91. *The Dehydration and Racemisation of* N-*Acyl*-L-*aspartic Acids by Acetic Anhydride.*

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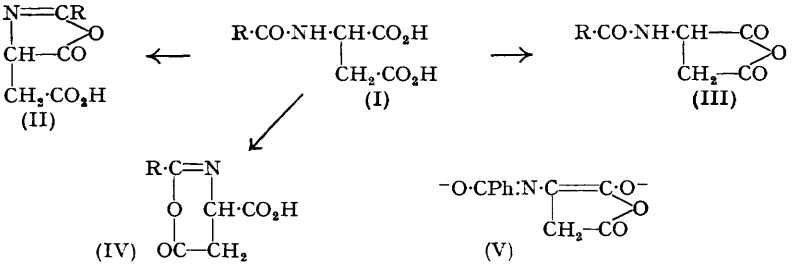
The salts of *N*-acetyl- and *N*-benzoyl-L-aspartic acids resist racemisation by aqueous acetic anhydride and evidence is adduced to show that they form internal anhydrides rather than oxazolones under these conditions.

Two crystalline products formed from *N*-acetyl-L-aspartic acid by acetic anhydride have been identified as the racemic and the optically active form of *N*-acetylaspartic anhydride, and not, as has been claimed by earlier workers, 2-methyl-5-oxazolon-4-ylacetic acid and *N*-acetyl-L-aspartic anhydride, respectively. The anhydride structure usually attributed to the dehydration product from *N*-benzoyl-L-aspartic acid has been confirmed.

The behaviour of *N*-acetyl- and *N*-benzoyl-L-aspartic anhydride with aqueous sodium hydroxide is described and discussed.

Optically active α-acetamido-acids are readily racemised by treating aqueous solutions of their salts with acetic anhydride (du Vigneaud and Meyer, *J. Biol. Chem.*, 1932, **98**, 295). This process, which is general, has been successfully applied by Barker, Hughes, and Young (*J.*, 1952, 1574) to a benzoylated hydrolysate obtained from gliadin in order to estimate the total glutamic acids (D plus L) by the method of isotope dilution. An attempt to apply the same process to the estimation of total aspartic acids revealed that neither *N*-acetyl- nor *N*-benzoyl-L-aspartic acid was readily racemised and thus led to the present investigation.

Because an *N*-acylaspartic acid (I) is both an α- and a β-acylamino-acid, it presents the possibility of dehydration to an oxazolone (II), a true anhydride (III), or a 5 : 6-dihydro-6-keto-1 : 3-oxazine (IV). Since the intermediate formation of (II) is the essential step in



the racemisation of an α-acylamino-acid by aqueous acetic anhydride (cf. *Adv. Protein Chem.*, 1948, **4**, 356), the preferential formation of (III) or (IV) would account for its failure in this case. The only recorded instance of the dehydration of a β-acylamino-acid to a 1 : 3-oxazine derivative is the conversion of β-benzamido*iso*valeric acid into 5 : 6-dihydro-6-keto-4 : 4-dimethyl-2-phenyl-1 : 3-oxazine by Baker and Ollis (*J.*, 1949, 345), who point out that this type of compound could play a part in the chemistry of aspartic acid. Some further observations on the dehydration of β-benzamido-carboxylic acids will be offered in a later paper.

The dehydration of *N*-acetyl-L-aspartic acid by acetic anhydride has been examined by Harington and Overhoff (*Biochem. J.*, 1933, **27**, 338), who reported the isolation of an oxazolone (II; R = Me), m. p. 145—146°, when the dry acid was boiled with acetic anhydride, and of a true anhydride (III; R = Me), m. p. 175°, from the moist acid and acetic anhydride at 95°. They obtained *N*-acetyl-L-aspartic acid as a microcrystalline solid, m. p. 142—143°, by acetylation of an aqueous, alkaline solution of L-aspartic acid with acetic anhydride, but in the present work this procedure invariably gave a syrup which contained traces of sodium acetate. Gordon, Martin, and Synge (*Biochem. J.*, 1943, **37**, 82) also record this acid as a syrup. Dehydration of this acetate-containing material gave an optically inactive compound, m. p. 143—144°, identical with that prepared by dehydrating crystalline, acetate-free *N*-acetyl-DL-aspartic acid. Dehydration of acetate-

free *N*-acetyl-L-aspartic acid, however, gave a material, m. p. 173°, which was hydrolysed by hydrochloric acid to give L-aspartic acid free from the D-isomer. The two dehydration products gave a mixed m. p. 156—160° and are clearly racemic and optically active forms of the same compound. The acetate-free *N*-acetyl-L-aspartic acid was produced as a syrup by acetylating an aqueous solution of L-aspartic acid without the addition of alkali. It was subsequently found possible to obtain it crystalline (m. p. 140—141°) by evaporating an aqueous solution of the optically active dehydration product. The crystalline acid induced crystallisation of the crude, non-crystalline acid produced by acetylation of L-aspartic acid, but recrystallisation of this acid was not a practicable means of purification.

The dehydration product, m. p. 143—144°, was given the oxazolone structure by Harington and Overhoff (*loc. cit.*) because it gave 2-methyl-5-oxazolon-4-ylacetyl chloride when treated with phosphorus pentachloride. Such behaviour, however, is not incompatible with the anhydride structure (II; R = Me) and this structure is now attributed to both the dehydration products for following reasons: (i) Both substances failed to react with diazomethane, implying the absence of carboxyl groups. (ii) Both substances reacted with ammonia to give amides, yielding acetaldehyde when treated with sodium hypobromite and then hydrolysed with 20% sulphuric acid. The acetaldehyde could only have arisen by decarboxylation of formylacetic acid produced by degradation of *N*-acetylaspartic α-amide. The 1 : 3-oxazine derivative (IV) could not give rise to an α-amide. (iii) The racemic dehydration product, m. p. 143—144°, gave with aniline a mixture of anilides from which the less soluble *N*-acetyl-DL-aspartic β-anilide, m. p. 198—199°, was readily isolated. The structure of this follows from its dehydration to a crystalline oxazolone, which gave with ammonia *N*-acetyl-DL-aspartic α-amide β-anilide. The latter was identified as an α-amide by the reaction previously used to identify *N*-acetylaspartic α-amide. The formation of mixed anilides is only compatible with the anhydride structure (III). (iv) The complete absence of racemisation when the optically active dehydration product was hydrolysed by hydrochloric acid is incompatible with the oxazolone structure (II) ("The Chemistry of Penicillin," Princetown Univ. Press, Princetown, New Jersey, p. 742).

A similar study of the action of diazomethane, ammonia, and hydrochloric acid, respectively, on the dehydration product, m. p. 204—205°, obtained from *N*-benzoyl-L-aspartic acid, has confirmed the anhydride structure (III; R = Ph) generally attributed to it.

It was previously reported that unpurified *N*-acetyl-L-aspartic acid retained 87% of its optical activity under the racemising conditions employed by du Vigneaud and Meyer (Barker, *Nature*, 1951, **168**, 908). It has now been found that an aqueous solution of the pure acid, prepared by dissolving recrystallised *N*-acetyl-L-aspartic anhydride in water, retained 96% of its optical activity when treated in the same manner. When the same racemising process was applied to *N*-benzoyl-L-aspartic acid only 76% of its optical activity was retained; when L-asparagine was benzoylated in aqueous sodium hydrogen carbonate and the resulting solution treated with acetic anhydride, the product retained only 5% of its optical activity. The last result is attributed to the ability of *N*-benzoyl-L-asparagine to form an oxazolone but not an anhydride.

Much more extensive racemisation occurred when pre-formed *N*-acylaspartic anhydrides were treated with aqueous alkali. *N*-Acetyl-L-aspartic anhydride retained 35% of its optical activity with N-sodium hydroxide at room temperature, while *N*-benzoyl-L-aspartic anhydride was completely racemised at the same temperature. The latter anhydride was insoluble in water but in N-sodium hydroxide immediately gave a faintly yellow solution which showed a strong, blue fluorescence under ultra-violet light. Both the colour and fluorescence faded as hydrolysis of the anhydride proceeded. The solubility, fluorescence, and racemisation can be explained by postulating the formation of the ion (V) in which the benzene ring is conjugated with two enolic double bonds. The absence of this stabilising conjugation in the corresponding ion derived from *N*-acetyl-L-aspartic anhydride can well explain its reduced tendency to racemise.

The isolation of crystalline anhydrides from these *N*-acylaspartic acids and acetic anhydride does not, of course, preclude the presence of other dehydration products in

equilibrium in solution. The partial racemisation produced by du Vigneaud and Meyer's method may take place through intermediate formation of small amounts of oxazolone, but it is pertinent that *N*-benzoyl-L-aspartic acid forms considerable amounts of the anhydride under these conditions as judged by the formation of a strong, blue fluorescence on addition of sodium hydroxide.

Experimental

M. p.s are uncorrected. Microanalyses are by Drs. Weiler and Strauss, Oxford.

N-*Acetyl*-L-*aspartic Acid.*—Acetic anhydride (25 c.c.) was added to a solution of L-aspartic acid (5 g.) in hot water (100 c.c.), the mixture cooled quickly to room temperature, more acetic anhydride (75 c.c.) added, and the whole stirred for 6 hours at 20° (cooling). A sample of the reaction product then gave no colour when buffered with sodium hydrogen carbonate and heated with ninhydrin. Removal of the solvents at 11 mm. gave *N*-acetyl-L-aspartic acid as a gum (6·7 g.), which, since it was free from metal acetates, could be used without purification in the preparation of *N*-acetyl-L-aspartic anhydride. This gum slowly crystallised when seeded and, after being washed with a little aqueous acetone and dried (P_2O_5), yielded microcrystalline *N*-acetyl-L-aspartic acid, m. p. 139—140°. Harington and Overhoff (*loc. cit.*) give m. p. 142—143°.

N-*Acetyl*-L-*aspartic Anhydride.*—Acetic anhydride (30 c.c.) and non-crystalline, acetate-free *N*-acetyl-L-aspartic acid (5 g.) were heated at 95—100° for 20 minutes. The resulting solution was filtered, reduced to half-volume at 11 mm., and cooled in ice for an hour. The crystalline *N*-acetyl-L-aspartic anhydride (2·7 g.) was washed with acetic anhydride, ethyl acetate, and finally light petroleum (b. p. 40—60°) and had m. p. 170—173°. Recrystallisation from acetic anhydride raised the m. p. to 173° [Harington and Overhoff (*loc. cit.*) give m. p. 175°] (Found: N, 8·8. Calc. for $C_6H_7NO_4$: N, 8·9%). Evaporation of an aqueous solution of the anhydride gave *N*-acetyl-L-aspartic acid as a syrup which slowly crystallised (m. p. 140—141°).

N-*Acetyl*-DL-*aspartic Anhydride.*—(*a*) *N*-Acetyl-DL-aspartic acid (5 g.) was prepared and dehydrated in the same manner as the optically active acid, giving *N*-acetyl-DL-aspartic anhydride (2·2 g.), m. p. 143—144° (Found: N, 8·7%). Harington and Overhoff (*loc. cit.*) describe this substance as 2-methyl-5-oxazolon-4-ylacetic acid and give m. p. 145—146°.

(*b*) *N*-Acetyl-L-aspartic acid, obtained as a syrup containing a little sodium acetate by aqueous alkaline acetylation of L-aspartic acid (Harington and Overhoff, *loc. cit.*), was dehydrated as in (*a*). The yield of *N*-acetyl-DL-aspartic anhydride, m. p. 143—144°, was similar.

N-*Acetyl*-DL-*aspartic* β-*Anilide.*—*N*-Acetyl-DL-aspartic anhydride (3·7 g.) was added to a solution of aniline (5 g.) in absolute ethyl alcohol (15 c.c.). The mixture became warm and gave a clear solution which quickly deposited crystals. Next morning the mixture was acidified with N-hydrochloric acid, the precipitate dissolved in dilute sodium hydroxide solution, the whole extracted with ether to remove aniline, and the aqueous layer acidified. After being kept at 0° overnight the precipitate was filtered off and washed with water. This crude material (4·3 g.), m. p. 180—182° (frothing), was boiled with 95% ethyl alcohol (40 c.c.), the mixture allowed to cool to room temperature, and the process repeated, giving N-*acetyl*-DL-*aspartic* β-*anilide* (2·9 g.), m. p. 197—198°. Recrystallisation from 95% ethyl alcohol raised the m. p. to 198—199° (Found: C, 57·3; H, 5·8; N, 11·4. $C_{12}H_{14}O_4N_2$ requires C, 57·6; H, 5·6; N, 11·2%). By evaporation of the combined alcoholic filtrates there was obtained a mixture, m. p. 176—179° (frothing), of *N*-acetyl-DL-aspartic α- and β-anilide (Found: C, 57·5; H, 5·8; N, 11·3%; equiv., 250. Calc. for $C_{12}H_{14}O_4N_2$: equiv., 250·3).

2-*Methyl*-5-*oxazolon*-4-*ylacetanilide.*—*N*-Acetyl-DL-aspartic β-anilide (1·0 g.) and acetic anhydride (5 c.c.) were heated at 95° for 20 minutes, the solvent removed at 11 mm., and the resulting syrup induced to crystallise by addition of a little ethyl acetate. The crystals were washed with benzene–ethyl acetate and then with light petroleum (b. p. 40—60°), giving 2-*methyl*-5-*oxazolon*-4-*ylacetanilide* (0·9 g.), m. p. 160° (from absolute ethyl alcohol) (Found: C, 61·6; H, 5·2; N, 11·7. $C_{12}H_{12}O_3N_2$ requires C, 62·1; H, 5·2; N, 12·1%).

N-*Acetyl*-DL-*aspartic* α-*Amide* β-*Anilide.*—2-Methyl-5-oxazolon-4-ylacetanilide (0·45 g.) was kept with aqueous ammonia (2 c.c.; *d* 0·88) for 12 hours and the resulting solid washed with water and dried. Recrystallisation from ethyl alcohol (63 c.c.) gave the α-*amide* β-*anilide* (0·38 g.), m. p. 216—218° (Found: C, 57·5; H, 6·0; N, 16·8. $C_{12}H_{15}O_3N_3$ requires C, 57·8; H, 6·1; N, 16·9%).

Ice-cold, 0·25N-sodium hypobromite was prepared by adding bromine (0·3 c.c.) to sodium hydroxide (1·4 g.) in water (12 c.c.) at 0°. *N*-Acetyl-DL-aspartic α-amide β-anilide (0·30 g.) was

added to 3·0 c.c. of this solution and stirred at 0° for 5 minutes and then at room temperature for 5 minutes, at the end of which the amide had dissolved. The solution was then kept at 50° for 5 minutes, sodium metabisulphite added until a negative reaction was obtained with starch–iodide paper, aqueous sulphuric acid (10 c.c.; 20%) added, and the solution slowly distilled into aqueous *p*-nitrophenylhydrazine hydrochloride. Acetaldehyde *p*-nitrophenylhydrazone (0·050 g.) was precipitated and, after crystallisation, had m. p. 129° alone and admixed with authentic material.

Degradation of N-*Acetyl- and* N-*Benzoyl-aspartic Amide.*—(*a*) *N*-Acetyl-DL-aspartic anhydride (0·70 g.) was added to aqueous ammonia (4 c.c.; *d* 0·88) and, after 30 minutes, the solution evaporated to dryness at 11 mm. Ice-cold, aqueous sodium hypobomite containing sodium hydroxide (2·0 g.) and bromine (0·62 c.c.) was added and the mixture worked up as in the previous degradation. Acetaldehyde *p*-nitrophenylhydrazone (0·29 g.) was obtained (m. p. and mixed m. p. 129°). Degradation of *N*-acetyl-L-aspartic anhydride gave similar results.

(*b*) *N*-Benzoyl-L-aspartic anhydride (1·0 g.) was added to aqueous ammonia (4 c.c.; *d* 0·88); after 2 hours the solution was diluted and acidified, and the precipitated amide(s) added to ice-cold, aqueous sodium hypobromite (11·5 c.c.; prepared as described for the α-amide β-anilide) and worked up as above. Acetaldehyde *p*-nitrophenylhydrazone (0·20 g.) was obtained (m. p. and mixed m. p. 129° .

N-*Benzoyl*-L-*aspartic Anhydride.*—*N*-Benzoyl-L-aspartic acid (5 g.; Fischer, *Ber.*, 1899, **32**, 2459) and acetic anhydride (25 c.c.) were heated at 100° for 30 minutes, reduced to 5 c.c. at 11 mm., and cooled in ice. The crystalline solid (3·5 g.) was recrystallised from a minimum of acetic anhydride and washed with dry acetone; it had m. p. 204—205° (Found: N, 6·2. Calc. for $C_{11}H_9O_4N$: N, 6·4%). Pauly and Weir (*Ber.*, 1910, **43**, 665) prepared this substance using acetyl chloride as dehydrating agent and gave m. p. 208—209° (corr.).

Racemisation of N-*Acetyl- and* N-*Benzoyl*-L-*aspartic Acid.*—(Specific rotations are calculated on the aspartic acid content.) (*a*) *N*-Acetyl-L-aspartic anhydride (1·238 g.), dissolved in water (5 c.c.), was kept overnight to ensure complete hydrolysis to *N*-acetyl-L-aspartic acid. The temperature was raised to 40°, hydrated sodium acetate (1 g.) and acetic anhydride (15 c.c.) were added, and the stirred mixture was kept at 40° for 8 hours. The resulting solution was evaporated to dryness over soda–lime in a vacuum desiccator, 6N-hydrochloric acid (15 c.c.) added, and the solution kept at 95° for 4 hours and then made up to 25 c.c. with 6N-hydrochloric acid. It then had $[\alpha]_D^{20}$ +23·8° (based on aspartic acid; $l = 2$) (duplicate, +23·2°). The two results correspond to an average racemisation of 4%. A control hydrolysis of *N*-acetyl-L-aspartic anhydride (0·7324 g. in 25 c.c. of 6N-hydrochloric acid) gave $[\alpha]_D^{20}$ +24·4° ($l = 2$).

(*b*) *N*-Benzoyl-L-aspartic acid (0·7608 g.) was treated as above, but the hydrolysate was extracted with benzene to remove benzoic acid and made up to 10 c.c. It gave $[\alpha]_D^{20}$ +18·5° (based on aspartic acid; $l = 1$), which corresponds to 24·5% racemisation. A control hydrolysis of *N*-benzoyl-L-aspartic acid (1·018 g.) gave $[\alpha]_D^{20}$ +24·5° (25 c.c.; $l = 2$).

Racemisation of N-*Acetyl- and* N-*Benzoyl*-L-*aspartic Anhydride.*—(*a*) *N*-Acetyl-L-aspartic anhydride (0·4652 g.) was kept in N-sodium hydroxide (8 c.c.) for 8 hours. Concentrated hydrochloric acid (11 c.c.) was added, and the whole kept at 95° for 4 hours, cooled to 20°, and made up to 25 c.c. with 6N-hydrochloric acid. It then gave $[\alpha]_D^{20}$ +8·6° (based on aspartic acid; $l = 2$), which corresponds to 65% racemisation.

(*b*) *N*-Benzoyl-L-aspartic anhydride (0·6512 g.) was dissolved in N-sodium hydroxide at room temperature, to give a faintly yellow solution which showed a strong, blue fluorescence under ultra-violet light. The colour and fluorescence faded completely during 3 hours. The solution was worked up as above with the inclusion of a benzene extraction to remove benzoic acid. It gave $\alpha_D^{20} = 0·00°$ ($l = 2$).

Racemisation of N-*Benzoyl*-L-*asparagine.*—L-Asparagine (1·40 g.; monohydrate) and sodium hydrogen carbonate (3·4 g.) were dissolved in water (15 c.c.), benzoyl chloride (2·3 c.c.) was added, and the whole stirred at 0—5° for 6 hours, whereafter acetic anhydride (15 c.c.) was added and stirring continued for a further 12 hours. Subsequent procedure followed that described for the racemisation of *N*-benzoyl-L-aspartic acid and gave $[\alpha]_D^{20}$ +1·1° (based on aspartic acid; 25 c.c., $l = 2$), which corresponds to 95·5% racemisation.

Grateful acknowledgment is made to the Distillers Co. Ltd. and to Imperial Chemical Industries Limited for grants.

UNIVERSITY COLLEGE, HULL. [*Received, September 27th*, 1952.]