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Reversible Blocking of Amino Groups with Citraconic Anhydride

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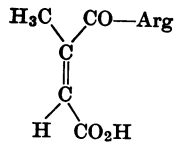
Butler, Harris, Hartley & Leberman (1967) showed that maleic anhydride could be used for the reversible blocking of amino groups. The maleyl group could be removed because the protonated form of the free carboxyl group catalysed the hydrolysis of the amide bond, presumably by intramolecular general acid catalysis (cf. Bender, Chow & Chloupek, 1958). The present paper reports the effects of introducing methyl groups into the molecule of maleic anhydride.

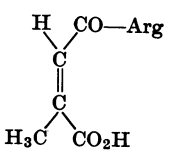
2,3-*Dimethylmaleic anhydride.* We had wished to investigate whether the use of maleylation to introduce glyoxyloyl groups (Dixon, 1968) could be extended to the introduction of pyruvoyl groups. We therefore treated arginine with 2,3-dimethylmaleic anhydride (Fluka A.-G., Buchs, Switzerland) as follows. To a solution of arginine hydrochloride (5M) in water was added, with stirring, dimethylmaleic anhydride to a final concentration of 5·5M, and the pH was maintained at 8 by the addition of N-NaOH. Uptake of base ceased after about an hour and suitable samples were then applied to paper for high-voltage electrophoresis in the buffer systems used previously (Perham, 1967). Material was detected on the paper by means of the ninhydrin–cadmium reagent (Heilmann, Barrollier & Watzke, 1957) and also by the Sakaguchi reaction (Jepson & Smith, 1953). It was immediately apparent that arginine alone and no product could be detected after electrophoresis at pH 3·5, but that at pH 6·5 some N^{α}-2,3-dimethylmaleylarginine was observed, together with a large streak of Sakaguchi-positive material that extended from the position of the product to that of arginine, presumably formed by degradation of the product to arginine during the electrophoresis. After electrophoresis at pH 8·9 [in 1% $(NH_4)_2CO_3$], no free arginine could be detected, the single Sakaguchi-positive spot corresponding to pure product. It therefore seemed likely that the breakdown of the protonated form of the product was extremely rapid and, indeed, after standing at pH 3·5 for 5min. at 20°, conversion of dimethylmaleylarginine into arginine was complete, as judged by paper electrophoresis at pH 8·9.

On the basis of these results, it appeared that the derivative would prove useful for the reversible blocking of protein amino groups because the slowness of removal of maleyl groups, which show a half-life of 11hr. at pH 3·5 and 37°, is a hindrance to their use. To test the reagent on the lysine residues of a protein, bovine insulin (Allen and Hanburys Ltd., London, W. 1) was oxidized with performic acid and treated with the reagent. The treated protein was desalted by gel filtration on Sephadex G-25 equilibrated with a buffer of 0·2M-trimethylamine quarter-neutralized with acetic acid, pH 10·3, and digested with trypsin (1%, w/w) in the same buffer for 3hr. at 20°. After being freeze-dried, the digest was exposed to pH 3·5 electrophoresis buffer and subjected to paper electrophoresis at pH 3·5 alongside a control sample of the tryptic digest of unmodified oxidized insulin. The appearance of the peptide Gly-Phe-Phe-Tyr-Thr-Pro-Lys and of free alanine is diagnostic of tryptic cleavage of the insulin B-chain at Arg-22 and Lys-29. From the tryptic digest of dimethylmaleyl-insulin, only the peptide Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala was isolated, proof that tryptic cleavage of the B-chain had been limited to Arg-22. However, attempts to carry out the gel filtration and tryptic hydrolysis at preferred pH values (e.g. pH 8) failed because of significant unblocking of the lysine residue during a period of several hours.

Citraconic anhydride. Thus, whereas maleyl groups are inconveniently hard to remove, 2,3-dimethylmaleyl groups are inconveniently hard to keep on. It seemed worth while to seek a group that might be hydrolysed off at an intermediate rate. 2-Methylmaleic anhydride (citraconic anhydride; British Drug Houses Ltd., Poole, Dorset) was therefore tried. It has the obvious disadvantage compared with the previous reagents that two products may be formed, according to which carbonyl group of the reagent is attacked. Arginine was treated with the reagent as outlined above and the product examined by paper electrophoresis. No free arginine remained and only one Sakaguchi-positive product, with the mobility expected of citraconylarginine, was visible at pH 6·5, whereas at pH 3·5 two products could be distinguished that had similar electrophoretic mobilities. The more cationic appeared to break down to arginine during



(I)



(II)

the run. HCl was added to the mixed reaction product (20mM) to give an HCl concentration excess of 40mM, and the solution was maintained at room temperature (20°). The concentration of free HCl should thus fall to 20mM as the product was hydrolysed. After various time-intervals samples were made alkaline with 0·1N-NH_3, and analysed both for the arginine formed and for the citraconylarginine that remained by paper electrophoresis at pH 8·9, followed by quantitative reaction with the ninhydrin–cadmium reagent. Both forms of citraconylarginine are ninhydrin-positive, presumably because of their hydrolysis during the mildly acidic ninhydrin reaction. Both methods gave a half-life of 1·1–1·4hr. under these conditions. No departure from first-order kinetics was observable up to 90% reaction. Nevertheless qualitative analysis of the reaction mixture, performed by electrophoresis at pH 3·5, which separates the two citraconylarginines, showed that the minor product, which was less cationic, was the more stable. It was stable enough on electrophoresis at this pH for the time-course of its breakdown in the 40mM-HCl to be followed and this yielded a half-life of 1·7hr. The difference in stability is not therefore very great. It is more marked at pH 3·5, presumably owing to the fact that the minor product is appreciably less protonated at this pH.

We cannot predict which of the expected products (I and II) is likely to be the more acidic, since α-methylacrylic acid and *trans*-crotonic acid have similar dissociation constants. To test whether the low yield of the minor products was due to steric hindrance by the methyl group to approach of the amine, anhydrides in which the methyl group was replaced with isopropyl and isobutyl groups were used. They did not lead to complete predominance of one product. They were prepared from ethyl acetoacetate (cf. Vaughan & Andersen, 1955; Rydon, 1936). It therefore seems unlikely that steric hindrance is a major cause of the asymmetry of reaction of the anhydrides.

The usefulness of citraconic anhydride as a reversible blocking reagent for protein amino groups was tested with the insulin system described above for dimethylmaleic anhydride. Performic acid-oxidized insulin (15mg./ml.) was treated with citraconic anhydride (100mg./ml.), with stirring, at room temperature and the pH was maintained at 8 by the addition of 5N-NaOH. Base uptake ceased after about 10min. and the protein was desalted by gel filtration on Sephadex G-25, in 0·5% NH_4HCO_3, pH 8·0. Tryptic digestion was carried out in the same buffer and the digest freeze-dried. One sample of the digest was submitted to paper electrophoresis at pH 6·5 and a second sample was suspended in pH 3·5 electrophoresis buffer at room temperature overnight before similar electrophoresis at pH 6·5. The results of these electrophoreses were entirely in accord with limitation of tryptic cleavage to Arg-22 in the B-chain of the citraconyl-insulin and with total release of the blocking group from Lys-29 on overnight incubation of the digest at pH 3·5 and 20°. It therefore seems that citraconylation provides an easy method for the reversible blocking of protein amino groups, in which both the introduction and removal of the citraconyl groups may be carried out under suitably mild conditions that most proteins should withstand.

Comparison with other reagents. The past few years have seen the development of several methods for the reversible blocking of protein amino groups. The derivatives produced vary widely in the conditions of their stability and their properties. Thus trifluoracetylation (Goldberger & Anfinsen, 1962) removes the positive charge of the modified amino groups, amidination (Hunter & Ludwig, 1962) maintains it, and maleylation (Butler *et al.* 1967), tetrafluorosuccinylation (Braunitzer, Beyreuther, Fujiki & Schrank, 1968) and, now, citraconylation convert it into a negative charge. These charge changes may be made the basis of diagonal electrophoretic methods for examining amino acid sequences around amino and thiol (aminoethylcysteine) groups (Perham & Jones, 1967; Perham, 1967) and, in favourable cases, help to establish the role of amino groups in macromolecular structure (Perham & Richards, 1968). The wide variety of pH ranges and temperatures over which the various derivatives are stable should enable a suitable derivative to be used for any polypeptide chain despite the widely differing behaviour and stability of individual proteins.

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