Flavianates of Lysine

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Whilst investigating the separation of the bases of protein hydrolysates, it seemed necessary to ascertain whether lysine is precipitated by flavianic acid (2:4-dinitro-1-naphthol-7-sulphonic acid) in the proportions (4 equivalents) recommended by Kossel & Gross (1924) and later by Cox (1928) for the precipitation of arginine as monoflavianate. Should lysine be precipitated under these conditions, the use of flavianic acid for the removal of arginine and histidine from hydrolysates of proteins must also involve some loss of lysine. This would inevitably affect the conclusions to be drawn from experiments such as, for example, those described by Bunney & Rose (1928) who endeavoured to prepare in this manner an arginine-free diet. According to Schmidt (1944) flavianates of lysine have not yet been isolated, although the preparation of a very soluble mono-salt was claimed by Kossel & Gross (1924). The experiments which follow show clearly that lysine is precipitated quantitatively in the presence of excess flavianic acid. The preparation and properties of both a mono- and a di-flavianate of lysine are also described in detail. A preliminary report on this work has already been made (Heathcote, 1948).

EXPERIMENTAL

Unless otherwise stated, melting points throughout are uncorrected.

C, H and S analyses were carried out by Drs Weiler and Strauss, Oxford. N was determined by the Pregl micro-Kjeldahl method as modified to deal with nitro groups.

Commercial flavianic acid (British Drug Houses Ltd.) was recrystallized before use; the recrystallized product had m.p. 150° (decomp.).

Precipitates formed from lysine and flavianic acid in weakly acid solution

Lysine dihydrochloride (0.38 g.) was dissolved in 6.25 ml. of water and freshly prepared Ag₂O was added, with continuous stirring, until the solution was just neutral to congo red. AgCl was filtered off and washed with water and a warm solution of flavianic acid (1.25 g. in 6.25 ml. of water) was added to the filtrate. The mixture was allowed to cool to about 5°. A yellow precipitate, P₁, consisting of clusters of yellow needles formed quickly and in good quantity. Another solution of lysine dihydrochloride of half the concentration

used in the previous experiment was made by dissolving 0.19 g. in 6.25 ml. of water. After adjustment of the pH and removal of AgCl as before, the filtrate was treated with 0.625 g. of flavianic acid dissolved in 3.1 ml. of water. Refrigeration produced a crop of crystals, P₂, similar in appearance to those of P₁. Each precipitate was collected separately on the centrifuge, washed with distilled water at room temperature and again centrifuged. The crystals of P₁, after drying, had m.p. 238° (decomp.) whilst those of P₂ melted at 248° (decomp.). Control experiments carried out with flavianic acid solutions, at least as concentrated as those used in the above experiments, showed that little flavianic acid was precipitated on cooling. The precipitates could not, therefore, consist of flavianic acid of varying degrees of purity. The formation of a salt of lysine with flavianic acid was indicated.

The nature of the precipitates. The precipitate, P₂ (m.p. 248°), on recrystallization from distilled water had m.p. 244°. The tendency for the melting point of the flavianate to drop on recrystallization from aqueous solution, or on washing with water, indicated the partial decomposition of a higher flavianate to a lower salt. This was confirmed by the appearance of orange specks on the yellow precipitate when ethanol was the washing fluid and also by the fact that recrystallization of a portion of P₂ from dilute flavianic acid solution elevated the melting point. Recrystallization of P₂ from stronger solutions of flavianic acid (e.g. >0.5%) resulted, however, in lowering of the melting point again. This behaviour closely simulated that of histidine diflavianate (Vickery, 1927) and suggested that P₂ was the corresponding salt of lysine. The behaviour of the crystals on washing with water would also account for the difference in melting point between precipitates P₁ and P₂. A N analysis, performed on P₂, confirmed that the salt was lysine diflavianate. (Found: N, 10.6%. Monoflavianate requires 12.2, diflavianate requires 10.8%.)

Preparation of pure lysine diflavianate

A sample of lysine diflavianate (m.p. 265°) prepared from pure lysine and flavianic acid was recrystallized from very dilute flavianic acid solution and washed carefully with an ice-cold, saturated, aqueous solution of diflavianate prepared from a smaller sample of identical material. The pure diflavianate had m.p. 275° (decomp.). (Found: C, 40.0; H, 3.4; N, 10.6; S, 8.5. C₆H₁₄O₂N₂.2C₁₀H₆O₈N₂S requires C, 40.3; H, 3.4; N, 10.8; S, 8.3%.) No water of crystallization was present. When pure, lysine diflavianate crystallizes in pale-yellow platelets (Fig. 1). The tendency towards needle-like cluster formation observed in the early preparations was found to be due to contamination with small amounts of flavianic acid.

Recovery of lysine from aqueous solution as diflavianate. Providing that excess flavianic acid is present, lysine is precipitated quantitatively as diflavianate from aqueous solu-

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tion as the following experiment shows. Anhydrous lysine monohydrochloride (0.9214 g.), m.p. 263° and $[\alpha]_D^{19} + 9.3^\circ$, was dissolved in 5 ml. of water and treated with excess flavianic acid (4.066 g.) dissolved in 25 ml. of hot water. On cooling to room temperature, lysine diflavanate precipitated; precipitation was allowed to continue at 5° for 18 hr. The salt was filtered, washed with a cold saturated aqueous solution of lysine diflavanate, dried and weighed. The m.p. 259° indicated a fair degree of purity for the diflavanate isolated. The yield of diflavanate was 3.783 g. corresponding to a recovery of lysine of 97%.

Fig. 1. Lysine diflavanate. Magnification, $\times 400$.

Recovery of lysine as diflavanate from strongly acid solutions. Anhydrous lysine monohydrochloride (0.9553 g.), m.p. 263°, was dissolved in 40 ml. of 5% (v/v) H_2SO_4 . The solution was warmed and solid flavianic acid (5 g.) was stirred in until all cloudiness had disappeared. On cooling to room temperature the yellow precipitate of lysine diflavanate settled out readily and was allowed to reach equilibrium at 5° overnight. The precipitate was then filtered into a weighed, sintered glass crucible, washed with ice-cold water, and finally dried *in vacuo*. The yield was greater than that required for theoretical recovery and the melting point (220°) suggested that contamination of the diflavanate with flavianic acid had occurred.

Recrystallization was effected from the minimum volume of 0.1% flavianic acid solution and washing of the precipitate of diflavanate was carried out with a solution of lysine diflavanate saturated at room temperature. The lysine diflavanate isolated finally had m.p. 270° and in duplicate experiments the yield averaged 98% of the theoretical.

Recovery of lysine from mixtures of lysine and ammonia. The greater solubility of ammonium flavianate in strongly acid as well as aqueous solution seemed to offer possibilities for the separation of lysine from a mixture consisting of lysine and NH_3 in the absence of arginine and histidine.

Lysine monohydrochloride (1.911 g.) and $(NH_4)_2SO_4$ (A.R.) (1.446 g.) were dissolved with warming in 80 ml. of 5% (v/v) H_2SO_4 and 10 g. of flavianic acid were stirred into the warm solution. When all the flavianic acid had dissolved, the solution was cooled in the refrigerator for 18 hr. The yellow crystals which precipitated were collected (Büchner) and washed with ice-cold water. The crystals,

when examined under the microscope, were substantial platelets of lysine diflavanate, but some needles resembling ammonium flavianate were also present. The precipitate was recrystallized from 50 ml. of 0.5% flavianic acid solution, collected as before and, after washing with saturated aqueous lysine diflavanate solution, dried *in vacuo*. The yield of diflavanate indicated a recovery of 98%, but analysis of the combined mother liquors showed that these contained only 78% of the NH_3 . A final recrystallization from the minimum volume of 0.1% flavianic acid solution yielded 7.555 g. of lysine diflavanate (m.p. 266°), a recovery of 93%. The mother liquor was made to volume and analysed as before for NH_3 . The total recovery of NH_3 now amounted to 95% of that present in the original mixture.

Mixtures of lysine and NH_3 , in similar proportions to those used in the experiment above, have been obtained by the author in the application of Rice's (1939) method to hydrolysates of casein and precipitation of lysine as diflavanate offers a means of separation of these bases.

Decomposition of lysine diflavanate and recovery of lysine. It was frequently necessary to decompose lysine diflavanate in order to recover the lysine as hydrochloride and also to check its purity. Decomposition was usually effected by means of conc. HCl in the manner described by Cox (1928) for the decomposition of arginine monoflavanate. Subsequently, however, the method employed by Pratt (1926), namely, solution of the arginine flavianate in hot dilute H_2SO_4 followed by extraction of the flavianic acid from the hot solution with *n*-butanol, was adapted to the decomposition of the lysine salt. After extraction, the H_2SO_4 was removed with baryta and the mono- or di-hydrochloride of lysine prepared as required.

Lysine monoflavanate

Evidence was produced above that washing crystals of lysine diflavanate with water or ethanol causes partial conversion to the monoflavanate. Attempts to prepare lysine monoflavanate by recrystallizing the yellow diflavanate from aqueous solution, however, always produced an impure salt with a melting point which varied around 244°. In view of these failures, an effort was made to crystallize the mono-salt from aqueous-ethanolic solution.

The preparation of lysine monoflavanate. About 5 g. of partially decomposed lysine diflavanate (m.p. 244°) were dissolved in the minimum amount of hot water, and absolute ethanol (100 ml.) was then added to the hot solution. On cooling, orange-yellow crystals separated, slightly paler than those of arginine monoflavanate, but tending to encrust the sides of the flask in much the same manner as the latter salt. These crystals, which appeared as stellate needles under the microscope (Fig. 2), were collected, washed with absolute ethanol and desiccated *in vacuo*. They had m.p. 213° (decomp.), which is identical with that reported by Kossel & Gross (1924) for lysine monoflavanate. The melting point was unaltered by recrystallization from aqueous-ethanolic solution. (Found: C, 40.8; H, 4.6; N, 11.6; S, 6.9. Calc. for $C_{16}H_{24}O_8N_2S$, $C_{16}H_{24}O_8N_2S$: C, 41.7; H, 4.4; N, 12.2; S, 7.0%.) As in the case of lysine diflavanate, the mono-salt was found to contain no water of crystallization.

The solubility of lysine monoflavanate. The preparation of lysine monoflavanate described above indicated that recovery of this salt from the diflavanate might not be strictly quantitative. This was confirmed by the following experiment.

Lysine diflavanate, m.p. 259°, (1.593 g.) was dissolved in about 25 ml. of hot water, and absolute ethanol (100 ml.) was poured into the solution. The mixture was cooled to 5°, and lysine monoflavanate precipitated on scratching the sides of the flask. The solution was stirred frequently to aid further precipitation and another 25 ml. of ethanol were added. After 7 hr. in the refrigerator, the crystals were filtered off, washed with absolute ethanol and finally with ether. The yield of lysine monoflavanate (m.p. 213°) obtained was only 53% of the theoretical, but slight further precipitation occurred on leaving the filtrate several days in the refrigerator.

Fig. 2. Lysine monoflavanate. Magnification, $\times 400$.

Lysine monoflavanate must therefore possess considerable solubility even in strongly ethanolic solution, and this fact may be responsible in some measure for the failure of other workers to isolate the salt. This solubility is in marked contrast to that of arginine monoflavanate which is almost completely insoluble even in aqueous solution.

DISCUSSION

The preparation of a monoflavanate of lysine has confirmed the earlier claims of Kossel & Gross (1924). Lysine monoflavanate, which melts at 213°, is apparently unstable in aqueous solution and is relatively difficult to prepare.

By contrast, the diflavanate of lysine (m.p. 275°) is easily prepared. This salt is precipitated quantitatively from aqueous or strongly acid solution, provided that sufficient, i.e. a stoichiometric, or slightly larger, amount of flavianic acid is present. In the absence of sufficient flavianic acid, however, a product intermediate in composition between the mono- and di-salts is precipitated. This intermediate product possesses the crystalline appearance of the diflavanate. The ease of precipitation of lysine diflavanate can be utilized to separate lysine from a mixture of ammonia and lysine in the absence of

other bases. On the other hand, this same property might be responsible for many hitherto unexplained, or even unrecognized, complications. Reference has been made to the removal of arginine and histidine by Bunney & Rose (1928) from hydrolysates of casein. It would now appear that excess flavianic acid must involve at least partial removal of the nutritionally essential lysine under the conditions employed by these workers.

Fürth & Deutschberger (1927) have reported that the procedure of Kossel & Gross (1924), in which arginine is precipitated from weakly acid solutions of protein hydrolysates by excess flavianic acid, yields arginine monoflavanate of insufficient purity. It follows also from the present work that, in the precipitation of arginine as diflavanate from strongly acid solutions of such hydrolysates (Vickery, 1940), the coprecipitation of lysine diflavanate is more than likely. The work of Chibnall, Rees & Williams (1943), who experienced some difficulty in applying Vickery's diflavanate technique to hydrolysates of certain proteins, supports this view, and some additional evidence is provided by the results of (unpublished) experiments carried out by the author on mixtures of basic amino-acids. The existence of lysine diflavanate, in varying degrees of purity, may account also for some of the unidentified flavianates which have been reported elsewhere, e.g. Beveridge & Lucas (1944*a, b*). In this connexion, the isolation of a flavianate of glycine from hydrolysates of gelatin (Heathcote, unpublished data) should be mentioned. These facts show that flavianic acid is a more general precipitant of amino-acids than has hitherto been suspected.

A further interesting point is that Beveridge & Lucas (1944*a*) reported a decomposition point of 259° for arginine monoflavanate although Vickery's (1940) work showed that no definite decomposition point is given by this salt. In agreement with Vickery, no definite decomposition point for arginine monoflavanate has been observed during the course of the present work. Finally, the existence of lysine diflavanate may account for the discrepancy between the value reported by Cox (1928) and that reported by Vickery (1940) for the melting point of arginine diflavanate.

SUMMARY

1. The claim of Kossel & Gross (1924), hitherto in dispute, with regard to the existence of a very soluble monoflavanate of lysine has been confirmed.

2. The existence of a diflavanate of lysine is reported, and the bearing of its preparation and properties upon some analytical problems is discussed.

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The Inhibition of Plant Phosphorylases by β -Amylase and the Detection of Phosphorylase in Barley

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The enzyme phosphorylase may readily be detected in aqueous extracts of a number of starch-storing organs by the iodine-staining properties of the polysaccharide produced by its action on glucose-1-phosphate (Hanes, 1940*a, b*). In extracts of ungerminated cereal grains (wheat, oats, rye and barley), however, no such iodine-staining material arises under similar conditions. It appeared possible that the high concentration of β -amylase in cereal extracts might lead to β -amylolysis, either of the product of phosphorylase action on glucose-1-phosphate, or of the carbohydrate primer necessary to initiate synthesis. In such circumstances the absence of iodine-staining material does not preclude the presence of phosphorylase. Release of free phosphate would also be prevented if synthesis is inhibited by destruction of the primer, but if only the product of synthesis is destroyed then phosphate release should be observed. Accordingly, the fate of glucose-1-phosphate has been examined in more detail, and the effect of β -amylase on phosphorylase action studied, using preparations from the broad bean (*Vicia faba*) and the potato (*Solanum tuberosum*) as test systems. These investigations show that β -amylase inhibits phosphorylase action, but by some mechanism other than primer destruction, and further that phosphorylase is present in extracts of ungerminated barley. A preliminary communication on these experiments has been made (Porter, 1949).

EXPERIMENTAL

Substrates

Glucose-1-phosphate was prepared as the dipotassium salt by the method of Hanes (1940*b*), modified according to a Practical Class Sheet from Dr R. Hill, Biochemical

Laboratory, Cambridge. The twice recrystallized product (40 g.) was then dissolved in 500 ml. of water, the solution passed through cation and anion exchange columns (McCready & Hassid, 1944), and the ester recovered from the final effluent and again recrystallized. This additional procedure served to remove small traces of carbohydrate impurity which acted as primer in the phosphorylase reaction.

Amylose, prepared from potato starch by the thymol method (Bourne, Donnison, Haworth & Peat, 1948), was supplied by Dr W. J. Whelan.

Primers for the phosphorylase reaction

Soluble starch was prepared from commercial potato starch by the method of Hanes & Cattle (1938), and used in 1% (w/v) solution.

A solution of *achroic dextrins* was made by heating 1 g. of soluble starch with 25 ml. of 5*N*-H₂SO₄ in a boiling-water bath until the mixture just failed to give a colour reaction with I₂ (about 15 min.). The cooled solution was neutralized with 5*N*-NaOH and diluted to 100 ml.

Amylose glycollate, supplied by Dr W. J. Whelan, was prepared as described by Peat, Bourne & Thrower (1947) and then exhaustively treated with soya-bean β -amylase.

Enzymes

Potato phosphorylase I was prepared by the method of Hanes (1940*b*). The final precipitate at 50% saturation with (NH₄)₂SO₄ was taken up in 25 ml. of 0.25*M*-citrate at pH 6.8, and the solution stored in the cold. The preparation was self-priming with respect to polysaccharide synthesis after a time lag of about 45 min. and contained a little α -amylase.

Potato phosphorylase II and broad-bean phosphorylase were prepared by the Pb precipitation technique (Barker, Bourne & Peat, 1949) and supplied by Mr P. Hobson in the form of freeze-dried powders. With these two preparations the time lag before the onset of polysaccharide synthesis, in the absence of added primer, was much longer than with potato phosphorylase I, and only traces of α -amylase were present.