Short Communications and Preliminary Notes

THE REACTION OF PROTEINS WITH ^{14}C-LABELLED N-CARBOXYLEUCINE ANHYDRIDE*

by

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The usefulness of N-carboxyamino acid anhydrides for the purpose of attaching amino acids to proteins by peptide linkages has recently been pointed out[1]. Similar studies had been independently initiated in this laboratory, with the particular ultimate aim of producing self-duplicating virus modifications (mutations) by such means.

N-carboxy-L-leucine anhydride labelled with ^{14}C in the chain** was used in the present study. The reagent, dissolved in dry dioxane, was added to a 20–100 fold volume of phosphate buffered protein solution (pH 7.0, occasionally 6.0 or 5.0) at 0°.

When dialysis and centrifugation is used as a means of isolating the protein derivatives[1], the reagent found associated with the protein may be present in one or more of the following forms: a. as contaminant (either nondialysable water soluble polymer mixed with the protein or free amino acid attached by secondary valences), b. as polyleucine chains built onto amino (and possibly other) groups of the protein, and c. as monomeric amino acid residues attached to ε-amino groups of lysine and/or α-amino groups of the chain ends of the protein. The last type of attachment appeared of main interest, since it introduced only typical α-peptide bonds which might be duplicated during virus synthesis.

The formation of non-dialysable polymer (reaction a) was definitely shown in model experiments. In fact, there is at present no conclusive evidence available to us that protein derivatives prepared with an excess of reagent and isolated by dialysis and centrifugation alone[1] are not mixtures of protein and polymer. However, when working with Tobacco mosaic virus (TMV), ultracentrifugation appeared to supply a tool for the quantitative separation of these two molecular species.

To resolve the question concerning the mode of attachment of the reagent residues (possibilities b and c, above), two methods of amino-end group analysis have successfully been employed in recent experiments. By means of fluoro-dinitrobenzene (FDNB) treatment of a protein derivative, all the terminal (*i.e.* not polymerized) amino acid residues can be tagged and isolated together with any unsubstituted original chain end residues, all of which can then be identified and determined by standard chromatographic methods[4,5]. Definite evidence for the total number of substituted protein amino groups is yielded by the decrease in the original chain end and lysine ε-amino groups (also determined with FDNB) of the derivative as compared to the original protein. Quantitation of the proportion of the bound radioactive leucine which is terminal, and thus isolated as the ether-soluble DNP-derivative, is possible also by means of the Geiger counter.

The recent application of EDMAN's method[6] to the endgroup analysis and stepwise degradation of proteins[7] has supplied similar evidence. The potential advantages of phenyl isothiocyanate (PITC) over FDNB in the present study lie in 1. supplying positive evidence of the extent of polymerisation of the reagent, and 2. obviating the high correction factors due to hydrolytic destruction of dinitrophenylamino acids under certain conditions[4,5].

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** This material was prepared by means of phosgene[2] from L-leucine to which was added a small amount of the leucine fraction of a hydrolysate of *Rhodospirillum rubrum*[3], kindly placed at our disposal by Dr H. TARVER.

TABLE I

REACTION OF PROTEINS WITH LABELLED LEUCYL CARBOXYANHYDRIDE

Protein	Reactions conditions**			Analyses of product* (Equivalents per 10^4 g)							
				Total leucine introduced		FDNB-method (4)			PITC-method (7)		
	Reagent/ amino-N (Mol-ratio)	Time	pH	From radioactivtiy*	From microbiol. assay	Original end-groups***	Terminal leucine†	Lysine decrease	Terminal amino acids***†	Radioactivity* Terminal amino acid†	Radioactivity* Non-terminal amino acid
Bovine Plasma Albumin	0.5	4 h	7	1.2		none	1.7	1.6			
	1.0	4 h	7	2.3		trace	2.3	2.4			
	0.5	1 min	7	1.0		none	1.3	1.8			
	1.0	1 min	7	1.6		none	2.0	2.8			
	0.5	4 h	5	1.0		none	1.1	1.1			
	1.0	4 h	5	1.6		n ne	1.4	2.4			
	0.8	2 h	7						1.7		
	2.4	2 h	7						3.0		
	7.2	2 h	7				2.3		2.8		
Insulin	1.5	1 min	7	3.0	2.9	0.6; 0.6	1.3	0.2			
	4.0	1 min	7	5.3	5.0	0.9; 0.5	1.6		3.3	1.5	4.5
	6.0	1 min	7	3.8	4.0	0.2; 0.5	1.6	0.7			
	8.0	1 min	7	7.3	7.5						
	untreated					1.4; 1.4			2.9		
Tobacco Mosaic Virus	2.5	1 min	7	0.4	0.3				0.5	0.3	0.4
	2.5	1 min	5	0.4	0.7				0.6		
	7.5	1 min	7	0.4	1.0				0.9		
	7.5	1 h	7	0.5	1.1					0.2	0.3

* Approximations on the basis of methods some of which are as yet under critical investigation. Radioactivity measurements in this preliminary series of experiments were of limited significance because of the low specific activity of the reagent, and high selfabsorption in protein and hydrolysate samples. All analyses with TMV were extremely erratic, probably owing to the limited extent of reaction of the virus protein.

** All experiments at o°, long term experiments at room temperature after one hour. Short-term experiments terminated by addition of excess alanine.

*** Identified as aspartic acid (less than one residue/mole protein) in the case of BSA, and phenylalanine and glycine in the case of insulin.

† Leucine introduced on the ε-amino groups of lysine, as well as at the original chain ends appears as "terminal" ether-soluble amino acid derivative in both methods.

Further, two methods were used for the determination of the total amount of ^{14}C-labelled amino acid associated with the protein, *i.e.*, the Geiger counter and microbiological assay. Such analyses, however, yield by themselves little information since the entire amount of added amino acid might be present in the form of contaminating polymer, or attached on only a single site of a protein molecule.

A considerable number of derivatives of TMV, insulin, lysozyme, egg and bovine serum albumin (BSA), ovomucoid, and conalbumin have been prepared by this technique and are being analysed by most of the above methods. The more definite findings, exemplified by the presented data, may be summarized as follows. 1. The reaction was almost complete after 1 minute at 0° and pH 7, and only slightly slower at pH 5. 2. With all proteins containing identifiable chain-end amino groups, these become partly substituted by leucine; however, in only a few instances (*e.g.* BSA) were the original end groups no longer detectable in the derivative. 3. The ε-amino groups of lysine were only partially blocked in all proteins, even with a great excess of reagent, which favoured polymerisation onto the protein. 4. When the reagent was not in excess, monomeric amino acid addition appeared to be the rule, but about half of the added reagent was not bound to the protein even under such conditions.

It appears of interest, and in line with the known role of the amino groups for the biological activities of insulin and lysozyme, that the former retained most of its activity even after extensive modification, while the latter was progressively inactivated*. The stable radioactive insulin derivative may prove a useful tool in studies of the mode of action of the hormone.

After subjecting TMV to the reaction with N-carboxyleucine anhydride under various conditions, the derivatives were purified by 3 or 4 cycles of differential centrifugation. The products contained rather little of the reagent, corresponding generally to only about one-third of the amino nitrogen of the virus protein (which is about 5000/Mole[8]). With a great excess of reagent, up to 4000 residues appeared to be bound, but over half of this was not terminal, indicating polymerisation. Unavailability or unreactivity of many of the amino groups of the virus represented an obstacle also in other reactions, notably that with FDNB. The virus modified by addition of about 1000 leucine residues was unchanged electrophoretically (in one preliminary test run), and retained its full activity, as tested by the half-leaf method on *Nicotiana glutinosa*[9]. Progeny of such virus, isolated from Turkish tobacco plants, was not distinguishably different from the original TMV, and certainly contained fewer (if any) N-terminal leucine residues, than the chemically modified parent preparation. Thus the introduced leucyl residues appear to be propagated no better than acetyl and other acyl substituents[8,9]. However, until difficulties in the analysis for the numbers of each type of amino group in TMV have been overcome**, no quantitative interpretation of their reaction with amino acid carboxy anhydrides is possible, nor a detailed analytical comparison of parent and progeny preparations.

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** At present, the occurrence of reactive (FDNB or PITC) chain-end amino groups in TMV appears very doubtful; their number is certainly far smaller than that of threonine, released by carboxypeptidase, and therefore presumed to be carboxyterminal[10].