THE QUANTITATIVE DETERMINATION OF TYROSINE, 3-IODOTYROSINE AND 3,5-DIIODOTYROSINE IN IODINATED INSULIN PREPARATIONS

D.M.HARRISON and C.J.GARRATT
Department of Chemistry, University of York, Heslington, York, U.K.

Received 9 September 1970

1. Introduction

In previous studies of the iodination of tyrosine in insulin the degree of iodination of particular samples has been calculated from the amount of iodine incorporated into the protein. In view of the uncertainty with which it has been hitherto possible to measure the concentration of insulin in solution [1] and the fact that in previous work on insulin no estimates have been made of the amount of uniodinated tyrosine residues interpretations of this early work are suspect. In this paper we describe methods for determining quantitatively the proportions of tyrosine, 3-iodotyrosine (MIT) and 3,5-diiodotyrosine (DIT) in any sample of insulin. Accurate molar extinction coefficients of Tyr, MIT and DIT have been determined and a method for hydrolysis of insulin and chromatographic separation of the amino acids is described. The methods we have used may have a wider applicability in studies of other iodinated proteins.

2. Molar extinction coefficients of tyrosine, MIT and DIT.

Commercially available MIT and DIT usually contain varying amounts of tyrosine or iodinated derivatives other than the major one. Highly purified samples of Tyr, MIT and DIT were obtained by eluting commercially available samples through 2.5 × 50 cm columns of Sephadex G-25 with 1 mM HCl. Halogen substituted phenols are retarded on these columns [2]. Accurate molar extinction coefficients based on nitrogen determination were obtained by the method previously described for insulin [1]. The values obtained which are believed to be the most accurate available are shown in table 1.

3. Separation of tyrosine, MIT and DIT using ion exchange

Amino acids were separated and quantitatively measured with a Technicon Auto Analyser (Technicon Instruments Co. Ltd., Chertsey) using a 130 cm column of Technicon Chromobeads type A at 60°. Citrate buffer pH 5.5 followed after 160 min by citrate buffer pH 8.5 was pumped through the column at 1 ml per min to elute the amino acids. The buffers were prepared as described in the Technicon Manual except that they were saturated with benzyl alcohol by adding 8 ml per litre of buffer. Using this system Tyr, MIT and DIT eluted in sharp peaks after 70, 160 and 250 min, respectively. When analysing amino acids obtained from protein hydrolysis it is important to remove excess ammonia by boiling the alkaline solution of the hydrolysate before applying it to the ion exchange column.

The molar extinction coefficients shown in table 1 were used to make up standard solutions of each amino acid which were applied to the column and the colour yield was determined.

4. Hydrolysis of insulin

Acid hydrolysis destroys MIT and DIT but these amino acids are stable to alkaline hydrolysis [3, 4].

North-Holland Publishing Company – Amsterdam

Table 1
The molar extinction coefficients of tyrosine, MIT and DIT.

	pH 2			pH 13		
	$\epsilon_M \pm$ S.E.M. $\times 10^{-3}$	Mean value	λ_{max}(nm)	$\epsilon_M \pm$ S.E.M. $\times 10^{-3}$	Mean value	λ_{max}(nm)
Tyr	1.39 ± 0.02 1.43 ± 0.02	1.41	274.1	2.42 ± 0.03 2.45 ± 0.03	2.43	292.0
MIT	2.46 ± 0.03 2.45 ± 0.03	2.45	282.2	3.82 ± 0.04 3.85 ± 0.04	3.83	305.5
DIT	2.73 ± 0.04 2.72 ± 0.03	2.72	286.0	5.83 ± 0.06 5.85 ± 0.06	5.84	310.7

Each nitrogen determination from which each extinction coefficient was determined was based on eight colorimetric measurements [1] and the standard errors take into account the errors arising at different stages of the calculation [4].

However the yield of tyrosine from insulin hydrolysed with 5 N NaOH for 16 hr at 110° was found to be low compared with the yield of glycine (table 2). This is presumably due to the slow rate of hydrolysis of peptide bonds between amino acids with bulky non-polar side chains [5]. In case this resistance to hydrolysis was in any way increased by hydrophobic bonding between such side chains we hydrolysed a sample of insulin with 2 N NaOH in 50% (v/v) methanol for 16 hr at 110°. The results (table 2) show that the yield of tyrosine was quantitative within the limits of error of the method. Table 2 also shows for comparison the analysis of a sample hydrolysed with 10 N HCl at 110° for 21 hr.

In another experiment we iodinated insulin with ^{131}ICI [6] which had been standarised by titration against sodium thiosulphate [4] and its specific activity measured by counting a sample in a well type scintillation counter with a sodium iodine crystal. Unreacted ^{131}I was removed by eluting the iodinated insulin

Table 2
Amino acid analysis of insulin hydrolysates

		Hydrolysed with:					
		Aqueous 5 M NaOH		Methanolic 2 M NaOH		HCl 6 M	
	Theoretical	μmoles found	no.*	μmoles found	no.*	μmoles found	no.*
Gly	4	0.121	4.00	0.364	4.00	0.194	4.00
Glu	7	0.165	5.50	0.492	5.40	0.342	7.05
Ala	3	0.096	3.10	0.277	3.10	0.160	3.30
Val	5	0.079	2.62	0.249	2.74	0.128	2.62
Leu	6	0.146	4.99	0.532	5.84	0.293	6.03
Tyr	4	0.093	3.10	0.351	3.86	0.158	3.26
Phe	3	0.074	2.40	0.251	2.75	0.141	2.91

* Number calculated assuming a yield of 4 for glycine.
Amino acid analyses were carried out with a Technicon Automatic amino Acid Analyser, using the standard buffer gradient recommended for a separation of amino acids in 21 hours. Only those amino acids which are not destroyed by alkaline hydrolysis are shown.

from a 2.5 X 30 cm column of Amberlite IRA-400 anion exchange resin with 1 M sodium borate buffer pH 9.0; the insulin was precipitated by adjusting the pH to 5.5 and adding sodium chloride; the precipitate was redissolved in 10 ml water by dropwise addition of 1 mM NaOH and the solution was dialysed against 3 changes of distilled water. The final concentration of insulin was determined by nitrogen analysis [1] and its specific activity determined. From this data it was determined that the insulin sample contained 5.92 atoms of iodine per molecule.

A sample of this insulin was hydrolyzed with 2 N NaOH in 50% methanol and the amino acids were analysed as described above. The sample was found to contain Tyr:MIT:DIT in the ratio 1:1.22:3.06. A molecule containing this ratio of these amino acids. would have to contain 0.81 residues of Tyr, 0.99 residues of MIT and 2.47 residues of DIT in order to contain 5.92 atoms of iodine. This adds up to a total of 4.25 tyrosyl residues which is in close agreement with the theoretical value of 4 for insulin. We therefor conclude that hydrolysis of insulin with 2 N NaOH in 50% methanol gives a quantitative yield of tyrosine.

5. Conclusions

We believe that it is only possible to obtain meaningful information about the iodination of proteins when proper care has been taken to measure accurately the precise degree of iodination of the protein and the distribution of iodine between MIT and DIT. This can only be achieved by amino acid analysis after complete hydrolysis of the protein. Standard methods of hydrolysis are not always adequate and hydrolysis with methanolic alkali has advantages.

The problems associated with measuring the distribution of iodine in particular tyrosine residues will be the subject of a later paper.

Acknowledgments

One of us (D.M.H.) was supported by the British Diabetic Association which we gratefully acknowledge.

References

[1] D.M.Harrison and C.J.Garratt, Biochem. J. 113 (1969) 733.
[2] A.J.W.Brook and S.Housley, J. Chromatog. 41 (1969) 200.
[3] J.W.H.Lugg, Biochem. J. 32 (1938) 775.
[4] D.M.Harrison, Ph.D. thesis (York) (1970).
[5] F.Sanger, Advan. Prot. Chem. 7 (1952) 1.
[6] A.S.McFarlane, Nature 182 (1958) 53.