degradation of simple peptides. Complications will certainly arise when amino acids such as lysine, tyrosine or histidine form part of the molecule.

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Alkaline decomposition of β-hydroxy-α-amino acids

Serine and threonine are decomposed when heated with strong alkali with the formation, among other products, of glycine and alanine from serine, and glycine and α-amino-*n*-butyric acid from threonine[1-3]. The mechanisms of the reactions leading to the formation of glycine and of the product corresponding to $R \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ by alkaline decomposition of these β-hydroxyamino acids have not been fully elucidated but plausible theories have been advanced by WIELAND AND WIRTH[2]. The work reported here was carried out to discover if the same reactions occur when other β-hydroxyamino acids are heated with strong alkali. The following amino acids were tested: serine, threonine, D-glucosaminic acid, DL-β-hydroxyaspartic acid, DL-β-hydroxyglutamic acid, DL-β-phenylserine, DL-β-*p*-hydroxyphenylserine, DL-α-amino-β-hydroxy-*n*-caproic acid (hydroxynorleucine), DL-α-amino-β-hydroxy*iso*caproic acid (hydroxyleucine), DL-α-amino-β-hydroxy-*n*-valeric acid (hydroxynorvaline) and DL-α-amino-β-hydroxy*iso*valeric acid (hydroxyvaline). Serine and threonine were obtained from British Drug Houses Ltd., DL-β-hydroxyaspartic acid (the least soluble of the two diastereoisomers obtained by the method of DAKIN[4]) from Prof. W. SHIVE, DL-β-hydroxyglutamic acid (synthesized by method of LEANZA AND PFISTER[5]) from Dr. K. PFISTER, DL-β-phenylserine and DL-β-*p*-hydroxyphenylserine (three forms synthesized by methods of BOLHOFER[6,7]) from Dr. W. A. BOLHOFER, and hydroxyleucine, hydroxynorleucine, hydroxyvaline and hydroxynorvaline (synthesized by methods of BUSTON *et al.*[8,9]) from Dr. H. W. BUSTON. D-glucosaminic acid was prepared by the method of PRINGSHEIM AND RUSCHMANN[10] modified according to WOLFROM AND CRON[11]. Paper chromatograms of these amino acids in various solvents revealed only one spot when sprayed with ninhydrin.

The procedure adopted was as follows. The amino acid (0.5 m*M*) was heated with 5*N* NaOH (2 ml) in a sealed glass tube at 105° C for 20 hours. The reaction mixture was then desalted by the method of CONSDEN, GORDON AND MARTIN[12], evaporated to dryness *in vacuo*, taken up in 1 ml of water and analysed by paper chromatography. Preliminary identification of the ninhydrin-reacting substances in the reaction products was made by two-dimensional chromatography, the chromatograms being run in one direction with phenol in an atmosphere of NH_3 and HCN and in the other with "collidine" in an atmosphere of diethylamine (DENT[13]). The "collidine" used was a mixture of equal parts of 2:4:6-collidine and the 2:4/2:5-lutidine supplied by Light and Co., the bases being redistilled before use. All chromatograms were run on Whatman No. 4 paper and sprayed with ninhydrin (0.1 %, w/v) in chloroform. Identifications were checked by marker experiments with several solvents, particularly *n*-butanol-acetic acid[14] and *tert.*-amyl alcohol-10 % (v/v) pyridine[15]. The latter separated isomeric amino acids that could not be distinguished with other solvents and proved valuable for identification of the products formed by alkaline decomposition of hydroxyvaline, hydroxynorvaline, hydroxyleucine and hydroxynorleucine. Several of the β-hydroxyamino acids could be readily distinguished from other amino acids on chromatograms by the colours of their spots with ninhydrin. For example, β-hydroxyglutamic acid gave a brownish purple spot, β-phenylserine gave a reddish brown spot, and β-hydroxyaspartic acid gave a spot that was initially greenish yellow and then changed through brown to purple. The positions of the β-hydroxyamino acids on two-dimensional phenol-"collidine" chromatograms are given in Fig. 1, and the ninhydrin-reacting substances detected on chromatograms of the products formed by heating the β-hydroxyamino acids with alkali are listed in Table I. It can be seen that all of the β-hydroxyamino acids investigated gave glycine when heated with alkali, that most also yielded the product $R \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, and that some gave small amounts of other ninhydrin-reacting substances. The latter could not be identified, but the substance obtained from serine resembled glutamic acid in its behaviour on paper chromatograms and that obtained from β-hydroxyglutamic acid behaved chromatographically like

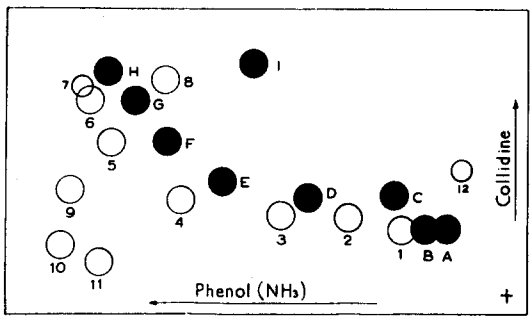

Fig. 1. Diagram showing positions of β-hydroxy-α-amino acids on two-dimensional phenol-"collidine" chromatograms. 1, aspartic acid; 2, glutamic acid; 3, glycine; 4, alanine; 5, valine and norvaline; 6, leucine, norleucine, *iso*leucine and methionine; 7, phenylalanine; 8, tyrosine; 9, proline; 10, arginine; 11, lysine; 12, cysteic acid. Black circles represent β-hydroxy-α-amino acids. A, β-hydroxyaspartic acid; B, β-hydroxyglutamic acid; C, glucosaminic acid; D, serine; E, threonine; F, β-hydroxyvaline and β-hydroxynorvaline; G, β-hydroxyleucine and β-hydroxynorleucine; H, β-phenylserine; I, β-*p*-hydroxyphenylserine.

TABLE I

NINHYDRIN-REACTING SUBSTANCES DETECTED ON CHROMATOGRAMS OF ALKALINE DECOMPOSITION PRODUCTS OF β-HYDROXYAMINO ACIDS

Amino acids were heated with 5*N* NaOH at 105° C for 20 hours. Strengths of spots detected are indicated by VS = very strong, S = strong, M = medium, W = weak, T = trace. A dash (—) indicates that the substance was not detected.

β-hydroxyamino acid	*Ninhydrin-reacting substances detected after heating with alkali*			
(R·CH(OH)·CH(NH_2)·COOH)	*Original*	*Glycine*	*R·CH_2·(NH_2)·COOH*	*Unidentified**
Serine	VS	S	S (alanine)	W (1)
Threonine	M	VS	W (α-amino-*n*-butyric acid)	—
β-Hydroxyaspartic acid	T	VS	—	—
β-Hydroxyglutamic acid	W	VS	M (glutamic acid)	T (1)
Glucosaminic acid	W	VS	?	T (3)
β-Phenylserine	—	VS	T (phenylalanine)	T (3)
β-*p*-Hydroxyphenylserine	—	VS	T (tyrosine)	T (1)
Hydroxyleucine	S	VS	W (leucine)	—
Hydroxynorleucine	VS	S	W (norleucine)	—
Hydroxyvaline	S	S	T (valine)	—
Hydroxynorvaline	VS	S	W (norvaline)	—

* Figures in parentheses indicate number of substances detected.

alanine. It may be noted that the β-hydroxyamino acids investigated differed greatly in their stability towards hot alkali and that only serine yielded an appreciable quantity of the product $R \cdot CH_2 \cdot CH(NH_2) \cdot COOH$. The presence of this product in alkali digests of glucosaminic acid could not be checked because a specimen for use as a marker was not available, but structural considerations suggest that, if formed, this product would not survive the alkali treatment. Products corresponding to $R \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ were not consistently detected in the alkaline decomposition products of β-phenylserine, β-*p*-hydroxyphenylserine and hydroxyvaline. For example, valine was detected in only two of five experiments with hydroxyvaline. Moreover, consistent results could not be obtained when attempts were made tc determine the amounts of ammonia and glycine formed by alkaline decomposition of several of the β-hydroxyamino acids. For example, in six experiments in which 50 mg samples of threonine were heated with 2 ml of 5*N* NaOH in sealed tubes at 105° C for 24 hours, the percentage of threonine nitrogen recovered as ammonia varied from 19 to 26 and the percentage recovered as glycine varied from 60 to 71. A possible explanation of these findings is that the decompositions of β-hydroxyamino acids when heated with alkali in glass vessels are affected by metals dissolved from the glass during the alkali treatment. This possibility has not been investigated but several workers have observed that the behaviour of amino acids and proteins on treatment with alkali is affected by metals. For example, VAN SLYKE[16] found that the amount of ammonia liberated when cystine was boiled with alkali in a copper flask was much greater than when a glass flask was employed and PLIMMER[17] noted the same effect with histidine. Also, GROH AND NYILASI[18] found that the poor reproducibility of the results they obtained in studies on the velocity of hydrolysis of proteins in alkali was due to catalytic effects caused by minute amounts of arsenic and iron in the glassware they employed.

In conclusion, it may be pointed out that the findings in this work with regard to the effect of molecular structure on mobility in phenol (Fig. 1) are in agreement with previous observations that mobility is decreased by the replacement of a hydrogen atom by a hydroxyl group.

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Preliminary Notes

Contribution of reducing agents to electrophoretic lack of homogeneity of crystalline ribonuclease

The heterogeneous nature of crystalline ribonuclease remains a current problem, despite recent studies which have shed much light on the mechanism of action of this enzyme. Crystalline ribonuclease has been shown to be resolvable into two or more components by partition[1], as well as by ion exchange resin[2] chromatography. Although electrophoresis[3] indicated apparent homogeneity of the enzyme protein, zone electrophoresis on starch[4] showed that crystalline ribonuclease is not electrophoretically homogeneous.

The present investigation with paper electrophoresis revealed that crystalline ribonuclease heterogeneity depends on the degree of the reduction of ribonuclease. The reduction products retained ribonuclease activity against yeast ribonucleic acid, although their electrophoretic mobilities were different.

Five mg of crystalline ribonuclease (Armour), recrystallized twice by the KUNITZ[5] procedure, were incubated separately with each of the following: reduced glutathione, cysteine, histidine, and ascorbic acid, in phosphate buffer at 50° C, for 1 hour. Aliquots of 15 μl were applied for electrophoretic mobility determination. Each sample was applied to three separate strips of filter paper run in parallel.

Electrophoresis was accomplished in the LKB 3276 electrophoresis equipment, using 40 × 410 mm Schleicher and Schüll No. 2043B (120 g/m^2) filter paper, or Whatman No. 1 filter paper. Phosphate buffers of pH 5.64, 6.50, 7.00, and 7.80, ionic strengths 0.36 or 0.10, were used.