A new reagent for the guanidination of proteins*

Among the reagents used for the chemical modification of the free amino groups of proteins, O-methylisourea[1] is useful since it replaces the positively charged NH_3^+ groups with positively charged $NHC(NH_2)_2^+$ groups causing thereby no change in the net charge of the protein molecules below about pH 8. Its main disadvantage is the high pH, about 10.5–11, required for its reaction, which may be too severe for some proteins. This preliminary note describes the use of a reagent 1-guanyl-3,5-dimethylpyrazole nitrate which guanidinates proteins at pH 9.5. This reagent was used by SCOTT *et al.*[2] and BANNARD *et al.* [3] for the preparation of mono and dialkyl guanidines from aliphatic and aromatic amines.

The reaction of GDMP with bovine serum albumin was carried out in an ice bath; the extent of reaction was studied as a function of pH, concentration of GDMP and time. The optimum reaction of free amino groups was obtained in 0.5 *M* GDMP. The reagent was dissolved in a few ml water, the pH was adjusted with continuous magnetic stirring to the desired value with 1 *N* NaOH and water added to make the solution 0.5 *N* with respect to GDMP. Serum albumin was dissolved in this solution to 5 % concentration and the pH readjusted if necessary. After stirring at 0° for the chosen time, the solution was dialysed exhaustively against phosphate buffer, pH 7.5 I 0.1. Protein concentrations were obtained by measuring the refractive-index increment of solutions with a Brice Phoenix differential refractometer. Ninhydrin colorimetric analyses were used to determine the free amino groups[4], with solutions of the unmodified protein as standard.

In Fig. 1 the percentage of amino groups reacted as a function of pH and time of reaction is compared with the results of HUGHES *et al.*[1] with O-methylisourea. It is seen that the lowest pH for the guanidination of bovine serum albumin with GDMP is pH 9.5. The guanidinated albumin sedimented as one symmetrical peak in the ultracentrifuge, at a rate similar to the albumin itself.

The reaction of β-lactoglobulin was more complicated. If β-lactoglobulin was reacted with 0.5 *M* GDMP gelling took place in few hours; similar results were obtained using O-methylisourea. As O-methylisourea and GDMP are chemically related to urea and guanidine, they may cause denaturation of proteins. No gelling took place if the reaction was carried out in 0.2 *M* GDMP and 3 % protein concentration. These conditions gave almost complete reaction of the ε-amino groups of lysine (Table I). Guanidinated β-lactoglobulin thus prepared showed an asymmetric peak that spread very rapidly upon ultracentrifugation.

HUGGINS *et al.*[5] observed gelling of various proteins in 8 *M* urea, attributed it to the formation of intermolecular disulphide bonds, and prevented it by sulphydryl group reagents such as *p*-chloromercuribenzoate. While *p*-chloromercuribenzoate protected lactoglobulin from aggregation on guanidination using O-methylisourea and the product showed one symmetrical peak in the ultracentrifuge, the mercurial precipitated with GDMP and did not protect lactoglobulin from aggregation on guanidination using this reagent.

N-ethylmaleimide[7] which combines rapidly and quantitatively with thiols, was reacted with lactoglobulin and the resulting solution allowed to react with 0.2 *M*

---

* Issued as N.R.C. No. 5183.

Abbreviations: GDMP, 1-guanyl-3,5-dimethylpyrazole nitrate; DNP, dinitrophenyl.

GDMP, pH 9.5, at 0° for 7 days. Ninhydrin analysis on this product are reported in Table I. The guanidated β-lactoglobulin thus prepared gave one symmetrical peak and negligible amounts of aggregates when examined in the ultracentrifuge.

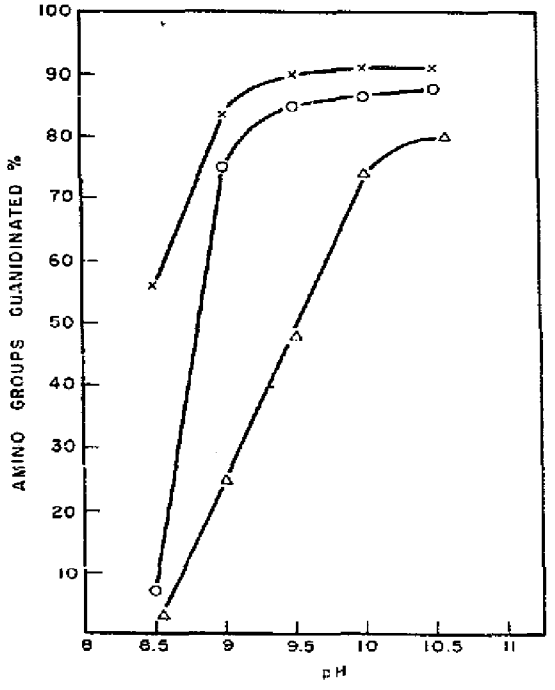

Fig. 1. Reaction of bovine serum albumin with GDMP. ×—× Reaction for 7 days. ○—○ Reaction for 3 days. △—△ Result of HUGHES *et al.*[1].

TABLE I

AMINO GROUPS, ε-DNP-LYSINE AND N-TERMINAL AMINO ACIDS IN NATIVE AND GUANIDINATED PROTEINS

Protein	*% Free amino groups by ninhydrin*	*No. of free amino groups*	*ε-DNP-Lysine moles amino acid/mole protein*	*N-terminal residue moles amino acid/mole protein*
Bovine serum albumin	100	(56)*	56.5 (55)*	Asp 0.96**
Guanidinated albumin	10	5.6	5.2	Asp 0.37**
β-lactoglobulin (denatured)	100	(32)	28 (29)	Leu 2.76***
Guanidinated lactoglobulin	10	3.2	0.7	Leu 1.98***

* Value in parentheses is from literature.
** Value corrected for 65% recovery[10].
*** Value corrected for 75% recovery[11].

The free amino groups of the native and guanidinated proteins were reacted with fluorodinitrobenzene as described by FRAENKEL-CONRAT, HARRIS AND LEVY[8]. The DNP-derivatives were hydrolysed for 16 h and examined quantitatively for the N-terminal amino acid as well as for ε-DNP-lysine by paper chromatography using the ethylbenzene system[9]. Results are given in Table I.

Five lysine amino groups in serum albumin appear not to react with GDMP

possibly because of steric hindrance. HUGHES *et al.*[1] found that exhaustive reaction with O-methylisourea led to the reaction of 54–57 out of 64–68 amino groups, determined by Van Slyke amino nitrogen analysis. Partial reaction with GDMP occurs with the N-terminal amino groups of these proteins. About 60 % of the N-terminal aspartic acid of serum albumin reacted, while about 30 % of N-terminal leucine of lactoglobulin appeared to react.

More complete details and further studies on this reagent will be reported later.

I wish to express my thanks to Dr. DAVID B. SMITH for his helpful suggestions and advice throughout the work.

Division of Applied Biology, A. F. S. A. HABEEB*
National Research Council of Canada, Ottawa (Canada)

1 W. L. HUGHES, H. A. SAROFF AND A. L. CARNEY, *J. Am. Chem. Soc.*, 71 (1949) 2476.
2 F. L. SCOTT, D. G. O'DONOVAN AND J. REILLY, *J. Am. Chem. Soc.*, 75 (1953) 4053.
3 R. A. B. BANNARD, A. A. CASSELMAN, W. F. COCKBURN AND G. M. BROWN, *Can. J. Chem.*, 36 (1958) 1541.
4 V. J. HARDING AND R. M. MACLEAN, *J. Biol. Chem.*, 24 (1916) 503.
5 C. HUGGINS, D. F. TAPLEY AND E. V. JENSEN, *Nature*, 167 (1951) 592.
6 E. FRIEDMANN, D. H. MARRIAN AND I. SIMMON REUS, *Brit. J. Pharmacol.*, 4 (1949) 105.
7 L. R. MACDONNELL, R. B. SILVA AND E. R. FEENEY, *Arch. Biochem. Biophys.*, 32 (1951) 288.
8 H. FRAENKEL-CONRAT, J. I. HARRIS AND A. L. LEVY, in D. GLICK, *Methods of Biochemical Analysis*, Vol. II, New York 1955, p. 359.
9 A. F. S. A. HABEEB, *J. Pharm. and Pharmacol.*, 10 (1958) 591.
10 T. PETERS, A. G. LOGAN AND C. A. SANFORD, *Biochim. Biophys. Acta*, 30 (1958) 88.
11 R. R. PORTER AND F. SANGER, *Biochem. J.*, 42 (1948) 287.

Received March 31st, 1959

* National Research Council of Canada Postdoctorate Fellow.

The C-terminal amino acid of carboxypeptidase-A

Although it has been shown that CPase-A consists of a single polypeptide chain with N-terminal asparagine[2] and a molecular weight of 34,000[3,4], no information has yet been made concerning its C-terminal residue. The C-terminal amino acid of CPase-A has now been found to be asparagine both by the enzymic method, in which the native crystalline CPase-A was allowed to act upon the denatured enzyme, and by the catalytic-hydrazinolysis method.

CPase-A used in this experiment was a specimen crystallized from frozen bovine pancreas and recrystallized 6–10 times according to NEURATH's method[5], but including one DFP treatment. It was shown to be homogeneous ultracentrifugally and to yield aspartic acid (0.8–0.9 mole/mole) and serine (0.05–0.1 mole/mole) in the N-terminal determination by the DNP method, supporting the result obtained by THOMPSON[2]. The amino acid composition determined by the DNP procedure[6] was almost in agreement with that obtained by SMITH *et al.*[4].

The CPase-A was denatured by incubating at room temperature (23–25°) for 2–3 h with 0.2 % aq. sodium dodecyl sulfate (pH about 7) in the presence of 0.04 *M* *β*-phenyl propionate, a strong competitive inhibitor of the enzyme[7]. After dialysis

Abbreviations: CPase-A, carboxypeptidase-A (ANSON's[1]); CPase-B, basic carboxypeptidase; DFP, diisopropyl fluorophosphate; DNP, dinitrophenyl.