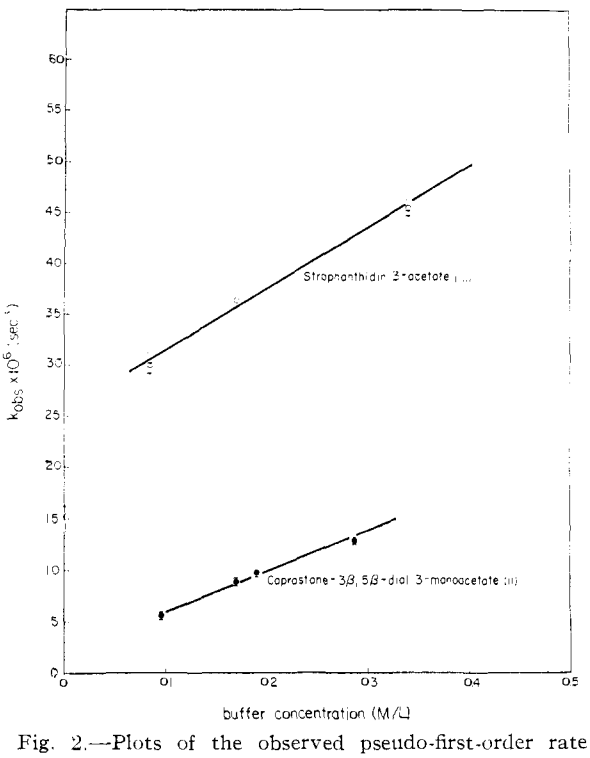
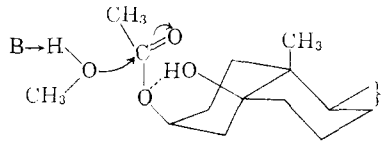


Fig. 2.—Plots of the observed pseudo-first-order rate constants (k_{obs}) for solvolysis at 40° (μ = 0.10 M), *vs.* concentration of triethylamine–triethylamine acetate at constant buffer ratio (3:1).

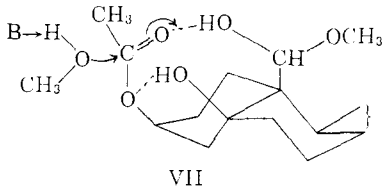
buffer ratio and constant ionic strength. The linear increase in rate conforms to the generally-accepted criterion for general base catalysis. To preclude the possibility of specific triethylamine catalysis, the solvolysis of II and III was repeated with another tertiary base of different base strength. The rates of solvolysis of II and III at constant buffer ratio and in the presence of varying concentrations of N-methylimidazole were found to be linear functions of the concentrations of base, strengthening the postulate that the solvolysis is indeed general base-catalyzed.

From the data in Table I, it is evident that introduction of a hydroxyl group at C-5 of coprostanol acetate (I), leading to the 1,3-diaxial hydroxyacetate (II), resulted in a 300-fold increase in the rate of solvolysis. The solvolysis of 1,3-diaxial hydroxyacetates, possibly to be regarded as in VI, appears to be the first recognized non-enzymatic example of general acid–general base-catalysis of ester solvolysis.[9,10,11]

Introduction of the 19-aldehyde group of strophanthidin 3-acetate (III) led to a four-fold increase in rate of solvolysis relative to II. Examination of molecular models reveals that the carbonyl group is too far from the acetoxy group to exert any direct facilitating effect. However, the model of the hemiacetal adduct (VII) shows that the acidic hemiacetal hydroxyl group is situated within hydrogen bonding distance of the carbonyl oxygen of the 3-acetate, as shown. The postulated hydrogen bonding shown would polarize the carbonyl group and facilitate attack of the nucleophile. In accordance with this view, the 19-alcohol, strophanthidol 3-acetate (IV) (with a less acidic 19-hydroxyl group), was found to be less labile toward solvolysis than strophanthidin 3-acetate (III), but more labile than coprostane-3β,5β-diol monoacetate (II). The rate of solvolysis of strophanthidinic acid methyl ester 3-acetate (V) was found to be the same as that of II, precluding an inductive effect by the C-10 substituent as an important factor in the facilitation.



VI



VII

Bruice, *et al.*, recently have studied the nature of neighboring hydroxyl group assistance in the alkaline hydrolysis of the ester bond in "systems of greater simplicity."[12] On the basis of kinetic isotope experiments (designed to test for possible general base-catalysis by the neighboring hydroxy group), the latter authors ruled out the possible involvement of general base-catalysis as the basis of the Henbest–Kupchan effect.[13] We suggest that the assumption that the nature of the hydroxyl-group assistance is the same in diverse systems is unwarranted. One is forced to conclude that the mechanism and magnitude of the rate enhancement of the solvolysis reaction will vary considerably with the detailed geometry and the conformational rigidity of hydroxy-acetates.[14]

We take pleasure in thanking Professor M. L. Bender for stimulating discussions.

(10) *Cf.* R. M. Krupka and K. J. Laidler, *J. Am. Chem. Soc.*, **83**, 1458 (1961).

(11) *Cf.* M. L. Bender, *ibid.*, **84**, 2582 (1962).

(12) See discussion following M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961).

(13) T. C. Bruice and T. H. Fife, *J. Am. Chem. Soc.*, **84**, 1973 (1962).

(14) This work was supported in part by a research grant from the National Institutes of Health (H-2275).

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REVERSIBLE BLOCKING OF PROTEIN AMINO GROUPS BY THE ACETIMIDYL GROUP[1]

Sir:

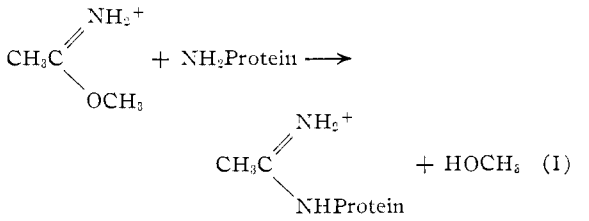
The limited degradation of proteins to a few large peptides is a usual preliminary step in the determination of amino acid sequences.[2] In

(1) This research was supported by a grant from the Division of Medical Sciences of the National Institutes of Health and by a Predoctoral Fellowship (HF-7714-C2) granted to R. B. by the National Institutes of Health.

(2) F. Sanger, *Advances in Protein Chem.*, **7**, 1 (1952); C. B. Anfinsen and R. R. Redfield, *ibid.*, **11**, 2 (1956); B. Witkop, *ibid.*, **16**, 221 (1961).

several cases, limited cleavage has been accomplished by tryptic digestion of proteins in which modification of lysyl residues has rendered the lysyl peptide bond resistant to trypsin.[3-6] For sequence analysis, the lysyl modification reactions of choice appear to be those, like dithiocarbamylation,[5] which later can be reversed without accompanying side reactions.

Methyl acetimidate reacts specifically with the amino groups of proteins to form acetamidino derivatives.[7,8] Since amidination of the ϵ-amino



group prevents tryptic digestion of the adjacent peptide bond,[7] large peptides can be prepared from amidinated proteins by the tryptic cleavage of arginyl bonds. To ascertain whether the amino groups might subsequently be regenerated from amidinated peptides, the present study of the removal of the acetimidyl group from acetamidino oxidized insulin has been carried out.

Acetamidino oxidized insulin was prepared as described previously (Preparation III).[7] Comparison of tryptic digests of this preparation with digests of oxidized insulin had demonstrated that amidination of the lysyl residue prevented tryptic digestion.[7] Trypsin normally splits oxidized insulin at the lysyl and arginyl bonds of the sequence . . arg - gly - phe - phe - tyr - thr - pro - lys - ala-COOH, the C-terminal region of the B chain.[9] The expected fragments were obtained from digests of oxidized insulin but only the arg-gly bond of acetamidino oxidized insulin was attacked (see Fig. 1).

The extent of removal of the acetimidyl group could be determined by measuring the alanine released during tryptic digestion. Alternatively the disappearance of modified octapeptide and reappearance of heptapeptide in tryptic digests could be followed electrophoretically since modified octapeptide could be distinguished from the gly. . .lys heptapeptide.

The known reactions of substituted amidines with the nucleophiles ammonia and hydrazine[12] suggested that these reagents might displace the acetimidyl group from proteins. One ml. of concd. NH_3, brought to an apparent pH of 11.3 by the addition of glacial acetic acid (ammonia: acetic acid, 30:2, v./v.), and 2–5 mg. of acetamidino oxidized insulin were mixed and allowed to stand in a closed vessel at room temperature for 8 hr. The reaction was terminated by diluting with water and lyophilizing. The material was digested with trypsin (1:50, w./w.; 37°; 2 hr.) and subjected to electrophoresis at pH 9[11]; the results are illustrated in Fig. 1. It is evident from the absence of modified octapeptide that this treatment sufficed to remove the acetimidyl group from the lysyl residue. Concurrent displacement of the acetimidyl group from the N-terminal amino acid residues was shown by the reappearance of the normal A chain and the normal N-terminal B chain peptide.

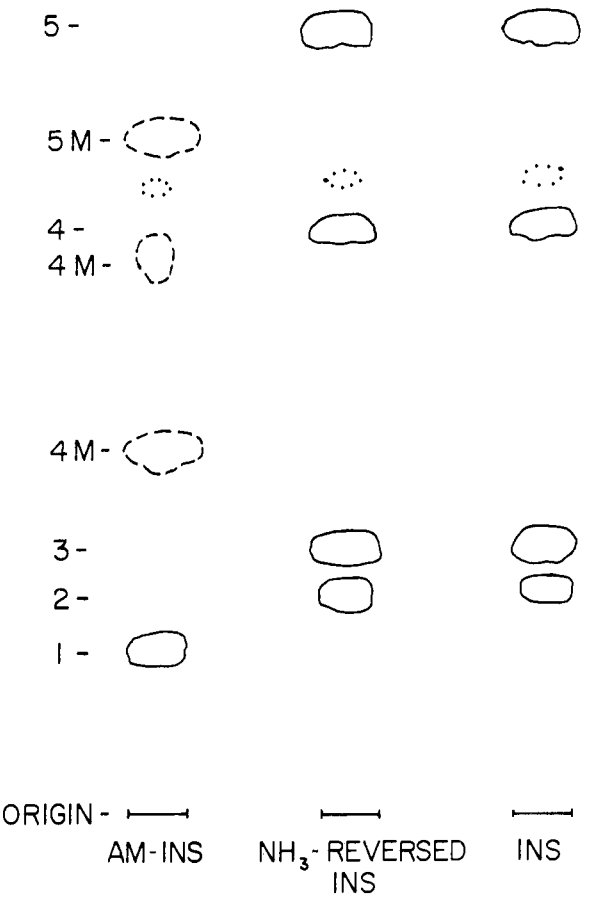


Fig. 1.—Diagram of electrophoretic patterns of tryptic digests. AM-INS: tryptic digest of amidinated oxidized insulin; NH_3-REVERSED INS: amidinated oxidized insulin reacted 8 hr. with NH_3–NH_4 acetate, pH 11.3, then digested with trypsin; INS: tryptic digest of oxidized insulin. Electrophoresis of 1 mg. samples performed in a Varsol-cooled tank[10] in 2% $(NH_4)_2CO_3$, pH 9,[11] at 20 v./cm. Ninhydrin positive bands, ———, are: 1, modified octapeptide, NH_2gly-phe-phe-tyr-thr-pro-amlys-alaCOOH; 2, heptapeptide, NH_2gly-phe-phe-tyr-thr-pro-lysCOOH; 3, alanine; 4, N-terminal B chain peptide, NH_2phe. . .arg-COOH; 5, A chain. Ninhydrin negative, diazo positive bands, ------, are: 4M, amidinated N-terminal B chain peptides (7); 5M, amidinated A chain. Minor bands,[7] . . . ., having the same amino acid composition as bands 4 or 4M were observed in each of the three patterns.

(3) R. R. Redfield and C. B. Anfinsen, *J. Biol. Chem.*, **221**, 385 (1956).

(4) C. B. Anfinsen, M. Sela and H. Tritch, *Arch. Biochem. Biophys.*, **65**, 156 (1956).

(5) T. C. Merigan, W. J. Dreyer and A. Berger, *Biochim. et Biophys. Acta*, **62**, 122 (1962).

(6) R. F. Goldberger and C. B. Anfinsen, *Biochemistry*, 1, 401 (1962).

(7) M. J. Hunter and M. L. Ludwig, *J. Am. Chem. Soc.*, **84**, 3491 (1962).

(8) L. Wofsy and S. J. Singer, to be published.

(9) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

(10) A. M. Katz, W. J. Dreyer and C. B. Anfinsen, *J. Biol. Chem.*, **234**, 2897 (1959).

(11) M. A. Naughton and F. Sanger, *Biochem. J.*, **78**, 156 (1961).

(12) R. L. Shriner and F. W. Neumann, *Chem. Rev.*, **35**, 351 (1944).

Although removal was complete in 8 hr., traces of octapeptide could still be found in tryptic digests after 4 hr. of treatment of the modified protein with NH_3-NH_4 acetate. A single study indicated that decreasing the reagent concentration slowed the reaction, as expected.

To confirm the absence of side reactions accompanying the reversal of amidination, both oxidized insulin and modified oxidized insulin were treated for 8 hr. at pH 11.3 with the ammonia reagent and aliquots digested with trypsin. Electrophoretic patterns of samples exposed to the reagent were compared with those of oxidized insulin and trypsin-treated oxidized insulin by staining with hypochlorite[13] and with reagents specific for tyrosine, arginine, and histidine.[14] None of the samples subjected to ammonia treatment displayed any bands not present in the corresponding untreated samples.

If, however, the acetimidyl group was removed by treatment with concd. ammonia at 100° for 10 min.,[15] the N-terminal B chain peptide was completely converted to another material of greater mobility.[16] After 8 hr. of reaction with concd. ammonia at room temperature or after 10 min. of heating at 100° in NH_3-NH_4 acetate mixtures at pHs 13, 12, or 11.3, partial conversion of the B chain peptide to side product occurred. The conditions finally chosen, pH 11.3 and room temperature, were mild enough to prevent this side reaction.

Hydrazine, 0.6–1.2 *M*, adjusted to pH 9 by the addition of acetic acid, displaced the acetimidyl group from the lysyl residue of acetamidino oxidized insulin in 24 hr. at room temperature. However, the absence of concomitant side reactions has not been fully established.

It thus appears feasible to regenerate completely the amino groups from large amidinated peptides. The tryptides included within the sequence of each large peptide may then be obtained by redigestion of the isolated unmasked peptides. The acetimidyl blocking group possesses some convenient properties. Essentially complete modification of amino groups can be obtained,[8] minimizing the number of peptides appearing in tryptic digests; the derivatives approximate the charge and solubility of the original proteins, unlike carbobenzoxy[4] or trifluoroacetyl[6] derivatives, which are much less soluble, especially in acid solution; and the relatively great stability of the amidino derivatives permits the separation of the large modified peptides by a variety of procedures. Moreover, since the degree of removal can be controlled by an appropriate choice of time and reagent concentration, redigestion of a partly unmasked peptide with trypsin can yield a mixture of peptides from which the alignment of tryptides comprising the large peptide may be deduced.

The present preliminary work on insulin therefore suggests that amidination may be generally useful in sequence determinations on larger proteins.

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(14) R. J. Block, R. LeStrange and G. Zweig, "Paper Chromatography," Academic Press, Inc., New York, N. Y., 1952.

(15) K. Satake, M. Tanaka and H. Shino, *J. Japan. Biochem. Soc.*, **50**, 6 (1961).

(16) E. J. Harfenist and L. C. Craig, *J. Am. Chem. Soc.*, **75**, 5528 (1953); F. Carpenter, *J. Biol. Chem.*, **237**, 404 (1962).

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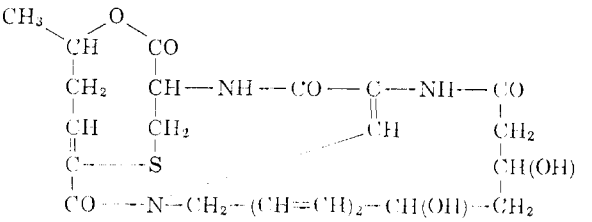
RECEIVED AUGUST 31, 1962

GRISEOVIRIDIN

Sir:

The isolation[1] and characterization[2,3] of griseoviridin, an antibiotic from a strain of *Streptomyces griseus*, have been reported previously. We now wish to present evidence leading to the assignment of the structure (1) to griseoviridin.



1

Griseoviridin has the molecular formula $C_{22}H_{27}N_3O_7S$.[2,4] It contains two acylable hydroxyl groups,[2] and on acid hydrolysis gives cystine[2] and 2-oxo-5-hydroxyhexanoic acid.[3] The latter is itself derived from the six carbon fragment: $CH_3CH(—O—)—CH_2—CH=C(—X—)—CO—$ (2) where X is O, N or S.[3] On reduction with Raney nickel the diacetate gives the saturated perhydrodethiogriseoviridin diacetate (**3**).[2] Acid hydrolysis of this gives two molecules of alanine, whilst reductive hydrolysis[2] leads to ω-aminodecanoic acid as the only other identified product. All carbon atoms in griseoviridin are thus accounted for.

Alkaline hydrolysis of **3** (uptake of three equivalents) and esterification (diazomethane) gave a neutral methyl ester, (**4**) (m.p. 125–127°).[5] Since no fragment is lost during the hydrolysis **3** is thus a lactone.

Reduction of **4** with lithium borohydride[6] followed by acid hydrolysis, neutralization and steam distillation gave alaninol.[7] Mild acid hydrolysis of **3** gave alanylalanine. Since, as will be shown, only the hydroxyl group in **2** is available for

(1) Q. R. Bartz, J. Standiford, J. D. Mold, D. W. Johannessen, A. Ryder, A. Maretzki and T. H. Haskell in "Antibiotics Annual" 1954–1955, Medical Encyclopedia, Inc., New York, N. Y., 1955, pp. 777–783.

(2) D. E. Ames, R. E. Bowman, J. F. Cavalla and D. D. Evans, *J. Chem. Soc.*, 4260 (1955); D. E. Ames and R. E. Bowman, *ibid.*, 4264 (1955); 2925 (1956).

(3) P. de Mayo and A. Stoessl, *Can. J. Chem.*, **38**, 950 (1960).

(4) Knowledge of the exact number of hydrogen atoms was not revealed by analyses, but awaited the final elucidation of structure.

(5) Satisfactory analyses have been obtained for all new compounds reported except those used without further purification. Substances have been characterized by ultraviolet, infrared, and, in most cases, n.m.r. spectra which are in accord with the assigned structures.

(6) Under the conditions used the ester carbonyl was reduced whilst the infrared spectrum indicated no reduction of the amide functions.

(7) We thank Dr. J. Walker (M.R.C., London) for an authentic specimen of *dl*-alaninol.