CHROMATOGRAPHY OF THE N-CARBAMOYL-AMINO ACIDS (HYDANTOIC ACIDS)

by

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A study (to be published later) of the action of barium hydroxide on α-amino acids and, in particular, on arginine has shown the formation of several N-carbamoyl-amino acids ($NH_2 \cdot CO \cdot NH \cdot CHR \cdot COOH$).

With the exception of citrulline[1] (δ-N-carbamoyl ornithine), the behaviour of these compounds on paper chromatograms has not been studied before, and it is the purpose of this communication to report the results of such studies.

Most of the compounds used in the present work have been prepared by other workers[2–10], and although no record of the histidine, threonine and lysine (NN'bis-carbamoyl lysine) derivatives could be found, it is likely that these were prepared by BOYD[11] during the conversion of protein hydrolysates into hydantoins ($\overline{NH \cdot CO \cdot NH \cdot CHR \cdot CO}$). Certain of the derivatives are possibly of biological significance; thus N-carbamoyl glycine is said to occur in rat liver[12], ε-N-carbamoyl lysine has been used in dietary studies[13], and N-carbamoyl glutamic acid has been postulated as an intermediate in the *in vivo* synthesis of citrulline[14], though this has been contested[15]. Moreover DAKIN[16] obtained some of the derivatives from the urine of animals injected with large amounts of the corresponding α-amino acid, so that present results may be of interest in the study of the nitrogenous constituents of liver and urine.

These compounds are also referred to in the literature as hydantoic acids, α-carbamido-acids, α-ureido-acids, α-uramido-acids, α-uramino-acids, N-carbaminyl-acids and aminoformyl-amino acids. N-carbamoyl alanine may therefore be described as α-methyl hydantoic acid, α-carbamido-propionic acid, α-ureido-propionic acid, α-uramido-propionic acid, α-uramino-propionic acid, N-carbaminyl alanine or N-amino-formyl alanine. This complexity of nomenclature is increased by the use of the term "carbamido" in referring to the group NH_2CO- (amino-formyl group) as in carbamido-arginine[4]. Moreover, the term "carbamino-acids" is also used[17] for acids such as $HOOC.NH.(CH_2)_3.CH(NH_2).COOH$ (δ-carbamino-ornithine). The term hydantoic acids is used, though not exclusively, in Chemical Abstracts (The American Chemical Society), and in Beilstein's Handbuch. The 1952 report on nomenclature[18], however, specifies that the grouping NH_2CO– shall be known as the carbamoyl radical, and this terminology is therefore used here.

EXPERIMENTAL

Preparation. The derivatives were, in most cases, prepared by dissolving about 20 mg of the amino acid together with up to 5 equivalents of potassium cyanate in 0.5 ml of water in $6'' \times 1/2''$ test-tubes, and heating at 100° C for 1 to 4 h[10]. N-carbamoyl cysteine was similarly prepared, but with a stream of oxygen-free nitrogen passing through the solution[6]. ε-N-carbamoyl lysine was prepared by the method of STEVENS AND ELLMAN[13]. Some preparations were also made using urea or urethane in place of potassium cyanate.

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Chromatography. Chromatography was carried out at room temperature on Whatman No. 1 filter paper with the solvents ascending the paper. As a rule the upper phase from the mixture: *n*-butanol 5 vol., acetic acid 1 vol. and water 4 vol. was used as first solvent, and phenol 90% saturated with water as the second solvent. After the runs the solvents were dried off at room temperature in a current of air. Several other solvents were tried, including (a) *n*-butanol saturated with water, (b) *tert*-amyl alcohol-water with a trace of diethylamine, (c) collidine-water and (d) 70 %and 80% acetone-water. All these were inferior to butanol-acetic acid and phenol.

Detection of the derivatives. To detect free amino acids or those N-carbamoyl derivatives still bearing a free amino group, the chromatograms were dipped in 0.2% ninhydrin in acetone[18]. The N-carbamoyl-amino acids were detected as yellow spots by a spray of 4% *p*-dimethylamino-benzaldehyde in 1 *N* HCl. Cystine, cysteine and their derivatives were detected by the nitroprusside test. A stock solution of 2% nitroprusside saturated with urea was mixed just before use with half its volume of 20% sodium carbonate. This spray detected sulphydryl groups immediately as impermanent red spots, and disulphide groups were detected by adding to the spraying mixture one-third of its volume of 2 *M* sodium cyanide. Histidine, tyrosine and their derivatives all gave the Pauly reaction when sprayed with a fresh alkaline solution of diazotised sulphanilic acid. In this test any phenol left in the chromatograms was first removed by thorough extraction with ether. Arginine and its derivative were detected as pink spots on a grey background by spraying first with a fresh mixture of 1 vol. 10% KOH, 1 vol. 60% urea and 2 vol. 0.1% α-naphthol in 50% ethanol. After drying at room temperature for a few minutes the sheet was sprayed with a solution of 2% bromine in 5% KOH. The sprays used were glass "Agla" atomisers (Burroughs, Wellcome & Co., Ltd.), preferably connected to a low pressure air-line (4 lbs/sq.in.).

Quantitative measurements. The yellow spots of the N-carbamoyl-amino acids were allowed to develop for 24 h after thoroughly spraying both sides of the paper, and were then cut out, eluted in 3 ml of pyridine and the optical density measured at 4420 A.U. Standards of citrulline should also be run for comparison, and a blank prepared from sprayed paper.

RESULTS

The positions of all derivatives and the corresponding amino acids on the chromatograms are shown in Fig. 1, as well as the positions of urea, thio-urea, allantoin, urethane and piperidine. The R_F values can be obtained from the co-ordinates, but as the temperature of chromatography was not controlled these values are means and may vary $\pm$ 0.04 unit. As with other chromatographic studies the pattern is often more useful for identification than R_F values. In general the N-carbamoyl-amino acids ran faster than the corresponding amino acids in butanol-acetic acid, but more slowly in phenol-water, although when ammonia vapour is present during the phenol run, the derivatives of the basic amino acids run faster in both solvents.

The aldehyde reagent for detecting the N-carbamoyl-amino acids has long been in use for urea and has been referred to in connection with N-carbamoyl-amino acids as the BARRENSCHEEN-WELTMANN reagent[21,4]. The test is fairly specific for ureas having one NH_2 group free. Urethane was detected by running for very short periods before spraying and biuret gave a colour only after an initial delay. On paper the spots are stable for several months, though the urea spot turned green and N-carbamoyl tryptophan gave a duller yellow spot which later acquired a grey or green tint. When a chromatogram of 18 free amino acids (those occurring in proteins) was sprayed with this reagent, tryptophan soon showed up as a dull yellow spot becoming violet (Ehrlich test), and on standing 24 h or more at room temperature most of the other amino acids gave very pale yellow spots. Presumably the amino groups react slowly with the aldehyde. The Barrenscheen-Weltmann reagent could be used after the chromatograms had been developed with ninhydrin. Most of the purple colours are then bleached by the acid, but the ε-carbamoyl lysine spot retained a reddish colour.

Cysteine and N-carbamoyl cysteine could only be detected by the alkaline nitroprusside spray when large amounts of these substances were present and the chromato-

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grams run for short periods (especially the phenol run). Moreover, the nitroprusside test could not be applied to chromatograms already developed with ninhydrin. A more complex nitroprusside reagent in which the chromatograms were dipped and which gave long colour stability has been described[22].

The derivatives of amino acids or amines with a secondary nitrogen, *e.g.* proline, hydroxyproline, sarcosine and piperidine, gave a weaker Barrenscheen-Weltmann reaction than, for example, N-carbamoyl alanine, and were difficult to detect on the chromatograms.

One property which is useful for identification in some cases is the regeneration of the corresponding α-amino acid from the N-carbamoyl derivatives by the action of alkali[2]. On a microscale this was conveniently done by heating the substance in a sealed tube with saturated (approx. 0.4 *N*) barium hydroxide at 110° C. Considerable regeneration occurs even in 2.5 h, though it must be borne in mind that hot alkali causes extensive breakdown of several amino acids. Regeneration was also achieved in 6 *N* HCl at 110° C for 12 h.

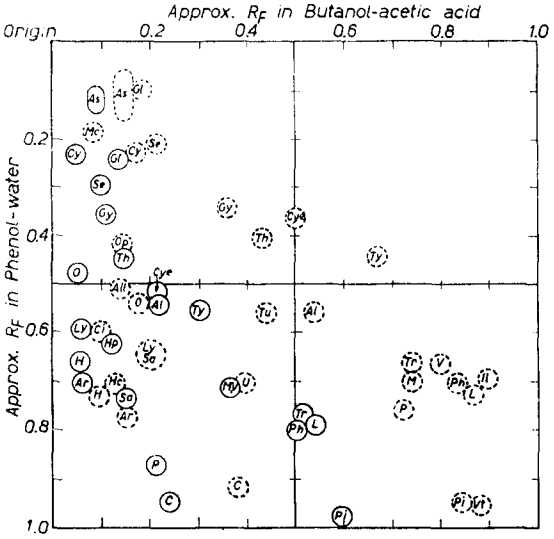

Fig. 1. Approximate positions of amino acids and amines (circles) and their N-carbamoyl derivatives (dotted circles) on chromatograms run with butanol-acetic acid, then with phenol. Abbreviations: Al, alanine. All, allantoin. Ar, arginine. As, aspartic acid. C, β-aminopiperidone. Ci, citrulline. Cy, cystine. Cye, cysteine. Gl, glutamic acid. Gy, glycine. H, histidine. HC, homo-citrulline (ε-N-carbamoyl lysine). HP, hydroxyproline. IL, isoleucine. L, leucine. Ly, lysine. M, methionine. MC, mono-N-carbamoyl cystine. O, ornithine. P, proline. Ph, phenyl-alanine. Pi, piperidine. Sa, sarcosine. Se, serine. Th, threonine. Tr, tryptophan. TU, thiourea. Ty, tyrosine. U, urea. Ut, urethane. V, valine. Cy, Ly and O (dotted circles) refer to the bis-NN′-carbamoyl derivatives.

The quantitative estimation of the derivatives on paper by elution of the yellow spots with pyridine is essentially the method used by HÜBENER *et al.* for urea[20]. Using citrulline and urea it was found that they gave the same molar extinction value. In pyridine $\varepsilon_{4420}^{1\,cm} = 2700$. Control chromatograms with known amounts of urea, citrulline or the derivative in question should be run at the same time.

The Barrenscheen-Weltmann spray reagent has a sensitivity comparable with the ninhydrin reagent for free amino acids. Thus 3 μg of citrulline or urea could be detected on two-dimensional chromatograms run in the manner described above.

Of the three N-carbamoyl-amino acids not apparently described before, N-carbamoyl DL-threonine was found to have a M.pt. of 185–6° C (with foaming) when recrystallised from water. The melting points of N-carbamoyl histidine and bis-NN′-carbamoyl lysine have not been determined, as crystals free of mother liquor impurities have not been obtained.

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SUMMARY

1. The N-carbamoyl derivatives (hydantoic acids) of 22 α-amino and α-imino acids have been prepared and their behaviour on paper chromatograms determined.

2. The preferred solvents for general use were (1) *n*-butanol-acetic acid-water and (2) phenol-water, and the derivatives could be detected down to 3 μg by a spray of 4% *p*-dimethylaminobenzaldehyde in 1 *N* hydrochloric acid.

RÉSUMÉ

1. Les dérivés N-carbamoyl (acides hydantoïques) de 22 acides α-aminés et α-iminés ont été préparés et leur mouvement sur les chromatogrammes bidimensionelles a été déterminé.

2. On préfère les deux solvants (1) *n*-butanol-acide acétique-eau et (2) phénol-eau, et les dérivés pouvaient être décelés jusqu'à 3 μg par la réaction au *p*-dimethylaminobenzaldehyde à 4% dans l'acide hydrochlorique 1 *N*.

ZUSAMMENFASSUNG

1. Die N-carbamoylderivate (Hydantoinsäuren) von 22 α-Aminosäuren und α-Iminosäuren sind hergestellt worden und ihre Bewegungen durch Verteilungschromatographie auf Papier gemessen.

2. Zwei Lösungsmittel, (1) *n*-Butanol-Essigsäure-Wasser und (2) Phenol-Wasser, wurden vorgezogen, und mit einem Reagens von 4% *p*-Dimethylaminobenzaldehyd in *N* Salzsäure können so wenig wie 3 μg der N-Carbamoylderivate papierchromatographisch nachgewiesen worden.

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