CXC. NOTE ON THE ACTION OF ACETYLATED AGENTS ON AMINO-ACIDS

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IN connexion with the development of a new method for the isolation of carbohydrate from protein hydrolysates [Neuberger, 1938] it became necessary to study certain aspects of the reactions of amino-acids and related compounds with pyridine-acetic anhydride and ketene.

Reactions of amino-acids with pyridine and acetic anhydride

If amino-acids are treated in glacial acetic acid solution with the theoretical amount of acetic anhydride, optically active acetamino-acids are obtained, while if a large excess of acetic anhydride is used, racemization and/or formation of azlactones takes place [Bergmann *et al.* 1926; Bergmann & Zervas, 1928]. Excess of acetic anhydride causes racemization even in aqueous solution in the presence of alkali [du Vigneaud & Meyer, 1932]. If the reaction with acetic anhydride, however, is carried out at 100° in the presence of pyridine, CO₂ is split off and acetaminoketones result [Dakin & West, 1928]. It has been found by the present author that this anomalous reaction is suppressed at low temperature. Thus two typical amino-acids, histidine and phenylalanine, which by treatment with pyridine and acetic anhydride at 100° are almost quantitatively converted into the corresponding acetaminoketones, give, when the reaction is carried out at 2°, very good yields of acetamino-acids; only in one case was there an indication that a minute trace of a ketone was formed. It seems, however, that at least partial racemization of some amino-acids is induced by this method of acetylation also.

Action of ketene on amino-acids and related compounds

Bergmann & Stern [1930] found that the alkali salts of simple amino-acids such as leucine and phenylalanine, or in some cases the amino-acids themselves, react with ketene to form *N*-acetylated compounds whilst tyrosine in presence of an excess of alkali gives *ON*-diacetyltyrosine. This method of acetylation which is quantitative and does not lead to side-reactions has been used to mask amino- and phenolic hydroxyl-groups in proteins [Herriott & Northrop, 1934; Stern & White, 1938] and it has been tacitly assumed that changes in the protein molecule, under the action of ketene, are in fact restricted to these two types of groups. The experiments of Bergmann & Stern [1930] were confined to racemic monoaminomonocarboxylic acids, *l*(-)-tyrosine and *l*(+)-glutamic acid and the question of racemization was not studied in detail by these authors; in the present work the study of the reaction has been extended to more complex amino-acids, in which cases, as will appear, partial or complete racemization may occur.

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As might be expected, the α -imino-group of proline is readily acetylated by ketene in alkaline solution. Basic amino-acids on the other hand react only with their amino-groups; thus from *l*(+)-arginine and *l*(-)-histidine the corresponding mono-acetyl compounds were obtained. Lysine, however, reacts with ketene to form a diacetyl derivative. It may be noted in passing that although the glyoxaline group of histidine does not react with ketene in aqueous solution, glyoxaline and 4-(or 5)-methylglyoxaline when dissolved in indifferent solvents are changed by ketene into non-crystalline unsaturated compounds not giving the Pauly reaction. Probably ring fission and simultaneous acetylation occur and the acetylated unsaturated diamines [Bamberger & Berl, 1893] formed in the first instance, undergo polymerization.

Aliphatic hydroxyl groups do not react readily with ketene even in indifferent solvents as shown by the fact that from ethyl *dl*- β -hydroxyglutamate in ether the *N*-acetyl compound only was obtained. In marked contrast to this is the behaviour of aliphatic thiol groups. If cysteine dissolved in 2 equiv. of alkali is treated with ketene both the amino- and thiol-groups are acetylated; α -thiol-propionic acid is likewise acetylated in aqueous alkali.

However, if α -thiolpropionic acid is treated with ketene in ether instead of water, a mixed anhydride of *S*-acetyl- α -thiolpropionic and acetic acids is formed. The mixed anhydride which can be distilled decomposes in the presence of water to form *S*-acetyl- α -thiolpropionic acid; this reaction is not surprising since ketene is known to form mixed anhydrides with carboxylic acids in indifferent solvents [Hurd & Dull, 1932]. Dicarboxylic acids, however, react with ketene in indifferent solvents to form internal anhydrides; thus from β -phenyl-glutaric acid the corresponding anhydride was readily produced.

It was noticed in several cases, especially when an excess of alkali was used, that the action of ketene on amino-acids resulted in partial or complete racemization. This is probably due to the action of acetic anhydride, which is almost certainly the first reaction product between ketene and water, and which, as mentioned above, racemizes acetamino-acids at alkaline reactions. Thus arginine is completely racemized, in agreement with the observation of du Vigneaud & Meyer [1932] who found that *N*-acetyl-*l*(+)-arginine is racemized by acetic anhydride even without addition of alkali.

From these results it can be concluded that if proteins are treated with ketene in alkaline solution acetylation may take place at amino-, thiol- and phenolic-groups, whilst guanidino- and aliphatic hydroxyl-groups will remain unaffected. The possibility that the glyoxaline group might react with ketene, when the carboxyl group is blocked, cannot be completely excluded; it is well known that the Bamberger fission does not take place with substituted glyoxalines containing free carboxyl groups in the side chain whilst the esters of such compounds undergo the fission in the normal manner [Windaus *et al.* 1921]; it is therefore possible that, although the glyoxaline ring of free histidine is not attacked by ketene in aqueous solution, the amino-acid may be so attacked when it is in peptide linkage.

EXPERIMENTAL

Action of pyridine and acetic anhydride on amino-acids in the cold

Preparation of acetyl-dl-phenylalanine. *dl*-Phenylalanine (1 g.) was suspended in a mixture of pyridine (5 ml.) and acetic anhydride (8 ml.) and the suspension was shaken mechanically for 2 hr. at 2°. The clear solution was left for 2 days at this temperature and then poured into ice-water. After standing for 4 hr. the solution was evaporated to dryness under reduced pressure,

the evaporation being twice repeated after addition of small amounts of water. The residue was taken up in a small amount of ethyl acetate, the ethyl acetate solution was dried and the substance was crystallized by addition of light petroleum. Yield was 85 % of the theoretical. Recrystallization was effected from water. At no stage of the preparation could a positive nitroprusside reaction for ketones be obtained. M.P. 148° (uncorr.). (Found: N (Kjeldahl), 6.85 %. $C_{11}H_{13}O_3N$ requires N, 6.76 %.) Titration: 20.9 mg. neutralized 5.10 ml. 0.02 *N* NaOH (cresol red). $C_{11}H_{13}O_3$, titrating as a monocarboxylic acid, requires 5.05 ml.

Preparation of acetylhistidine. *l*(-)-Histidine (1 g.) was suspended in pyridine (5 ml.) and acetic anhydride (8 ml.) and the mixture was shaken at 2° for 2 hr. and then left for 2 days at this temperature. Since part of the solid still remained undissolved the suspension was shaken for 6 hr. more at room temperature. The solution, which now contained only a small amount of solid matter, was poured into ice-water. A very faint reaction with nitroprusside was obtained. Removal of pyridine and acetic acid was carried out as described above. The residue obtained after evaporation was placed in a dish in a desiccator over absolute alcohol at a pressure of 60 mm. After a few hours the product crystallized out and was filtered off and dried. Yield 80 % of the theoretical. Recrystallization was effected by addition of acetone to a concentrated aqueous solution. (Found: N (Kjeldahl using SeO_2 as catalyst), 19.35 %. $C_8H_{13}O_4N_3$ requires N, 19.53 %.) M.P. 155° (not sharp) $[\alpha]_D = +22.5^\circ$. Acetyl-*l*(-)-histidine [Bergmann & Zervas, 1928] has M.P. 169° and $[\alpha]_D = +44.7^\circ$. It is clear therefore that partial racemization has occurred during acetylation.

Action of ketene on amino-acids and related compounds

Ketene was generated from acetone as described by Herriott [1934] in an all-glass apparatus in order to avoid the formation of polymerization products.

Preparation of acetyl-l(-)-proline. *l*(-)-Proline (250 mg.) was dissolved in 25 ml. 0.4 *N* NaOH and treated with ketene for 45 min. 5 ml. 2 *N* HCl were then added and the solution was evaporated to dryness under reduced pressure. The residue was taken up in alcohol, the solution filtered and again evaporated. The residue was taken up in ethyl acetate and crystallized by addition of light petroleum and recrystallized from chloroform and light petroleum. Yield was 80 % of the theoretical. M.P. 115° (uncorr.). (Found: N, 8.84 %. $C_7H_{12}O_3N$ requires 8.86 %. Titration: 12.6 mg. neutralised 4.01 ml. 0.02 *N* NaOH. $C_7H_{12}O_3N$ requires 3.98 ml.) $[\alpha]_D = -104^\circ$. *N*-acetyl-*l*(-)-proline as described by du Vigneaud & Meyer [1932] has M.P. 117° (uncorr.) and $[\alpha]_D = -106.7^\circ$.

Preparation of acetyl-dl-arginine. 1 g. of crystalline arginine prepared [Bergmann & Zervas, 1926] from the hydrochloride of $[\alpha]_D = +17^\circ$, was dissolved in 25 ml. CO_2 -free water and ketene was passed into the solution for 2 hr. The solution was evaporated to dryness and the residue was dried over KOH in a desiccator. The syrup was dissolved in a small quantity of water and crystallized by addition of alcohol. The product had M.P. 253° (decomp.) and lost no water on drying over P_2O_5 at 60° at 1 mm. (Found: N, 26.03 %. $C_8H_{11}O_3N_4$ requires 25.93 %.) A 5 % solution was optically inactive.

An inactive preparation of the acetyl compound was also obtained when a solution of *l*(+)-arginine hydrochloride ($[\alpha]_D +17.0^\circ$) containing 2 equiv. of NaOH was treated with ketene at 0°.

Preparation of acetylhistidine. *l*(-)-Histidine, crystallized according to Vickery & Leavenworth [1927], was treated with ketene and the resulting

solution was worked up as described for arginine. The product was only partly racemized. It had $[\alpha]_D = +28.5^\circ$; pure acetyl *l*(-)-histidine has $[\alpha]_D = +40.5^\circ$.

Action of ketene on lysine. No crystalline acetyl derivative was obtained from lysine, but on treating 1 g. of *l*(+)-lysine dihydrochloride dissolved in water containing 3 equiv. of alkali with ketene a product was obtained which was soluble in alcohol and ethyl acetate. A solution, after ketene treatment, containing 5.7 mg. of total nitrogen per ml. gave 0.31 mg. of amino-nitrogen, in the Van Slyke apparatus, after shaking for half an hour, indicating that almost quantitative acetylation of both amino-groups had taken place.

Action of ketene on glyoxalines. Ketene was passed into a solution of 1 g. glyoxaline in anhydrous ether (20 ml.) which was kept at -15° to avoid formation of polymerization products of ketene. An amorphous product was obtained which was easily soluble in water, slightly soluble in alcohol and insoluble in ether and did not give a reaction with diazobenzenesulphonic acid in Na_2CO_3 even after acid hydrolysis. A chloroform solution of the substance took up bromine and decolorized alkaline permanganate. Boiling the product with methyl alcoholic HCl [Windaus *et al.* 1921] led to an amorphous product which did not take up bromine any more and from which only a partly crystalline *p*-nitrophenylosazone was obtained; the latter could not be further purified. The product obtained by the action of ketene took up hydrogen in the presence of Pd but only to the extent of 25% of the amount calculated for an ethylenediamine.

Similar products were obtained by the action of ketene on 4(or 5)-methylglyoxaline.

Preparation of ethyl N-acetyl-dl- β -hydroxyglutamate. Ethyl *dl*- β -hydroxyglutamate hydrochloride (2.2 g.) was dissolved in a small quantity of water; to the chilled solution ether and solid K_2CO_3 were added until the aqueous layer formed a thick paste. The paste was extracted several times with ether and the combined extracts were dried first with K_2CO_3 and then with freshly prepared BaO. Into the ethereal solution ketene was passed at -15° for 1 hr. After removing the ketene by a stream of nitrogen the ethereal solution was concentrated to a syrup, which crystallized after standing in a desiccator at 0° for 2 days. Recrystallization was effected from ether. Yield was 53% of the theoretical. M.P. 46° (uncorr.). (Found: N, 5.29%. $\text{C}_{11}\text{H}_{19}\text{O}_6$ requires N, 5.32%.) Acetyl content: found 17.1%. $\text{C}_{11}\text{H}_{19}\text{O}_6$ requires N, 16.47%.

Reaction of ketene with thiol groups

Preparation of NS-diacetylcysteine. *l*(+)-Cysteine hydrochloride (1 g.) was dissolved in a small quantity of water containing 3 equiv. of NaOH; ketene was passed into the solution for 1 hr. The solution was then acidified with 3 equiv. of H_2SO_4 and extracted with ethyl acetate; the residue from the evaporation of the combined and dried extracts was dissolved in a small quantity of ethyl acetate and, on addition of light petroleum (B.P. $120-140^\circ$), a crystalline precipitate was obtained. Yield 50% of the theoretical. M.P. $111-112^\circ$ (uncorr.). (Found: N, 6.84%. $\text{C}_7\text{H}_{11}\text{O}_4\text{NS}$ requires 6.83%.) Titration: 20.3 mg. neutralized 4.75 ml. 0.02*N* NaOH. $\text{C}_7\text{H}_{11}\text{O}_4\text{NS}$, as a monobasic acid, requires 4.95 ml. Acetyl estimation: acetyl found 41.3%; calculated for 2 acetyl groups 41.95%.

The substance is soluble in water, alcohol and most organic solvents. The nitroprusside reaction develops very slowly but the colour remains constant for many hours indicating a slow splitting of the thioester linkage by the ammonia employed in the reaction.

Preparation of S-acetyl- α -thiolpropionic acid. To a solution of freshly distilled α -thiolpropionic acid (5 g.) in freshly boiled out water (50 ml.) 30 ml. of 5*N* NaOH were added with exclusion of air. Ketene was passed into this solution for 2 hr. at 0°. The bulk of the acetic acid was then removed by extraction with ether; the solution was acidified with 30 ml. of 5*N* HCl and again extracted with ether. After removal of the ether an oil was obtained which could be distilled under very low pressure. It had b.p. 132–133° at 1 mm.; it was soluble in water, alcohol, ether and chloroform. (Found: S (Carius), 21.95%. $C_5H_8O_3S$ requires S, 21.62%.) The oil titrates as a monobasic acid; 142.4 mg. neutralized 9.69 ml. 0.1*N* NaOH (cresol red); calc. for $C_5H_8O_3S$, 9.62 ml. Saponification: 10 ml. 0.1*N* NaOH were added to the neutralized solution, and the mixture heated at 70° for 3 hr.; 1 ml. 0.1*N* HCl was then added and the solution was boiled to remove CO_2 and titrated with 0.1*N* NaOH to pH 7.5. Total amount of alkali required 9.65 ml.; calculated for 1 mol. of acid liberated 9.62 ml.

Preparation of a mixed anhydride of S-acetyl- α -thiolpropionic and acetic acids. 5 g. of α -thiolpropionic acid (freshly distilled) were dissolved in absolute ether (25 ml.) and treated with ketene. The solution was then evaporated to dryness and the oily residue which had a very pungent odour was dried in a desiccator over KOH and conc. H_2SO_4 . It was finally obtained as a water-clear mobile liquid by distillation at 0.25 mm. with a bath-temperature of 77–83°. Yield was 5.59 g. (Found: S, 17.1%. $C_7H_{18}O_4S$ requires S, 16.84%.) The oil is soluble in most organic solvents, and insoluble in water; on prolonged contact with water, however, it dissolves and loses its pungent odour.

Preparation of S-acetyl- α -thiolpropionic acid from the mixed anhydride. 2.5 g. of the mixed anhydride were shaken with 5 ml. of water for 5 hr. when the oil had completely dissolved; the solution was then evaporated to dryness and the residue was left in a desiccator over KOH. It was then distilled; b.p. 133°/1 mm. (Found: S, 22.05%. $C_7H_8O_3S$ requires S, 21.62%.) Titration: 123.2 mg. required 8.36 ml. 0.1*N* NaOH for neutralization to cresol red; calculated for $C_5H_8O_3S$ as a monobasic acid 8.37 ml.

Preparation of β -phenylglutaric acid anhydride. β -Phenylglutaric acid (2 g.) dissolved in absolute ether (25 ml.) was treated with ketene. The oil obtained crystallized after 2 days' standing in a desiccator, and was recrystallized from benzene. Yield 77% of the theoretical. m.p. 101°; a mixture with a sample prepared by the action of acetyl chloride showed no depression of m.p. (Found: C, 69.64; H, 5.35%. $C_{11}H_{10}O_3$ requires C, 69.47; H, 5.30%.)

REFERENCES

- Bamberger & Berl (1893). *Liebigs Ann.* **273**, 352.
 Bergmann & Stern (1930). *Ber. dtsh. chem. Ges.* **63**, 437.
 ——— & Witte (1926). *Liebigs Ann.* **449**, 277.
 ——— & Zervas (1926). *Hoppe-Seyl. Z.* **152**, 282.
 ——— (1928). *Biochem. Z.* **203**, 280.
 Dakin & West (1928). *J. biol. Chem.* **78**, 91, 743.
 Herriott (1934). *J. gen. Physiol.* **18**, 69.
 ——— & Northrop (1934). *J. gen. Physiol.* **18**, 35.
 Hurd & Dull (1932). *J. Amer. chem. Soc.* **54**, 3427.
 Neuburger (1938). *Biochem. J.* **32**,
 Stern & White (1938). *J. biol. Chem.* **122**, 371.
 Vickery & Leavenworth (1928). *J. biol. Chem.* **76**, 701.
 du Vigneaud & Meyer (1932). *J. biol. Chem.* **98**, 295.
 Windaus, Dorries & Jensen (1921). *Ber. dtsh. chem. Ges.* **54**, 2745.