The Reaction between Proteins and Reducing Sugars in the 'Dry' State. Relative Reactivity of the α - and ϵ -Amino Groups of Insulin

By HELEN M. SCHWARTZ* AND C. H. LEA

*Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge
and Department of Scientific and Industrial Research*

(Received 5 June 1951)

In a study of the reaction between casein and glucose in freeze-dried systems Lea & Hannan (1950) obtained an indication that the few terminal α -amino groups present in casein possess a reactivity towards glucose of the same order as that of the ϵ -amino groups of the lysine residues. Gottschalk & Partridge (1950), on the other hand, found that in the reaction between amino-acids and reducing sugars the reactivity of the amino group increases as its distance from the carboxyl group increases. Since this latter observation leads to the expectation that the ϵ -amino group of lysine will react with aldoses much more rapidly than α -amino groups, it was considered desirable to make a further examination of the reactivity of free amino groups in different amino-acid residues in proteins.

The nature and reactivity of the free amino groups of casein were first re-investigated by the fluorodinitrobenzene (FDNB) method (Sanger, 1945), using paper chromatography (Davis, 1950) and reverse-phase partition chromatography on chlorinated rubber (Partridge & Swain, 1950) for the separation and identification of the 2:4-dinitrophenyl-(DNP-) amino-acids. Terminal α -amino groups of aspartyl, glutamyl, lysyl, phenylalanyl and valyl residues were identified, and these α -groups were found to react at approximately the same rate as the ϵ -amino groups of the lysine side chains. The original observations of Lea & Hannan (1950) were thus confirmed and extended.

The results with casein, however, have not been reported in detail since (a) the casein used had been purified from a commercial source and (b) the very small proportion of α -amino groups present in casein made a sufficiently accurate determination of their relative activities impossible. For the latter reason, attention was turned towards the reaction of glucose with insulin, a protein in which the proportion of free α -amino groups is exceptionally high, and in which the number, nature and position of all the residues carrying free amino groups have now been established with some certainty (Sanger, 1945; Sanger & Tuppy, 1951).

* Research officer, South African Council for Scientific and Industrial Research. Present address: C.S.I.R. Fats and Protein Unit, University of Cape Town, South Africa.

EXPERIMENTAL

Determination of optimum relative humidity for the reaction. Crystalline insulin in approximately 3% solution at pH 7 was mixed with 1.5 equiv. of glucose (amino N basis), and part of the solution retained for determination of total N content. The remainder, after dividing equally between a number of glass tubes, was freeze-dried and kept at 37° over solutions of H_2SO_4 which provided a range of constant relative humidities (R.H.). Samples were removed at suitable intervals for determination of amino N by the Van Slyke method, using a reaction time of 30 min. at 20°.

Preparation and standardization of the DNP derivatives of insulin and of insulin-glucose. Two portions of the insulin-glucose mixture (approx. 150 mg. each) were allowed to react at 37° and 65% R.H. for 4 and for 16 days, and immediately treated with FDNB (Sanger, 1945). DNP-insulin (130 mg.) was prepared similarly as a control. The protein content of the DNP derivative was determined in each case by estimating its glutamic acid content by the specific decarboxylase method (Gale, 1945) after hydrolysis with 5.8N-HCl for 24 hr., the glutamic acid content of insulin being taken as 18.60 g./100 g. dry ash-free protein (Chibnall, 1946). This method requires considerably less material than the more usual one based on the determination of amide N (Sanger, 1945).

Chromatographic separation of the DNP-amino-acids. The three DNP proteins (30–55 mg.) were hydrolysed with 10 ml. 5.8N-HCl for 4 hr. and the hydrolysates extracted with ether. DNP-glycine and DNP-phenylalanine were determined in the ether extracts after separation by partition chromatography on silica gel- $CHCl_3$ columns. The silica gel used failed to give a satisfactory separation of the DNP-phenylalanine band from the artifact band, and the latter was therefore removed before running the chromatogram by the following method due to Isherwood & Cruickshank (1951). The ether-soluble DNP-amino-acid fraction was dissolved in 90% (w/v) H_2SO_4 and extracted with benzene to remove dinitrophenol. The H_2SO_4 solution was then diluted with ice water and extracted with benzene containing 10% (v/v) *tert.*-amyl alcohol. The DNP-amino-acids and any dinitroaniline present passed into the benzene-*tert.*-amyl alcohol solution, from which the DNP-amino-acids were extracted with 1% (w/v) $NaHCO_3$ solution. The DNP-glycine and DNP-phenylalanine were recovered from the $NaHCO_3$ solution by acidification and extraction with ether. They were separated from each other by partition chromatography on a silica gel- $CHCl_3$ column (Sanger, 1945) and estimated spectrophotometrically in 1% $NaHCO_3$ solution.

The HCl-soluble residue from ether extraction of the original hydrolysate was evaporated to dryness in a rotary

film vacuum evaporator (Lea, Hannan & Rhodes, 1951) and refluxed with 10 ml. 5.8*N*-HCl for a further period of 8 hr. ϵ -DNP-lysine was estimated spectrophotometrically in *N*-HCl solution after separation on a silica gel-66% methyl ethyl ketone-ether column (Sanger, 1945).

Recovery experiments. Recovery experiments were carried out, after acid hydrolysis, on a mixture containing DNP-glycine, DNP-phenylalanine, ϵ -DNP-lysine and protein in the proportions present in DNP-insulin. Both casein and a casein-glucose complex were used as proteins, the latter being included to test whether the larger amount of humin formed during the hydrolysis of protein-sugar complexes affects the recovery of DNP-amino-acids.

The values obtained in the presence of protein were very close to those of Sanger (1945), indicating that the extra steps involved in removal of the artifacts do not result in any appreciable loss of the DNP-amino-acids. The recoveries, moreover, were not significantly affected by hydrolysis with the protein-sugar complex in place of protein (Table 1).

Table 1. *Recovery of DNP-glycine, DNP-phenylalanine and ϵ -DNP-lysine after hydrolysis with protein or with a protein-sugar complex*

	Hydrolysis time (hr.)	Recovery (%)	
		With casein	With casein-glucose
DNP-Glycine	4	53.8, 52.6	51.9
DNP-Phenylalanine	4	85.3, 88.7	83.3
ϵ -DNP-Lysine	12	88.4, 88.2	92.4

RESULTS

Influence of activity of water on the amino group-glucose reaction. The effect of storage in equilibrium with atmospheres at 15–85% R.H. on the rate of disappearance of the amino groups of insulin, as determined by the Van Slyke method, is shown in Fig. 1. The proportion of the total amino groups which had reacted with glucose after a given period at 37° was found to increase with increasing humidity to a maximum at about 70% R.H., and then to decrease again at higher humidities. The browning of the reaction mixture which accompanied the loss of amino groups, on the other hand, did not show any evidence of an optimum humidity, but increased progressively with humidity throughout the range covered.

Reaction of insulin with glucose at 65% R.H. Fig. 2 shows the course of the reaction, as followed by the loss of Van Slyke amino N, of insulin with glucose (1.5 equiv.) at pH 7 and 65% R.H. during 32 days at 37°. Approximately one-third and two-thirds of the total amino groups can be seen to have reacted after 4 and 16 days, respectively. At 4 days no browning of the stored material was detectable, but at 16 days the reaction mixture was perceptibly brown and at 32 days more noticeably so.

On the basis of these results larger samples of insulin were allowed to react with glucose under

similar conditions for 4 and 16 days for subsequent determination by the FDNB method of the nature and quantity of the free amino groups remaining unreacted.

Fig. 1. Influence of humidity on the rate of reaction of insulin with glucose. (Insulin reacted with 1.5 equivalents of glucose/amino group for 16 days at 37°.)

Fig. 2. Loss of free amino N in insulin stored with glucose at 65% R.H. and 37°.

Rates of reaction with glucose of the three types of amino group present in insulin. The results of examination by the FDNB method of insulin and of the 4- and 16-day insulin-glucose reaction products are presented in Table 2 and Fig. 3. The numbers of free amino groups of the three types found in insulin are only approximately equal to the theoretical value of 2.0 each, but the figures are quite close to those previously obtained by Sanger (1945).

The reactivity towards glucose of the three types of amino group obviously varies very considerably, those of phenylalanine being most and those of lysine

Table 2. Reaction of the α - and ϵ -amino groups of insulin with glucose at 37° and 65 % R.H.

	Free amino groups/submolecule of mol.wt. 12,000	Amino N (mg./g. total N)	
		Calc. from FDNB data	Found by Van Slyke method
Insulin			
Glycyl α -	2.31	23.3*	
Phenylalanyl α -	1.81	13.6	
Lysyl ϵ -	1.91	14.3	
Total	6.03	51.2	49.4
Insulin-glucose (reacted 4 days)			
Glycyl α -	1.46	14.8*	
Phenylalanyl α -	0.55	4.1	
Lysyl ϵ -	1.52	11.4	
Total	3.53	30.3	30.2
Insulin-glucose (reacted 16 days)			
Glycyl α -	0.77	7.8*	
Phenylalanyl α -	0.36	2.7	
Lysyl ϵ -	0.86	6.5	
Total	1.99	17.0	16.9

* Calculated on the assumption that glycine gives values 135% of theoretical in the Van Slyke reaction in a 30 min. reaction period at 20°.

least reactive. In the 4-day reaction product, for example, the free α -amino groups attached to the terminal phenylalanyl residues had reacted approximately twice as extensively as the α -amino groups attached to the terminal glycyl residues, and about

glucose products as determined by the Van Slyke method and as calculated from the FDNB data on the assumption that glycine gives values equal to 135 % of theoretical in a 30 min. reaction period in the Van Slyke determination (cf. Sanger, 1945).

Fig. 3. Reaction of the amino groups of insulin with glucose at 37° and 65% R.H. $\circ$ — $\circ$, glycyl α -amino groups (terminal); $\triangle$ — $\triangle$, phenylalanyl α -amino groups (terminal); $\square$ — $\square$, lysyl ϵ -amino groups; $\bullet$ — $\bullet$, total amino N (Van Slyke).

3.5 times as extensively as the ϵ -amino groups of the lysyl residues. After 16 days the differences between the various groups were becoming less marked as the phenylalanine reaction slowed down on approaching completion (cf. Fig. 2).

Excellent agreement was obtained between the values for the total amino N content of the insulin-

DISCUSSION

The existence of an optimum R.H. for reaction of the amino groups appears to be a characteristic of the protein-sugar reaction in the 'dry' state. Insulin resembles casein (Lea & Hannan, 1949) in showing maximum reactivity of the amino groups at about 70 % R.H., in contrast to the more complex dried blood-plasma system where maximum reactivity is at about 55 % R.H. (Lea, Hannan & Greaves, 1950).

The results obtained from the reaction of the α - and ϵ -amino groups of casein, and particularly of insulin with glucose are quite different from what might be expected from Gottschalk & Partridge's (1950) observations on the reactivity of simple amino-acids with glucose. It is not unreasonable to suppose, however, that the influence of a neighbouring free-carboxyl group will not be the same as that of a carbonyl group in a peptide linkage; the apparent dissociation constant of the amino group of an amino-acid is known to be altered considerably when the amino-acid becomes part of a peptide.

In proteins, moreover, steric factors might be expected to influence the relative ease with which the rather bulky glucose molecule can approach and react with the amino groups. In the case of insulin it is perhaps not without significance that the most reactive (phenylalanyl) α -amino groups are situated at the ends of the longer 'B' chains which are

probably on the outside of the protein molecule, and the glycyl α -amino groups are at the ends of the shorter 'A' chains (Sanger, 1948) and perhaps therefore less accessible, while the least reactive ϵ -amino groups of the lysyl side chains are now known to be located, one in each 'B' chain, adjacent to the alanine residue which provides the terminal carboxyl group of these chains (Sanger & Tuppy, 1951).

It is hoped to extend these investigations to other proteins and peptides with the object of obtaining some insight into the factors influencing the reactivity of amino groups towards reducing sugars.

SUMMARY

1. The reaction at 37° between insulin and glucose freeze-dried together from solution at pH 7 has been investigated.

2. The rate of reaction has been shown to increase with increasing activity of water to a maximum at a water content in equilibrium with a relative humidity of approximately 70 %, and thereafter to

decrease again with further increase in water content.

3. The reactivities towards glucose of the α -amino groups attached to the terminal phenylalanyl and glycyl residues, and of the ϵ -amino groups attached to the lysyl residues of insulin have been determined by the fluorodinitrobenzene method. After 4 days at 65 % relative humidity, 70 % of the phenylalanyl amino groups, 37 % of the glycyl amino groups and only 21 % of the lysyl amino groups had reacted. After 16 days the corresponding figures were 80, 66 and 55 %.

The authors wish to express their thanks to Dr R. S. Hannan for helpful discussions on the fluorodinitrobenzene method, to Dr E. F. Gale for glutamic acid decarboxylase, and to Dr F. A. Isherwood for the method used for the removal of interfering substances from hydrolysates of 2:4-dinitrophenyl proteins.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

- Chibnall, A. C. (1946). *J. int. Soc. Leath. Tr. Chem.* **80**, 1.
 Davis, H. F. (1950). Private communication.
 Gale, E. F. (1945). *Biochem. J.* **39**, 46.
 Gottschalk, A. & Partridge, S. M. (1950). *Nature, Lond.*, **165**, 684.
 Isherwood, F. A. & Cruickshank, D. (1951). Private communication.
 Lea, C. H. & Hannan, R. S. (1949). *Biochim. Biophys. Acta*, **3**, 313.
 Lea, C. H. & Hannan, R. S. (1950). *Biochim. Biophys. Acta*, **4**, 518.
 Lea, C. H., Hannan, R. S. & Greaves, R. I. N. (1950). *Biochem. J.* **47**, 626.
 Lea, C. H., Hannan, R. S. & Rhodes, D. N. (1951). *Biochim. Biophys. Acta*, **7**, 366.
 Partridge, S. M. & Swain, T. (1950). *Nature, Lond.*, **166**, 272.
 Sanger, F. (1945). *Biochem. J.* **39**, 507.
 Sanger, F. (1948). *Rep. Progr. Chem.* **45**, 283.
 Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463, 481.

A Micromethod for the Serial Determination of Organically Bound Halogen

BY S. J. PIRT AND E. B. CHAIN

International Research Centre for Chemical Microbiology, Istituto Superiore di Sanità, Rome

(Received 10 September 1951)

The main methods for the determination of organically bound halogen are: (1) combustion with nitric acid in the presence of silver nitrate and gravimetric determination of silver halide, (2) dry combustion yielding the free halogen which may be determined gravimetrically or volumetrically, (3) fusion with potassium and volumetric determination of the ionized halide. The authors were faced with the problem of having to follow at frequent time intervals the metabolic fate of

halogen-containing compounds in micro-organisms. For this purpose methods (1) and (2) were found to be too long and cumbersome, whilst in the potassium-fusion method difficulty was experienced in bringing all of the organic substance into intimate contact with the potassium. The following alternative method was therefore devised allowing the rapid determination of small amounts of organically bound halogen (1.5–15 μ mol.) in series.