The Amino Acids and other Ampholytes of Urine

2. THE ISOLATION OF A NEW SULPHUR-CONTAINING AMINO ACID FROM CAT URINE

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The application of two-way paper chromatography to the study of the amino acids excreted in human urine (Dent, 1947) has facilitated the recognition of the amino acidurias which are associated with certain pathological conditions. The method has now been applied for several years and a large number of urine samples from normal and pathological subjects has been examined (Dent & Harris, 1951; Crumpler, Dent, Harris & Westall, 1951). Datta & Harris (1951) also investigated the urinary amino-acid patterns of other mammals and it was apparent from this study that there were wide variations in the pattern of amino-acid excretion to be observed between different species. This finding was, perhaps, not unexpected in view of the different diets involved. It was, however, surprising to find that a ninhydrin-reacting substance excreted by the domestic cat in quantity and by the ocelot in lesser amount was a hitherto undescribed amino acid.

This substance, now named 'felinine' but referred to as 'cat-spot' by Datta & Harris (1951), partially overlays leucine and isoleucine on the phenol-ammonia collidine-lutidine two-way paper chromatogram, but it can be readily separated from them on a one-way chromatogram run with *tert.*-amyl alcohol. After oxidation with hydrogen peroxide (Dent, 1948) the spot no longer appeared in its original position on two-dimensional chromatograms but in the presence of other amino acids it was difficult to decide whether it had moved to a new position or whether it had been degraded to such an extent that it failed to give a ninhydrin colour. However, the suspicion remained that felinine was a new sulphur-containing amino acid. The following sulphur-containing amino acids were run on two-way chromatograms: homocysteine, homomethionine, lanthionine, $\beta\beta$-dimethylcysteine, $\beta\beta\beta'\beta'$-tetramethylcystine and the series of normal C_1—C_5 *S*-alkylcysteines. However, the ninhydrin-sprayed spots due to these substances failed, in every instance, to match the position of felinine.

The pure amino acid has now been isolated from cat urine by fractionation on ion-exchange columns (Westall, 1952). Felinine was recovered from the primary fractionation on Zeo-Karb 215 mixed with other amino acids. Treatment of this mixture on columns of the anion-exchange resin Dowex 2 yielded fractions containing felinine contaminated only with a small proportion of another unknown amino acid designated as 'peach-spot' (on account of its ninhydrin colour reaction) and which moves to a position just above glutamic acid in the diagram of Dent (1948). Felinine and 'peach-spot' could not be separated by fractionation on either Zeo-Karb 215 or Dowex 2 resin columns at room temperature, but separation was achieved on a Zeo-Karb 215 column operated at 65° (Partridge & Brimley, 1951). Under these conditions the 'peach-spot' substance was largely degraded and a decomposition product was found in the early fractions whilst felinine was recovered from the later fractions.

The amino acid has not been crystallized. It was readily soluble in water and an aqueous solution, on evaporation, formed a syrup which could not be induced to crystallize. Addition of ethanol in small amounts gave a clear thixotropic gel and further additions yielded no crystals. An opaque white gelatinous precipitate was formed on addition of ether to the ethanol solution, but this material had no apparent crystalline structure. Precipitation with acetone from the aqueous-ethanolic solution provided the best method of isolating the substance. It is well known that small amounts of impurities inhibit the crystallization of certain amino acids but, in this case, such an explanation would appear unlikely. Samples from three separate isolations of the amino acid from different collections of cat urine have all yielded analyses which agree within very narrow limits.

The simplest empirical formula that could be derived was $C_8H_{17}O_3NS$ (mol.wt. 207) and a determination of the molecular weight (240) by freezing-point depression in water indicated that this formula was reasonable. Since this substance was retained (in quantity) by both cation- and anion-exchange resins it was inferred that it possessed a basic group and at least one acidic group. The total nitrogen (Dumas) could be entirely accounted for as NH_2-N by the nitrous acid method and an equivalent of carbon dioxide was liberated using the ninhydrin-carbon dioxide method with a citrate

buffer at pH 2·5. The material when run on the two-way chromatograms in the presence of copper carbonate (Crumpler & Dent, 1949) formed a copper complex and failed to give a ninhydrin spot. It was thus reasonable to suppose that the substance was an α-amino acid.

Oxidation of the amino acid

As the felinine contained sulphur, the change in position on a chromatogram of the ninhydrin spot after treatment with hydrogen peroxide was probably due to oxidation of a sulphur atom. Fig. 1

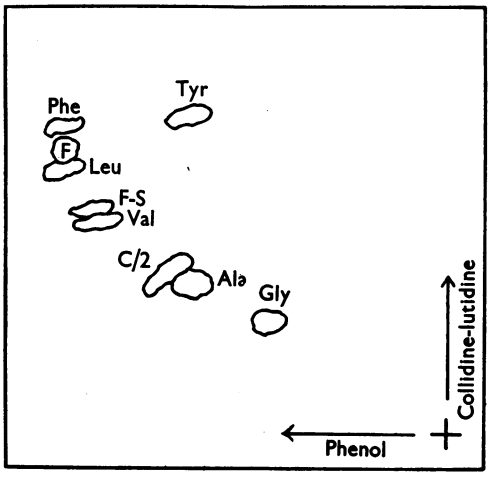

Fig. 1. A two-way paper chromatogram showing the relative positions of felinine and its derivatives. Ala, alanine; C/2, C/2 substance; F, felinine; F-S, felinine sulphone; Gly, glycine; Leu, leucine; Phe, phenylalanine; Val, valine; Tyr, tyrosine.

shows the position of its oxidation product in relation to several other amino acids. The substance was partly oxidized by air during chromatography, and faint spots were frequently observed on the chromatograms after treatment with ninhydrin. One of these spots appeared to be due to oxidation during the drying off of phenol before rerunning in the collidine-lutidine solvent mixture, whilst another could be due to the formation of a sulphoxide. A compound $C_8H_{17}O_5NS$ formed by oxidation of felinine with hydrogen peroxide has been isolated. Analysis indicated that the original substance had taken up two atoms of oxygen with the probable formation of a sulphone. This idea was supported by the observation that equimolar quantities of felinine and methionine took up the same amount (2·2 atoms/mol.) of oxygen from potassium permanganate. The oxidized derivative decomposed vigorously at 120–125° and slowly darkened on storage at room temperature.

Treatment with Raney nickel

Distillation of an aqueous solution of felinine in the presence of Raney nickel yielded water, together with a small quantity of a colourless liquid which formed an upper layer. This substance was identified as *iso*amyl alcohol by formation of a 3:5-dinitrobenzoate and by oxidation with dilute chromic acid to *iso*valeraldehyde which gave a crystalline derivative with 2:4-dinitrophenylhydrazine. Alanine was isolated from the reaction residue and proved to be the only ninhydrin-reacting substance present. From the evidence gained in the reaction with Raney nickel it is reasonable to assume that felinine contains cysteine linked to *iso*amyl alcohol by its sulphur atom (I). This structure is provisional.

$$HO_2C.CH(NH_2).CH_2.S.C(CH_3)_2.CH_2.CH_2OH \quad (I)$$

Degradation of the amino acid

When felinine was heated with concentrated hydrochloric acid some of the original substance was found unchanged, together with traces of cystine and cysteine, whilst a second unknown sulphur-containing amino acid was produced in fair yield (approx. 40 %). A brown oily substance was also liberated. The amino acids were fractionated first on a column of Zeo-Karb 215 and then on a column of Dowex 2 and the hydrolysis product (C/2) crystallized. Analyses gave the empirical formula $C_{11}H_{22}O_4N_2S_2$. When run on a two-way paper chromatogram using phenol-ammonia and collidine-lutidine the C/2 substance moved to a position close to alanine (Fig. 1). No ninhydrin-colouring spot was observed in this solvent system after oxidation of the substance with hydrogen peroxide. The oxidation product decomposes in phenol but it survived after running on paper with a butanol-acetic acid mixture (Partridge, 1948) and had R_F 0·10. Treatment of an aqueous solution of the C/2 substance with Raney nickel gave alanine in good yield as the only surviving ninhydrin-reacting substance.

Treatment of felinine with 2N-sodium hydroxide at 100° destroyed the amino acid and no substance remained which would yield a ninhydrin-reacting spot. All the nitrogen, presumably as ammonia, was trapped in acid when the substance was distilled in the presence of 2N-sodium hydroxide.

In an attempt to explain the series of degradations which occur when felinine is hydrolysed with acid the following scheme, based on the assumption that formula I represents the general structure, is provisionally suggested.

Under the treatment with acid some of the felinine breaks down with the liberation of free cysteine (II). The cysteine then condenses with the unchanged felinine eliminating water and forming a di-cysteine derivative (C/2) (III). This derivative would have

the found empirical formula ($C_{11}H_{22}O_4N_2S_2$) and would liberate alanine and *iso*pentane on treatment with Raney nickel. Whilst the formation of alanine has been confirmed, it has not, so far, been possible to characterize the hydrocarbon. However, some further evidence for the above scheme was provided by increased production of the dicysteine substance by the addition of L-cysteine to the mixture before hydrolysis. The production of a brown oil during the treatment with acid probably arose by polymerization of the C_5 residue remaining after the liberation of free cysteine. The identity of this substance is unknown, but analysis has shown that it contained neither nitrogen nor sulphur.

$$HO_2C.CH(NH_2).CH_2.S.CMe_2.CH_2.CH_2OH \quad (I)$$

$$\downarrow$$

$$(II) \quad HO_2C.CH(NH_2).CH_2SH + CH_2{:}CMe.CH_2.CH_2OH \rightarrow \text{Polymer}$$

$$\downarrow + (I)$$

$$(III) \quad HO_2C.CH(NH_2).CH_2.S.CMe_2.CH_2.CH_2.S.CH_2.CH(NH_2).CO_2H + H_2O.$$

EXPERIMENTAL

M.p.'s are given uncorrected.

Cat urine (5·2 l.) was concentrated under reduced pressure at 50° to 1 l. Ethanol (3 l.) was added and the mixture was left at 0° overnight. The precipitate was removed and washed with 80% (v/v) ethanol and the filtrate and washings were evaporated to a syrup. Water (2 l.) was added and this solution was shaken with 100 g. of charcoal powder (previously washed with 10% (v/v) aqueous acetic acid and then with water). The charcoal was removed by filtration and the clear solution, diluted to 10 l. with water, was run through a system of large columns containing the cation-exchange resin Zeo-Karb 215. The details of these columns and the method used for their operation were described in Part 1 of this series of papers (Westall, 1952). The amino acids were displaced with 0·25 N-ammonia and 40 fractions (each 50 ml.) were collected. The amino-acid composition of these fractions, determined by paper chromatography, is illustrated in Fig. 2. It will be observed that felinine occurred in fractions 5–20 (inclusive) along with a number of other amino acids. These fractions were bulked (800 ml.), boiled for 1 min. to expel CO_2 and transferred to a series of columns containing the anion-exchange resin Dowex 2. The amino acids were displaced with 0·1 N-HCl and collected in 55 fractions (25 ml. each). The amino-acid composition of these fractions is illustrated in Fig. 3. Fractions 27–45 contained felinine contaminated only with a trace of an unknown substance which gave a peach-coloured ninhydrin spot on the paper chromatograms. These two substances formed mixed bands when fractionated on both the Zeo-Karb 215 and Dowex 2 columns. However, a separation was achieved by using a heated Zeo-Karb 215 column (Partridge & Brimley, 1951). The series of small columns was jacketed and water at 65° was circulated continuously. The mixture (fractions 27–45 bulked) was applied to the columns in the usual way and the amino acids were displaced with 0·1 N-ammonia. Under these conditions the 'peach-spot' substance was concentrated in the early fractions, whereas the later fractions contained felinine only. Thirty fractions (each 25 ml.) were collected and nos. 5–28 were selected for the isolation. These fractions were combined and evaporated under reduced pressure to a pale-yellow syrup. Ethanol (10 ml.) was added, followed by 250 ml. of acetone which was added gradually with occasional shaking. After leaving overnight at 0° the white flocculent precipitate was filtered, washed with acetone and dried over H_2SO_4; yield 6·25 g., m.p. 177°, from 5·2 l. of cat urine. (Found: C, 46·2; H, 8·2; N (Dumas), 6·7; S, 15·4%. $C_8H_{17}O_3NS$ requires C, 46·4; H, 8·2; N, 6·8; S, 15·4%. Amino N (Van Slyke nitrous acid), 6·8%; carboxyl N, 6·7%; $[\alpha]_D^{20}$ −23° (*c*, 2·2 in water).)

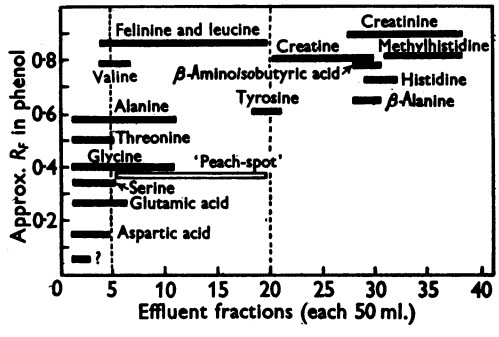

Fig. 2. Primary fractionation of cat urine on Zeo-Karb 215. Displacement chromatography using ammonia.

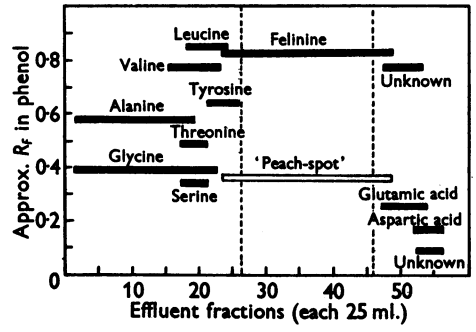

Fig. 3. Secondary fractionation on Dowex 2. Displacement chromatography using HCl.

Preparation of felinine sulphone

To 0·20 g. felinine in 25 ml. of water was added 2 ml. of 30% (v/v) H_2O_2 and the mixture was left at room temperature for 30 min. The solution was evaporated under reduced pressure at 40° to 10 ml. in order to remove excess H_2O_2. Paper-chromatographic analysis indicated that the original felinine had reacted completely. The aqueous solution was evaporated to a syrup and, after the addition of 1 ml. ethanol, a white flocculent precipitate was obtained by the gradual addition of 20 ml. of acetone. Yield 0·15 g.

m.p. 120–125° (decomp.). (Found: C, 40·7; H, 7·1; N (Dumas), 5·6; S, 12·9%. $C_8H_{17}O_5NS$ requires C, 40·2; H, 7·1; N, 5·8; S, 13·4%.)

Treatment with Raney nickel

Felinine (0·5 g.) was dissolved in 5 ml. water in a micro-distillation flask and 1 g. of Raney nickel (Bougault, Cattelain & Chabrier, 1938) was added, after 1 hr. the mixture was distilled and the first few drops of distillate were collected. Two layers were observed and the upper layer (alcohol) was transferred by means of a micropipette to a dry tube. Successive additions of about 0·2 g. of Raney nickel were made followed by distillation until the distillate failed to give two layers.

Isolation of alanine. The aqueous solution from the above experiment was filtered free from Raney nickel and transferred to a small column of Zeo-Karb 215. The column was washed with water and 0·1 N-NH_3 was applied to displace the alanine. As soon as a positive ninhydrin reaction was obtained on the effluent, fractions (approx. 5 ml.) were taken and the collections proceeded until the breakthrough of ammonia. Paper-chromatographic analysis of these fractions showed that alanine was the only amino acid present. The fractions were combined, evaporated to 3 ml. and the alanine was crystallized by addition of 3 vol. of acetone. Yield, 0·55 g. (72% of theoretical). (Found: N (Dumas), 15·6%. Calc. N, 15·73%.)

Preparation of the 3:5-dinitrobenzoate of the alcohol. 1 ml. of a solution of 1 g. 3:5-dinitrobenzoyl chloride (in 2 ml. ligroin + 8 ml. dry benzene) and 0·5 ml. of pyridine were added to the tube containing the unknown alcohol. The mixture was refluxed on a water bath for 30 min. After cooling, the mixture was treated in the usual manner and the resulting crystals after recrystallization from ethanol-ether (4:1, v/v) had m.p. 63·5°. A derivative prepared similarly from *iso*amyl alcohol gave m.p. 64°; mixed m.p. 63·5°.

Preparation of the 2:4-dinitrophenylhydrazone of the aldehyde obtained by mild oxidation of the alcohol. An aeration train of four tubes was connected in series: the first tube contained conc. H_2SO_4, the second 0·5 g. Raney nickel and 0·05 g. felinine in 1·5 ml. water, the third 1·5 ml. 2 N-H_2SO_4 with a small crystal of $K_2Cr_2O_7$ and the last tube contained 2 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl. A gentle stream of air was passed through the solutions and the tube which contained dilute chromic acid was kept at 50°. After allowing aeration to continue for 1 hr., the orange-coloured precipitate which formed in the last tube was filtered off and recrystallized from aqueous ethanol (m.p. 122·5°). *iso*Valeraldehyde 2:4-dinitrophenylhydrazone prepared similarly had m.p. 123°. Mixed m.p., 122·5°.

Action of acid on felinine and isolation of substance C/2

The amino acid (0·5 g.) was heated with 10 ml. 5 N-HCl in a sealed tube at 105° for 36 hr. 50–100 µl. of a brown oil separated out on the surface. Its composition is at present unknown but N and S were shown to be absent. After removing the oil, the solution was evaporated under reduced pressure and the resulting syrup was taken up in water (100 ml.). This solution was transferred to a column of Zeo-Karb 215 resin (two tubes in series; diam. 1·5 and 0·9 cm.; contents 8 and 2 g. resin respectively). The column was washed with water to remove the remaining traces of HCl from the hydrolysate and the amino acids were displaced with 0·12 N-NH_3. The effluent was collected in nine fractions (each 5 ml.). Paper-chromatographic analysis of these fractions showed that only a partial separation of the component amino acids had been obtained. These fractions, now free of HCl, were combined and transferred to a set of columns of Dowex 2 (two tubes in series; diam. 1·2 and 0·7 cm.; contents 5 and 1·5 g. of resin respectively). The amino acids were displaced with 0·05 N-HCl and nine fractions (each 5 ml.) were collected. Fractions 1–4 contained unchanged felinine, 2–9 the C/2 substance, whilst cystine and cysteine were present in 8 and 9. Fractions 4–7, which apparently contained C/2 substance only, were combined (20 ml.) and evaporated to 2 ml. A crop of white crystals was obtained. Yield, 103 mg.; m.p. 230–232°. (Found: C, 43·1; H, 7·1; N (Dumas), 9·0; S, 21·0%. $C_{11}H_{22}O_4N_2S_2$ requires C, 42·6; H, 7·1; N, 9·05; S, 20·6%.)

DISCUSSION

The urine used for this and other isolations was collected from small colonies of cats kept in metabolic cages but samples of urine from cats kept under domestic conditions have also been examined by two-way chromatograms and these have shown similar urinary patterns. Felinine is usually the most abundant amino acid excreted. The 24 hr. output (approx. 100 ml.) may contain as much as 100–120 mg. of the substance. Examination of cat blood-plasma ultrafiltrate by two-way paper chromatograms has shown no trace of felinine, but this evidence alone is insufficient to rule out the probability of its presence in low concentration.

The significance of the occurrence of this sulphur amino acid in cat urine is at present unknown. It is improbable that it occurs as such in the diet, as the amount excreted is relatively high; moreover, as the food given to the cats is similar to that consumed by man, it is unlikely that the amino acid would have escaped detection in these foods. Even so, the type of diet may have some influence on the excretion of felinine since in one pair of cats transferred from a mixed diet to one of fish only there was a marked decrease in the excretion of felinine with a simultaneous increase in the taurine output (Datta, S. P. & Harris, H., unpublished). In fermentation by yeast, *iso*amyl alcohol is known to arise from leucine by deamination and decarboxylation. In rats, Bloch (1944) found that, after feeding leucine or *iso*valeric acid labelled with deuterium, he obtained identical isotopic concentrations in the tissue cholesterol. From this he concluded that *iso*valeric acid is an intermediate in leucine metabolism. It is possible therefore that *iso*amyl alcohol occurs in the series of reactions leading from leucine to *iso*valeric acid. In cats, a partial block in this mechanism might lead to an accumulation of *iso*amyl alcohol which, being toxic, would probably conjugate with some other substance before being excreted.

Cysteine would be available for this reaction and felinine might be the non-toxic end product. On the other hand the new amino acid could be a normal product of mammalian metabolism and take part in the transfer of C_5 units (i.e. isoprene-type) or C_3 units (*iso*propyl units found by oxidation and removal of C_2 as acetate) in the synthesis of such substances as sterols or carotenes which require a branched-chain carbon skeleton. Whilst such a substance need, in most animals, occur only transiently, it is possible that in the cat, owing to some specific peculiarity, this substance is accumulated and excreted.

The C/2 substance which is formed on treatment with acid is not believed to occur naturally. No trace of it was observed on the two-way paper chromatograms of cat urine and none was encountered in the course of the large-scale fractionation.

The structure proposed for felinine must remain provisional until further proof becomes available. Some attempts have already been made to synthesize the new amino acid and also the C/2 substance derived from it. This work will be reported in a later paper.

SUMMARY

1. A new sulphur-containing amino acid (felinine) has been isolated from cat urine following a series of fractionations on ion-exchange resins.

2. This substance is excreted by the cat in amounts as high as 100–120 mg./day, which is greater in amount than that of any other single amino acid.

3. Degradative studies with Raney nickel led to the identification of alanine, *iso*amyl alcohol and hydrogen sulphide indicating that felinine is a *S*-hydroxyalkylcysteine, the alkyl group having the carbon skeleton of *iso*pentane.

4. On treatment with mineral acid felinine gives rise to a second sulphur-containing amino acid (C/2), which is believed to be a dicysteine derivative. This substance does not occur as such in cat urine.

The author wishes to record his thanks to Prof. F. Challenger and Dr C. E. Dent for their advice and interest during the course of this work. The micro-analyses for C, H, N and S were carried out by Weiler and Strauss, Oxford. I should also like to thank Dr S. P. Datta of the Biochemistry Department, University College, London, and Dr E. Reid of the Biochemistry Department, Cambridge, for supplies of cat urine.

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A Chemical Examination of Connective Tissue in Rheumatic Fever

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A characteristic feature of subcutaneous nodules in rheumatic fever and also in rheumatoid arthritis is the central core of eosinophil fibrillar material, usually termed 'fibrinoid' from its histological resemblance to fibrin. However, there is much conflict of opinion regarding the presence of fibrin in such lesions, and these opinions are based mainly on histological grounds (cf. Glynn & Loewi, 1952), and few chemical or physical studies have been made on such tissue. Much stronger evidence for the presence of degraded collagen in rheumatoid arthritis is afforded by electron-micrograph and X-ray studies of Kellgren, Ball, Astbury, Reed & Beighton (1951), and chemical analyses of rheumatoid nodules have shown that the collagen present has its normal content of hydroxyproline (Bien & Ziff, 1951). The purpose of the present investigation was to examine chemically rheumatic fever nodules in order to ascertain what difference, if any, existed between them and normal connective tissue. A preliminary report of this work has been given by Consden, Glynn & Stanier (1952).