of it. To shed some light on the mutual interactions existing in insulin between the tyrosyl residues and their environment, the iodination has now been studied under different reaction conditions in water and in organic solvents.

EXPERIMENTAL

Crystalline pig insulin was supplied from Novo Research Institute, Copenhagen, Denmark (lot S23276). Zinc-free insulin was prepared by the procedure of Sluyterman (1955); the material so obtained contained less than 0·04% of Zn; [^{125}I]iodine and [^{131}I]iodine were supplied by CEA–CEN–SORIN. All other chemicals were of reagent grade.

Iodination techniques. (*a*) Constant-current electrolysis. Constant-current electrolysis (Rosa *et al.* 1967) was used for iodination of insulin in aqueous solution at pH 7·5, in alcohol–water mixtures and in 8M-urea. The following procedure was used for iodination of insulin at pH 7·5: insulin (30mg.) was dissolved in 25ml. of a solution 0·1M with respect to NaCl and 0·2–1·25mM with respect to KI (labelled with 1mC of ^{125}I) at pH 7·5; 10ml. of this solution was electrolysed at a constant current of 100μA. At suitable times 1ml. samples were taken from the electrolytic cell, diluted with 1ml. of a carrier insulin solution (2mg./ml.) and exhaustively dialysed against four changes of twice-distilled water at pH 7·5. The amount of the initial iodine bound to the insulin was evaluated by paper radioelectrophoresis of samples of the reaction mixture before dialysis. The insulin concentration in the final solution was measured by a micro-Kjeldahl method and the ID* was calculated and expressed as the number of iodine atoms bound/monomer unit, assuming that insulin has mol.wt. 6000. Essentially the same procedure was followed for iodination in 8M-urea at pH 7·5 and in methanol–water or ethanol–water solutions. Urea was removed by exhaustive dialysis against several changes of twice-distilled water at pH 7·5; iodinated insulin was precipitated from the water–alcohol mixtures by adjusting the pH to 4·5–4·7.

(*b*) Iodination with ICl. ICl (Springell, 1962) was used for iodination of insulin at pH values lower or higher than 7·5 and in ethylene glycol–water or propylene glycol–water

* Abbreviations: ID, degree of iodination; MIT, monoiodotyrosine; DIT, di-iodotyrosine.

mixtures. A standard titrated solution of highly purified ICl (0·02 M) was prepared in 1 M-HCl; enough NaCl to give a final concn. of 2 M was then added. A typical experiment consisted of dissolving 10 mg. of insulin in 1 ml. of 0·1 M-NaOH, adding 1 ml. of buffer (1 M-glycine–1 M-HCl, pH 1–3, or 1 M-KH_2PO_4–1 M-NaOH, pH 6–7, or 1 M-glycine–1 M-NaOH, pH 8–10, as appropriate) followed by 6 ml. of the organic solvent and ICl (0·1–0·2 ml.). [^{125}I]Iodine was mixed with ICl immediately before its addition to the buffered insulin solution. After 180 min. the reaction was stopped by acidification and the iodinated insulin was exhaustively dialysed and then precipitated by adjusting the pH to 4·5–4·7.

Oxidative sulphitolysis of the iodoinsulins. Splitting of disulphide bonds and conversion of insulin into the *S*-sulphonated chains was performed with sulphite and tetrathionate (De Zoeten *et al.* 1961*a*). A solution containing 30 mM-sodium tetrathionate and 75 mM-sodium sulphite in 8 M-urea was prepared and the pH was adjusted to 7·6 with acetic acid; 0·5 ml. of this solution were used for a 2 mg. sample of iodinated insulin. The reaction mixture was incubated at 5° for 10 hr. in a sealed glass vial; 10 μl. portions of the reaction mixture were subjected to paper electrophoresis at pH 3·2 in a 8 M-urea–20% aq. (v/v) acetic acid buffer (Whatman 3MM paper, 16 hr., 4 v/cm.). The spots of the *S*-sulphonated A and B chains were identified by radioautography; the paper strip was cut into 0·5 cm. widths and the radioactivity of each width was measured in a well-type scintillation counter.

Isolation of the tyrosyl groups. The procedure followed to evaluate the distribution of the radioactive iodine among the four tyrosyl groups of the insulin monomer was essentially that described by De Zoeten *et al.* (1961*a*,*b*). *S*-Sulphonated A and B chains were eluted from the electrophoresis strips with 0·02 M-ammonium acetate buffer, pH 4·1, and with 0·02 M-phosphate buffer, pH 8·4, and then freeze-dried. The extraction yields ranged from 40 to 80%. The A chain was split into the fragments A_{1-14} and A_{15-21} by incubation with α-chymotrypsin (pH 8·4, 30° for 30 min.) The B chain was split into the fragments B_{1-22} and B_{23-29} by incubation with trypsin (pH 8·4, 5° for 60 min.). A carrier of unlabelled *S*-sulphonated A or B chain was added to the reaction mixture. The A-chain fragments were separated by electrophoresis in 30% acetic acid, pH 1·7; the spots were identified by radioautography and the paper was cut into 0·5 cm.-wide strips for the radioactivity measurement. The B-chain peptides were separated by electrophoresis in a 0·2 M-phosphate–8 M-urea buffer, pH 6·5.

Determination of monoiodinated and di-iodinated tyrosyl groups. The separation of monoiodinated from di-iodinated tyrosyl groups was carried out on the insulin chains and on the chain fragments isolated as described above. The enzymic digestion of the iodinated insulin was carried out with Pronase, in 0·15 M-tris–HCl buffer, pH 8, for 5 hr. at 37°; the relative Pronase concentration used was 100 μg./mg. of insulin and enough methylmercaptoimidazole was added (to prevent deiodination) to give a final concentration of 0·01 M (Covelli & Wolff, 1967). The enzymic digestion of the chains or of the chain fragments was carried out with porcine pancreatin for 48 hr. at 37°. The chain fragments were eluted from the electrophoresis strips with a phosphate buffer, pH 8·4. Samples (0·1 ml.) of the eluate of each peptide were transferred to 2 ml. sterile glass vials containing 0·2 ml. of the pancreatin solution (4% pancreatin in tris–maleate buffer, pH 8·4), a few drops of xylene and enough methylmercaptoimidazole to give a final concentration of 0·01 M.

The hydrolysates were analysed by descending paper chromatography with butan-1-ol saturated with 2 M-acetic acid (Whatman no. 1 paper, 16 hr.). Spots of MIT and DIT were identified by radioautography, and the paper was cut into 0·5 cm.-wide strips for radioactivity measurements.

Under the conditions given above less than 1% of the radioactivity remained at the origin and 90–95% was accounted for as radioactive amino acids (MIT or DIT); 5–8% of the radioactivity was found as inorganic iodine and as an iodinated organic compound of unknown composition. Chromatography with pyridine–2 M-acetic acid (4:1, v/v) was carried out in some cases to check the absence of iodinated histidine (Wolff & Covelli, 1966).

Ultracentrifugal studies. Sedimentation analyses of insulin in various solvents were performed by centrifugation in a Spinco model E analytical ultracentrifuge, with a schlieren optical system and synthetic-boundary cell.

Centrifugation was carried out for 30 min. at 56 100 rev./min. at 20°. Sedimentation coefficients were corrected for density and viscosity of solvents.

Apparent diffusion constants ($D_{0,w}$) were calculated from sedimentation diagrams as described by Van Holde (1960). Partial specific volume, $\bar{v}$, was evaluated from the amino acid composition.

Bioassay of the iodinated insulins. The biological activity of the various iodinated insulins was measured by the rat epididymal fat-pad assay (Rosa *et al.* 1968).

RESULTS

Distribution of iodine between the A and B chains. Insulin samples containing from 0·2 to 4 iodine atoms/molecule (mol.wt. 6000) were prepared at pH 7·5, then submitted to oxidative sulphitolysis; the iodine content of each chain was measured as described above, and the results were expressed in terms of $R_{B/A} = {}^{125}$I radioactivity of the B chain/^{125}I radioactivity of the A chain. The dependence of $R_{B/A}$ on the ID is illustrated in Fig. 1, showing that at pH 7–8 the iodination of insulin up to a degree of substitution of about 4 iodine atoms/molecule occurs predominantly on the tyrosyl groups of the A chain. At an average ID of 1 iodine atom/molecule, the iodine content of the A chain is about 7·5 times that of the B chain. That iodine is selectively taken up by the tyrosyl groups was confirmed by the absence of iodinated histidine; even at ID 4·2 iodine atoms/molecule the MIT and DIT spots accounted for 94% of the iodine content of the insulin sample, in agreement with the results reported by Covelli & Wolff (1967).

The distribution of the iodine between the A and B chains is strongly modified when iodination is carried out in methanol–water (3:1, v/v) or ethanol–water (3:1, v/v) solution; the progressive iodination of insulin was studied up to ID 3·9 iodine atoms/molecule. This was done in single experiments, by withdrawing samples of the reaction mixture at

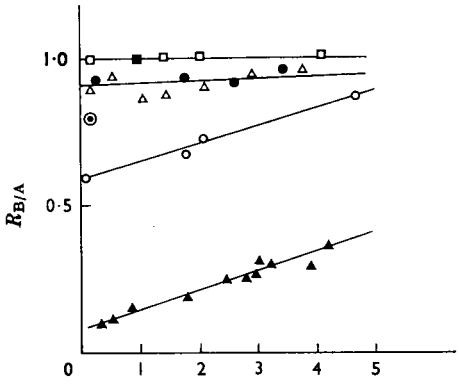

Fig. 1. Effect of various solvents on the distribution of the iodine between the A and B chains of insulin ($R_{B/A}$=^{125}I radioactivity on the B chain/^{125}I radioactivity on the A chain). ▲, In water at pH 7·5; ○, in 8M-urea at pH 7·5; ⊙, in dioxan–water (3:1, v/v); △, in methanol–water (3:1, v/v); ●, in ethanol–water (3:1, v/v); □, in ethylene glycol–water (3:1, v/v); ■, in propylene glycol–water (3:1, v/v).

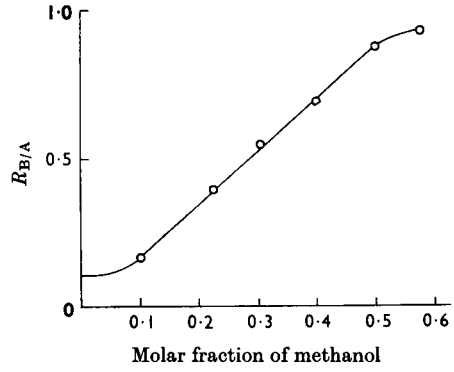

Fig. 2. Effect of increasing concentration of methanol on the distribution of iodine between the A and B chains of insulin. $R_{B/A}$ is defined in Fig. 1.

suitable times. Fig. 1 shows that the B chain carries about 40% of the radioactivity even at the earliest stages of the iodination. The chromatography of the hydrolysate showed that the iodine is selectively taken up by the tyrosyl groups.

During the experiment of progressive iodination in methanol–water (3:1, v/v), a larger sample was withdrawn at the time corresponding to ID 2 iodine atoms/molecule (the actual ID was found to be 1·97). After precipitation and washing the iodinated insulin was divided into two portions; one was submitted to sulphitolysis and gave an $R_{B/A}$ value of 0·93 based on ^{125}I counting. The second was dissolved in 0·9% sodium chloride and 5mM-potassium iodide labelled with ^{131}I, and iodinated again by electrolysis at pH 7·5 in the absence of methanol up to ID 2·17. The iodinated insulin was precipitated and submitted to sulphitolysis and electrophoresis. The ^{131}I radioactivity of each chain was easily separated from that of the ^{125}I by counting in a γ-ray spectrometer. The results revealed that ^{131}I radioactivity was mainly concentrated on the A chain; the $R_{B/A}$ relative to the ^{131}I radioactivity was found to be 0·25, showing that most of the additional 0·20g.atom of iodine bound/mole of insulin in aqueous solution had been taken up by the tyrosyl groups of the A chain. This result suggests that the effect exerted by methanol on the protein is reversible, as far as the reactivity of the tyrosyl groups is concerned.

The effects of 8M-urea, of dioxan–water (3:1, v/v), of ethylene glycol–water (3:1, v/v) and of propylene glycol–water (3:1, v/v) in promoting the iodination of the B chain are illustrated in Fig. 1. Comparison of insulin samples of the same iodine content but iodinated in the different solvents shows that the corresponding $R_{B/A}$ value is greater in 8M-urea than in water; glycols are more effective agents than methanol in promoting iodination of the B chain, since they actually abolish any difference in iodine content existing between the chains ($R_{B/A}$ 1). The effect of dioxan appears to be intermediate between those of urea and methanol. The influence of the methanol concentration on the distribution of the iodine between the chains was studied in separate experiments; to several portions of a solution of insulin (2mg./ml.) and potassium iodide (0·5μmole/ml.) in 0·05M-phosphate buffer, pH 7·4, increasing amounts of methanol were added and iodination by electrolysis was carried out on each sample up to ID about 1 iodine atom/molecule. The dependence of $R_{B/A}$ on the molar fraction (X) of methanol in the reaction mixture is depicted in Fig. 2; for X=0·10, corresponding to 20% (v/v) of methanol, the distribution of the iodine between the chains is the same as that occurring in water. As the methanol concentration increases, a linear relation is observed between $R_{B/A}$ and X, for X values ranging from 0·15 to 0·50, i.e. up to a methanol concentration of 65–70% (v/v).

The dependence of $R_{B/A}$ on the pH at which the iodination is carried out is illustrated in Fig. 3. The distribution of the iodine between the chains remains the same up to pH 8, but the $R_{B/A}$ increases rapidly beyond pH 10. At pH 10·5 the $R_{B/A}$ value is of the same order as that measured in 8M-urea at pH 7·5. These results show that only at extremely alkaline pH values is the B chain iodinated to an appreciable extent. De Vries (1962) showed that the increased iodine content of the B chain beyond pH 9–10 is mainly accounted for by the iodination

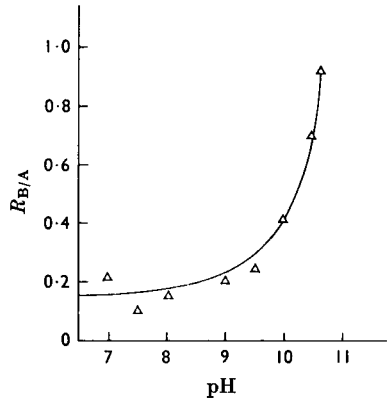

Fig. 3. Effect of the pH at which the iodination is carried out on the distribution of the iodine between the A and B chains of insulin. $R_{B/A}$ is defined in Fig. 1.

Table 1. *Sedimentation coefficients and apparent molecular weight of insulin in various solvents*

$S_{20,w}$ values are referred to a insulin concentration of 1·2 mg./ml., the same as that used in iodination experiments.

Solvent	$S_{20,w}$	$10^7 \times D_{app.}$	Apparent mol.wt.
Aqueous solution*			
pH 7·95	3·18	12·5	23500
pH 9·10	3·12	12·5	23000
pH 10·30	2·03	—	—
8M-Urea, pH 7·5	0·99	—	—
Methanol–water†	1·07	9·31	7700
Ethylene glycol–water†	1·30	1·48	10000

* pH 7·95: 0·1 M-phosphate buffer; pH 9·10 and pH 10·30: 0·1 M-glycine buffer.

† 3 vol. of methanol or ethylene glycol and 1 vol. of 0·1 M-phosphate buffer, pH 7·5.

of Tyr-B-26; we have found that at pH 10·5 Tyr-B-26 contains 84% of the total iodine bound to the B chain. At pH 1·2 and 2·5 (not shown in Fig. 3) the $R_{B/A}$ values are respectively 0·15 and 0·18.

Some iodination experiments at pH 1·2 were carried out at different insulin concentrations (from 0·02 to 2 mg./ml.) and ionic strength (from I 0·05 to I 0·4). No difference was found in the corresponding $R_{B/A}$ values. The same was found when zinc–insulin was iodinated at pH 9 and the corresponding $R_{B/A}$ value was compared with that obtained for zinc-free insulin.

The iodination at pH 7·5 of a fully methylated insulin sample (containing 5·83 methoxyl groups/molecule) was also investigated. The $R_{B/A}$ value at ID 0·8 iodine atom/molecule was found to be 0·30, about twice that measured on insulin at a similar degree of iodination ($R_{B/A}$ 0·16 at ID 0·75). It is noteworthy that the increased iodine content of the B chain in esterified insulin is accounted for mainly by Tyr-B-16, which contains 75% of the total iodine bound to the B chain.

The state of aggregation of insulin in various solvents was evaluated from sedimentation coefficients and apparent diffusion coefficients (Table 1); apparent molecular weights were calculated from the Svedberg equation. It is evident that organic solvent–water mixtures promote dissociation into monomers.

Reactivity of the tyrosyl residues. The distribution of iodine among the tyrosyl groups of the insulin when iodination is carried out in aqueous solution or in methanol–water (3:1, v/v) was measured at increasing ID values. Table 2 shows the results for the B-chain tyrosyl groups. When iodination is carried out in aqueous solution Tyr-B-16 and Tyr-B-26 show about the same reactivity, since they take up similar amounts of iodine. Also, the relative amounts of MIT and DIT found in the hydrolysates of the two fragments of the B chain are rather similar, suggesting that the k_1/k_2 ratio (where k_1 and k_2 are the rate constants for the first and the second iodination reactions respectively) is about the same for Tyr-B-16 and Tyr-B-26. As shown in Fig. 1 the organic solvents increase the reactivity of the B-chain tyrosyl groups in comparison with those of the A chain. The results reported in Table 2 show that in methanol Tyr-B-16 and Tyr-B-26 have similar reactivity and are converted into similar amounts of MIT and DIT, suggesting that the organic solvent affects to the same extent and in the same way the iodination of both residues.

The distribution of iodine among the tyrosyl groups of the A chain is reported in Table 3. In aqueous solution and at the earliest stages of the iodination, the iodine uptake by Tyr-A-19 is higher than that of Tyr-A-14; as the ID increases, the iodine content of Tyr-A-19 becomes similar to that of Tyr-A-14. No evidence was found of the large fall in reactivity of Tyr-A-19 at higher ID values reported by De Zoeten *et al.* (1961*b*). Experiments carried out on insulin samples iodinated by iodine monochloride showed that the behaviour of Tyr-A-19 is independent of the iodination procedure. On the other hand De Zoeten *et al.* (1961*b*) suggested that entrance of the second iodine atom is completely inhibited in Tyr-A-19. Our results show that this is not the case, since DIT was found in the hydrolysate of the A-chain fragment containing Tyr-A-19. However, the entrance of the second iodine atom appears to take place on Tyr-A-19 with more difficulty than on the other residues; this is shown in Fig. 4, where the evolution of the conversion into DIT of each tyrosyl group is represented. In aqueous solution the DIT content of the

Table 2. *Distribution of iodine between the tyrosyl groups of the B chain in insulin iodinated in aqueous solution or in methanol–water*

The results represent the averages of three separate analyses with 90% confidence limit.

Expt. no.	ID (iodine equivalents/ mole of B chain)*	Distribution of iodine (%)		Distribution of total iodine as MIT and DIT between Tyr-B-16 and Tyr-B-26 (%)			
		Tyr-B-16	Tyr-B-26	MIT-16	DIT-16	MIT-26	DIT-26
	In aqueous solution						
1	0·21 (1·45)	48·5	51·5	42·8	5·7	46·6	4·9
2	0·50 (2·44)	50·0	50·0	35·9	14·1	41·6	8·4
3	0·64 (3·25)	51·8	48·2	39·0	12·8	34·4	13·8
4	0·96 (3·91)	54·2	45·8	31·2	23·0	31·2	14·6
	In methanol–water (3:1, v/v)						
5	0·30 (0·63)	48·0	52·0	46·5	1·5	49·9	2·1
6	0·52 (1·10)	47·2	52·8	42·0	5·2	47·0	5·8
7	1·05 (2·20)	48·1	51·9	32·7	15·4	35·2	16·7
8	1·88 (3·82)	47·6	52·4	26·7	20·9	28·0	24·4

* Average values for ID of insulin are given in parentheses.

Table 3. *Distribution of iodine between the tyrosyl groups of the A chain in insulin iodinated in aqueous solution or in methanol–water*

The results represent the averages of three separate analyses with 95% confidence limit.

Expt. no.	ID (iodine equivalents/ mole of A chain)*	Distribution of iodine (%)	
		Tyr-A-14	Tyr-A-19
	In aqueous solution		
1	0·15 (0·17)	41·5	58·5
2	0·44 (0·49)	41·3	58·7
3	1·15 (1·36)	46·4	53·6
4	1·81 (2·24)	51·0	49·0
5	1·94 (2·44)	47·7	52·3
6	2·60 (3·25)	49·4	51·6
	In methanol–water (3:1, v/v)		
7	0·59 (1·10)	38·3	61·7
8	1·15 (2·20)	35·0	65·0
9	1·94 (3·82)	36·0	64·0

* Average values for ID of insulin are given in parentheses.

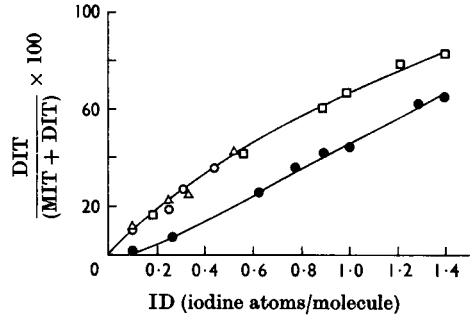

Fig. 4. Iodination of insulin in aqueous solution at pH 7·5: % of DIT found in fragments A_{1-14} (□), A_{15-21} (●), B_{1-22} (△) and B_{23-29} (○) as a function of the iodine content of each fragment. In each fragment the iodine was selectively bound to the corresponding tyrosyl group (tyr-A-14; Tyr-A-19; Tyr-B-16; Tyr-B-26). The results are expressed as the DIT/(MIT + DIT) percentage ratio for each fragment.

fragments corresponding to Tyr-B-16, Tyr-B-26 and Tyr-A-14 is about the same for the same ID, suggesting that the k_1/k_2 ratio has about the same value for the three tyrosyl groups. The DIT content of the Tyr-A-19 fragment is systematically lower, suggesting that entrance of the second iodine atom must be in some way hindered by the topology of the protein near Tyr-A-19. In methanol also the DIT content of the Tyr-A-19 fragment was found to be systematically lower in comparison with the other residues. Fig. 5 clearly shows that methanol does not abolish the anomalous behaviour of Tyr-A-19 as far as the second iodination reaction is concerned. It is noteworthy that in methanol Tyr-B-16, Tyr-B-26 and Tyr-A-14 again show a similar degree of conversion into DIT and therefore a similar k_1/k_2 ratio; since in methanol the three residues show about the same uptake of iodine, they must have similar rate-constant values.

Biological activity of iodinated insulins. The biological activity of insulins containing various amounts of iodine, prepared in aqueous solution or in methanol–water (3:1, v/v), was measured by using the fat-pad assay.

The biological activity of the insulin samples iodinated in methanol does not differ significantly

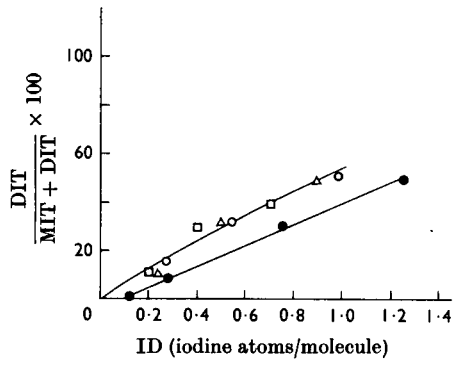

Fig. 5. Iodination of insulin in methanol–water (3:1, v/v): % of DIT found in fragments A_{1-14} (□), A_{15-21} (●), B_{16-22} (△) and B_{23-29} (○) as a function of the iodine content of each fragment. In each fragment the iodine was selectively bound to the corresponding tyrosyl group (Tyr-A-14; Tyr-A-19; Tyr-B-16; Tyr-B-26). The results are expressed as the DIT/(MIT + DIT) percentage ratio for each fragment.

Table 4. *Biological activity of insulin iodinated in aqueous solution or in methanol–water*

Expt. no.	ID (iodine equivalents/ mole of insulin)	Biological activity (% of original)
	In aqueous solution	
1	1·5	92·1±13
2	2·5	55·6±4·3
3	3·2	45·6±3·6
4	4·0	22·0±2·6
	In methanol–water (3:1, v/v)	
5	1·4	94·4±9·1
6	3·1	55·7±5·7
7	4·4	27·8±3·7

from that of the samples iodinated in aqueous solution (Table 4). The bioassays were carried out in duplicate, comparing the iodinated insulin sample with two blanks, one of native insulin directly dissolved in aqueous solution, the other of native insulin dissolved in methanol, then subjected to the same purification procedure used for the iodinated sample.

DISCUSSION

Studies on the iodination of insulin have shown that the four tyrosyl residues display different reactivities toward iodine (De Zoeten *et al.* 1961*a*,*b*; Springell, 1962). In aqueous solution between pH 1 and pH 8 the iodination of insulin up to ID 3–3·5 iodine equivalents/mole (mol.wt. 6000) occurs predominantly on the tyrosyl groups of the A chain (Tyr-A-14 and Tyr-A-19); the two tyrosyl residues in the B chain (Tyr-B-16 and Tyr-B-26) are iodinated at a considerably lower rate.

In the present experiments evidence has been given that, in contrast with the situation in aqueous solution, all four tyrosyl groups show similar reactivity when iodination is carried out in presence of some organic solvents.

It is noteworthy that the solvents used in this study are differentiated in their capacity of altering the conformation of native proteins. It is fairly certain (Singer, 1962) that intrapeptide hydrogen bonds are stronger in dioxan than in methanol, and in methanol than in water solution, whereas the hydrogen-bonding capacity of glycols and of water is very similar. Therefore the net helical content of the molecule is probably not significantly changed on transfer from water to glycol solution. On the other hand the efficacy of these compounds in increasing the reactivity of the B-chain tyrosyl groups towards the iodine is in the order: ethylene glycol and propylene glycol $\simeq$ methanol and ethanol > dioxan. This could suggest that the lack of reactivity of Tyr-B-16 and Tyr-B-26 towards the iodine in aqueous solution is mostly due to a hydrophobic environment. It is noteworthy that Tyr-B-16, Tyr-B-26 and Tyr-A-14 show similar k_1 and k_2 values in methanol and similar k_1/k_2 ratios in water at pH 7·5. This means that the environment of the B-chain tyrosyl groups does not affect the relative amounts of MIT and DIT that are formed; the difference between Tyr-A-14, which in aqueous solution is probably external and freely accessible to the solvent, and the B-chain tyrosyl groups seems to reside solely in a different degree of accessibility to the iodine when the reaction occurs in aqueous media.

It is noteworthy that the effect of methanol on the reactivity of the B-chain tyrosyl groups is largely reversible; when insulin is transferred from methanol to water Tyr-B-16 and Tyr-B-26 are no longer exposed to the iodine, despite the fact that insulin had been previously iodinated in the organic solvent up to 2 iodine equivalents/mole, half of which was bound to the B-chain tyrosyl groups. This indicates that *ortho* positions of the two tyrosine residues are not sterically constrained by the environmental structure, suggesting that the behaviour of Tyr-B-16 and Tyr-B-26 on iodination is mostly due to their location in non-polar areas of the molecule.

In aqueous media between pH 1 and pH 9 the reaction with iodine is almost exclusively confined to Tyr-A-14 and Tyr-A-19. Beyond pH 9–10 there is additional participation of Tyr-B-26; this is reflected by increasing values of the $R_{B/A}$ (Fig. 1). At pH 10·5, Tyr-B-26 becomes fully exposed, and is then as reactive as Tyr-A-14. The reactivity of Tyr-B-16 increases with pH at a much lower rate,

since at pH 10·5 80% of the total iodine bound to the B chain is still accounted for by Tyr-B-26. These results suggest that the environment of the two tyrosyl groups is affected to a different extent by changes in conformation occurring at alkaline pH values. On the other hand Tyr-B-16 is clearly part of the protein core and the sequence Leu-$Tyr_{(16)}$-Leu-Val probably represents a compact area of strong hydrophobic interactions that is difficult to destroy unless organic solvents are used. The abnormally high pK of Tyr-B-16 (De Vries, 1962) reflects the stability of this portion of the molecule. The behaviour of Tyr-B-26, on the other hand, suggests that its non-polar environment depends to some extent on the integrity of the overall conformation.

In 8M-urea at pH 7·5 the uptake of iodine by the B chain is greater than in water at the same pH but lower than in methanol and glycols. Urea has a stronger effect on Tyr-B-26 than on Tyr-B-16; the reactivity of Tyr-B-26 in 8M-urea is not far from that exhibited in methanol.

The action of urea could result from the combination of separate effects; whereas the unfolding of the molecule is likely to be the prevailing effect with Tyr-B-26, the ability of urea to weaken hydrophobic interactions (Bruning & Holtzer, 1961) could be of major importance with Tyr-B-16.

A contribution to the low reactivity of Tyr-B-16 and Tyr-B-26 in aqueous medium probably comes from the hydrogen-bonding of their hydroxyl groups (Laskowski, Leach & Scheraga, 1960), which decreases the availability of the phenolate form required for the iodination reaction. Although the hydrogen bonds postulated to involve the phenolic hydroxyl group are probably not stable enough to exist on their own in aqueous medium, a non-polar area around the tyrosyl group will play a stabilizing role, by providing a medium of low dielectric constant or by decreasing the entropy losses in bond formation, assuring a juxtaposition of the bonded groups. The increased reactivity of Tyr-B-16, found in esterified insulin, could correspond to the loss of the hydrogen-bonding contribution after the methylation of the acceptor group (Laskowski *et al.* 1960), i.e. the carboxyl group of Asp-B-13.

The behaviour of Tyr-A-14 and Tyr-A-19 does not apparently differ in water or methanol; either in water or in methanol Tyr-A-19 shows an abnormally high k_1/k_2 ratio in comparison with the other residues. Although the experimental evidence rules out the hypothesis of De Zoeten *et al.* (1961*b*) that di-iodination does not occur on Tyr-A-19, the entrance of the second iodine atom to the phenolic ring of this residue is hindered to some extent. A possible explanation may include the effect of the proximity of the CyS-A-20–CyS-B-19 disulphide bridge; Herskovits & Laskowski (1962) have suggested that disulphide bonds adjoining tyrosyl and tryptophyl residues are capable of producing a partial blocking of the residue against free access of the solvent. In this connexion, extensive iodination of the A-chain tyrosyl groups causes one of the interchain disulphide bonds to become unreactive towards the sulphite (Rosa *et al.* 1967). Evidence has been given that the effect is associated with the formation of di-iodinated tyrosyl groups on the A chain (Mancini, Rosa & Rossi, 1967) and some results (A. Massaglia, unpublished work) suggest that the disulphide bridge that does not react in iodinated insulin is CyS-A-20–CyS-B-19. This suggests the existence of structural interactions between Tyr-A-19 and the disulphide bond; the presence of a structure highly constrained by the disulphide bridge could make entrance of the second iodine atom difficult. The persistence of the effect in methanol, however, implies that neither electrostatic nor hydrophobic interactions are involved in the stability of the structural situation existing in the vicinity of Tyr-A-19, favouring the hypothesis of a direct steric effect.

When working with organic solvents, it is difficult to discriminate among the various effects they may exert on the protein. Methanol, ethanol and dioxan drive the association equilibria of insulin towards the monomer; their effect on the reactivity of the B-chain tyrosine residues could imply that Tyr-B-16 and Tyr-B-26 are inaccessible to the iodine in the insulin dimer but accessible in the monomer. Although such a possibility cannot be completely excluded, in the sense that iodination of the B chain could be actually facilitated in the monomer, there is a large body of evidence against the hypothesis that the effect of the organic solvents might be solely accounted for by their influence on the state of aggregation of the protein: (*a*) the B-chain tyrosyl groups show the same behaviour in the pH range 1–9 despite the effect of pH on the state of aggregation of the insulin; (*b*) at pH 1–2 the distribution of iodine between the tyrosyl groups is not affected by insulin concentration or ionic strength, which determines the relative amount of monomer present in solution (Jeffrey & Coates, 1966); (*c*) as far as the distribution of iodine between the chains is concerned there is no difference between zinc–insulin and zinc-free insulin at pH 8–9 although the latter dissociates into the monomer at this pH (Marcker, 1960); (*d*) although the monomer is the prevalent form in methanol and in dioxan the latter solvent is less effective than the former in promoting the iodination of the B chain.

It is therefore rather unlikely that the effect of the solvents on the association equilibria could explain the increased reactivity of Tyr-B-16 and Tyr-B-26.

The results of the bioassays carried out on the insulin samples that have been iodinated in methanol are noteworthy. Studies to correlate the extent of iodination with the biological activity have been all performed on insulins iodinated in aqueous media at pH ranging from 7·5 to 8·5–9 (Fraenkel-Conrat & Fraenkel-Conrat, 1950; De Zoeten & Van Strik, 1961; Izzo, Bale, Izzo & Roncone, 1964; Rosa *et al.* 1967). The inactivation occurs beyond an average ID of 0·8–1·5 iodine equivalents/mole (mol.wt. 6000) depending on the bioassay technique, and it is essentially complete at about 4 iodine equivalents/mole. Insulin iodinated in an alcohol–water mixture shows essentially the same behaviour. On the other hand the iodine was mainly located on the A chain in insulin iodinated in aqueous media whereas it was equally distributed between A and B chains in insulin iodinated in alcohol. It may be concluded that the loss of hormonal activity associated to the iodination is virtually independent of the intramolecular location of the substituent or that different inactivating mechanisms are operative in the two cases.

The results discussed in the present paper suggest that, of the four tyrosyl groups in insulin, each has a different environment: Tyr-A-14 is fully exposed to the solvent; Tyr-A-19 is sterically influenced by the environmental structure; Tyr-B-16 is embedded into a non-polar area whose stability is virtually independent of the molecular conformation; and Tyr-B-26 is probably in a situation similar to that of Tyr-B-16 with the difference that its non-polar environment depends on the preservation of the native structure. However, the non-polar areas would seem to be mainly localized on the B chain. This view could be also supported by other facts, namely the high self-association tendency of the isolated B chain in water, in contrast with the monomeric form assumed by the A chain (Leach & Scheraga, 1956–58), the high association constant of the dimer, in which the two monomeric units are probably coupled through the B chains, the high helical content of the B chain, which probably corresponds to the rod-shaped regions of high electron density found in insulin by X-ray crystallography (Joly, 1965); a helical structure probably requires a non-polar area to be stable in water.

Further, the finding that the antigenic sites in insulin are selectively located on the A chain, as shown by the experiments of Dixon & Wardlaw (1960) on hybrid insulins, suggests that in aqueous media a large part of the B chain is not exposed to the solvent.

These considerations could support the idea that the B chain or a part of it may be envisaged as a sort of non-polar moiety of the insulin molecule.

The transition from the dimeric (or polymeric) form to the monomeric form could correspond in insulin to the acquisition of a particular affinity towards the non-polar solvents; this could explain the solubility of insulin in non-aqueous solvents and could perhaps play a role in interaction of the hormone with the lipid matrix of the cellular membrane.

This work was supported by the Euratom contract 04865/1 BiOI and the NATO research grant N.359. We are grateful to Dr Schlichtkrull, of the Novo Research Institute, for the generous gift of highly purified pig insulin.

REFERENCES

Bruning, W. & Holtzer, A. (1961). *J. Amer. chem. Soc.* **83**, 4865.

Covelli, I. & Wolff, J. (1967). *J. biol. Chem.* **242**, 881.

De Vries, J. Th. (1962). Ph.D. Thesis: Leiden University, E. Ijdo.

De Zoeten, L. W., De Bruin, O. A. & Everse, J. (1961*a*). *Rec. Trav. chim. Pays-Bas*, **80**, 907.

De Zoeten, L. W., Havinga, E. & Everse, J. (1961*b*). *Rec. Trav. chim. Pays-Bas*, **80**, 917.

De Zoeten, L. W. & Van Strik, R. (1961). *Rec. Trav. chim. Pays-Bas*, **80**, 927.

Dixon, G. H. & Wardlaw, A. C. (1960). *Nature, Lond.*, **188**, 721.

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

Herskovits, T. T. & Laskowski, M., jun. (1962). *J. biol. Chem.* **237**, 2481.

Izzo, J. L., Bale, W. F., Izzo, M. J. & Roncone, A. (1964). *J. biol. Chem.* **239**, 3743.

Jeffrey, P. D. & Coates, J. H. (1966). *Biochemistry*, **5**, 3820.

Joly, M. (1965). In *Molecular Biology*, vol. 6, p. 279. Ed. by Horecher, B. New York: Academic Press Inc.

Laskowski, M., Leach, S. J. & Scheraga, H. A. (1960). *J. Amer. chem. Soc.* **82**, 571.

Leach, S. J. & Scheraga, H. A. (1956–58). *C. R. Lab. Carlsberg, Ser. chim.*, **30**, 211.

Mancini, P., Rosa, U. & Rossi, C. A. (1967). *Proc. 4° Congr. naz. Soc. ital. Biofisica e Biologia Moleculare; Ric. sci. (Quaderni)*, **47**, 87.

Marcker, K. (1960). *Acta chem. scand.* **14**, 2071.

Rosa, U., Massaglia, A., Pennisi, F., Cozzani, I. & Rossi, C. A. (1967). *Biochem. J.* **103**, 407.

Rosa, U., Rossi, C. A. & Donato, L. (1968). In *Pharmacology of Hormonal Polypeptides and Proteins*, p. 336. Ed. by Paoletti, R. New York: Plenum Press Inc.

Singer, S. J. (1962). *Advanc. Protein Chem.* **17**, 40.

Sluyterman, L. A. F. (1955). *Biochim. biophys. Acta*, **17**, 169.

Springell, P. H. (1962). *Biochim. biophys. Acta*, **63**, 136.

Van Holde, K. E. (1960). *J. phys. Chem.* **64**, 1582.

Wolff, J. & Covelli, I. (1966). *Biochemistry*, **5**, 867.