

On the Mechanism of the Phenyl Isothiocyanate Degradation of Peptides

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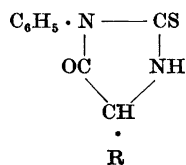
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A study of the reaction mechanism has revealed that the N-terminal amino acid is split off in the form of its 2-anilino-5-thiazolinone derivative. The latter is very unstable and can be converted along two different routes — thermal or hydrolytic — to the isomeric 3-phenyl-2-thiohydantoin derivative. The bearings of these findings on the degradation method are discussed.

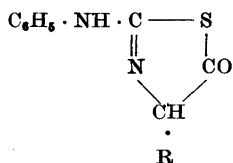
A formal analogy has earlier been assumed for the phenyl isocyanate^{1,2} and the phenyl isothiocyanate^{3,4} degradation of peptides. However, some observations and in particular the great difference in the rate of formation of phenyl thiohydantoin (PTH) in aqueous^{5,6} and non-aqueous media^{4,7} indicated a peculiarity in the mechanism of the phenyl isothiocyanate degradation which required closer investigation. With the introduction of the paper chromatographic procedure for the identification of phenyl thiohydantoin⁸ this became possible.



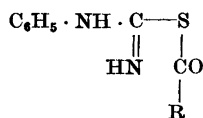
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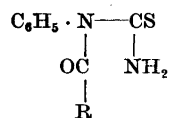
II



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IV



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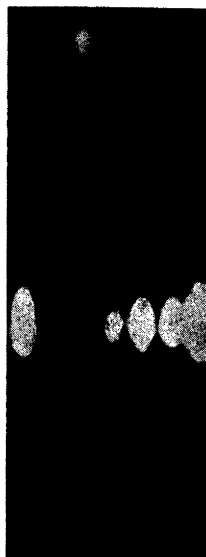


Fig. 1. Paper chromatogram of products resulting from the cleavage of PTC-leucylglycine in nitromethane-hydrogen chloride. From left to right: PTH-leucine, "crude product", PTC-leucylglycine, 1st, 2nd and 3rd fraction of recrystallised product, PTH-leucine. Solvent A.

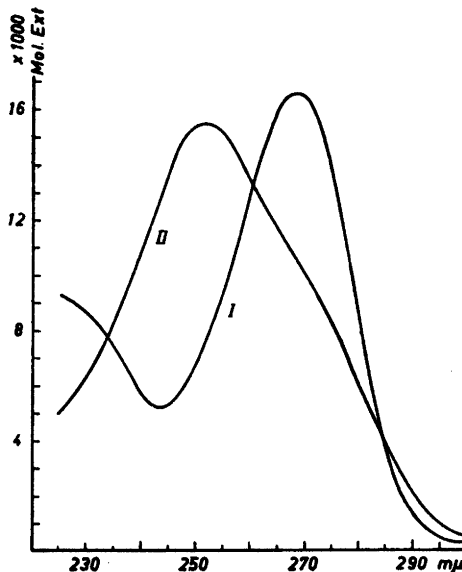


Fig. 2. Ultraviolet absorption curves of 5-isobutyl-3-phenyl-2-thiohydantoin (I) and 2-anilino-4-isobutyl-5-thiazolinone (II) in ethanol.

Paper chromatography of the reaction mixture resulting from cleavage of phenyl thiocarbamyl (PTC)-DL-leucylglycine (I; $R = CH_2 \cdot CHMe_2$; $R' = H$) in nitromethane-hydrogen chloride, revealed a hitherto unobserved substance, whereas the expected PTH-leucine (II; $R = CH_2 \cdot CHMe_2$) was lacking (Fig. 1). However, on mere recrystallization the new compound was completely transformed into PTH-leucine. Obviously the N-terminal amino acid of the peptide was split off in the form of a labile derivative which was easily convertible to the phenyl thiohydantoin. The nature of this intermediate and the conditions for its conversion have been studied.

Avoiding heat and moisture the intermediate could be recrystallized as its salt with strong acids. Elementary analyses showed it to be an isomeride of the corresponding phenyl thiohydantoin from which it however differed in paper chromatographic behaviour (Fig. 1) and in ultraviolet absorption (Fig. 2).

It could be demonstrated that two different ways are open for the conversion of the intermediary compound into the corresponding phenyl thiohydantoin. At elevated temperature the transformation takes place as an intramolecular rearrangement (Figs. 3 and 4). On the other hand, in the presence of water, the intermediate compound is rapidly hydrolysed to the correspond-

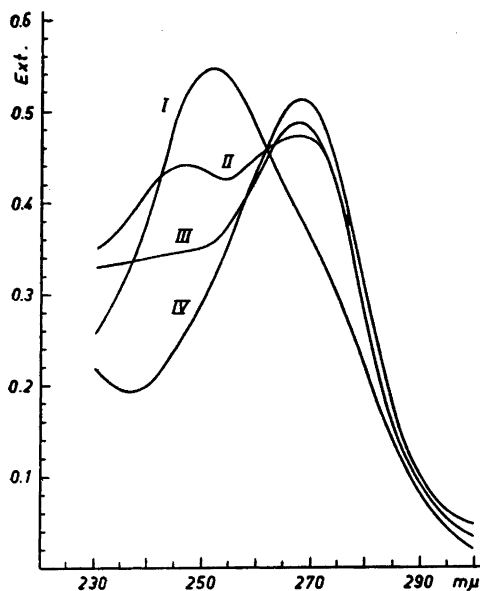


Fig. 3. Conversion of 2-anilino-4-isobutyl-2-thiazolinone hydrochloride into PTH-leucine in the dry state at 115° C. Ultraviolet absorption curves of samples at zero time (I) and after 10 min. (II), 25 min. (III) and 60 min. (IV).

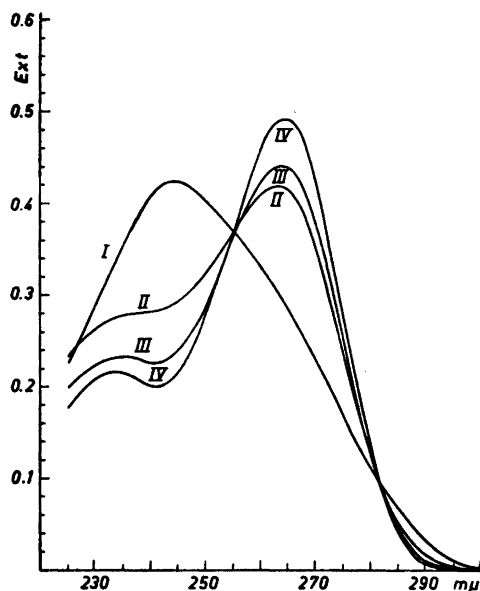


Fig. 4. Conversion of 2-anilino-4-isobutyl-5-thiazolinone hydrochloride into PTH-leucine in glacial acetic acid at 93° C. Ultraviolet absorption curves of samples at zero time (I) and after 10 min. (II), 19 min. (III) and 39 min. (IV).

ing PTC-amino acid which then slowly undergoes ringclosure to the PTH-amino acid (Fig. 6). The rate of this ringclosure was followed at pH 3 and the temperatures 30° C and 50° C (Fig. 5).

A striking similarity exists between these reactions and those displayed by S-acylated thioureas. The latter have been extensively investigated by Dixon and his coworkers⁹⁻¹¹. They obtained the hydrochloride of S-acetyl-N-phenyl thiourea* (IV; R = Me) by reacting phenyl thiourea with acetyl chloride in the cold. On heating, this compound was rapidly converted first to the N-acetyl-N-phenyl thiourea (V; R = Me) and later to the N'-acetyl-N-phenyl thiourea. On the other hand water hydrolysed the thiolester linkage with the formation of phenyl thiourea and acetic acid. By analogy our intermediate should then be formulated as a 2-anilino-5-thiazolinone (III).

Several attempts were made at an independent synthesis of the labile intermediate compound but of these only an approach analogous to the one used by Dixon *et al.* for preparing the open-chain S-acylated thioureas was successful. The acid chloride of phenyl thiocarbamyl-L-leucine was prepared and

* Dixon *et al.*¹⁰ prefer to formulate these compounds as sulfonium chlorides.

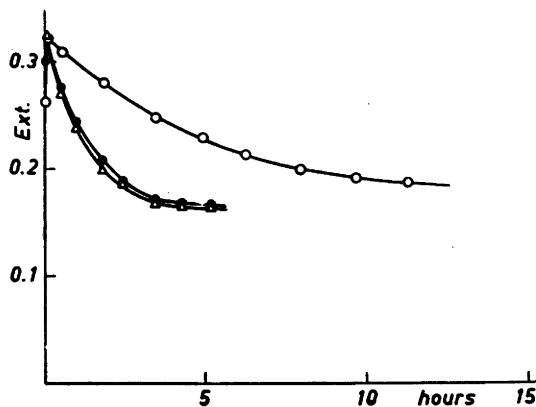


Fig. 5. Conversion of 2-anilino-4-isobutyl-5-thiazolinone hydrochloride via PTC-leucine (initial rapid rise) into PTH-leucine in aqueous solution at pH 3 and 30° C (—○—○—) and 50° C (—●—●—) followed through change in extinction at 243 mμ. For comparison the conversion of PTC-leucine into PTH-leucine at pH 3 and 50° C (—△—△—).

this spontaneously converted itself to the hydrochloride of 2-anilino-4-isobutyl-5-thiazolinone:

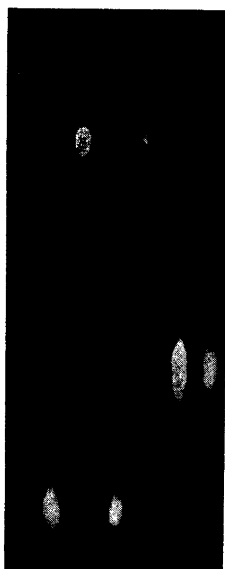
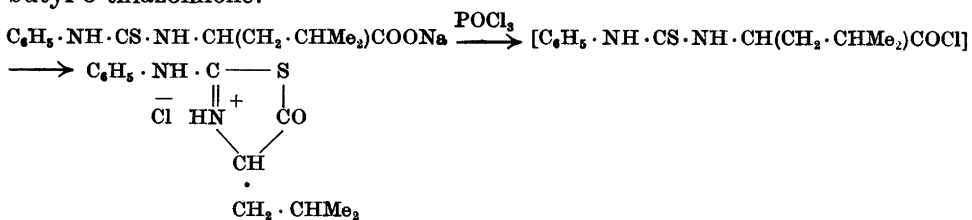


Fig. 6. Paper chromatogram of products formed in the cleavage of PTC-leucylglycine in 4 N hydrochloric acid. From left to right; PTC-leucylglycine, 2-anilino-4-isobutyl-5-thiazolinone hydrochloride, PTC-leucine, samples of reaction mixture after 1 min., 15 min., 80 min., and PTH-leucine. Solvent A.

EXPERIMENTAL

Elementary analyses (except micro-Kjeldahl) were made at the Analytical Laboratory, Department of Chemistry, University of Lund, and at the Chemical Laboratory, University of Copenhagen.

Melting points were determined on a heating block (Fisher-John) and are uncorrected.

Paper chromatography was carried out as described by Sjöquist⁸.

Cleavage of phenyl thiocarbamyl-DL-leucylglycine in nitromethane-hydrogen chloride. Phenyl thiocarbamyl-DL-leucylglycine⁴ (200 mg) was suspended in dry nitromethane (10 ml) saturated with dry hydrogen chloride. The reaction mixture was left at room temperature for 15 min. and the crystals of glycine hydrochloride were filtered off. The mother liquor was evaporated *in vacuo* to dryness without heating. The crude, crystalline product (157 mg) was subjected to paper chromatography in solvent A (Fig. 1) and the U.V.-absorption was measured in abs. ethanol* where a broad absorption with a maximum at 252 m μ ($\epsilon_{252} = 59$; $C = 1$ g/l) was observed.

Part of the crude product (70 mg) was dissolved in abs. ethanol (1 ml) and a small insoluble residue filtered off. The preparation was then fractionally crystallized in the cold by the gradual addition of petroleum ether. Melting points, R_F -values in solvent A (Fig. 1) and U.V.-absorption curves were determined on the separate fractions (Table 1).

Table 1.

	M. p.	R_F -value in solvent A	Abs. max.
Crude product	126–135°	0.80	$\epsilon_{252} = 59$
Fract. I (10 mg)	178°	0.59	$\epsilon_{268} = 63$
Fract. II (31 mg)	178°	0.59	$\epsilon_{268} = 63$
Fract. III (4 mg)	140–144°	0.59	$\epsilon_{268} = 59$
PTH-leucine	178°	0.59	$\epsilon_{268} = 64$

*Cleavage of phenyl thiocarbamyl-DL-leucylglycine in glacial acetic acid-hydrogen chloride*⁷. Phenyl thiocarbamyl-DL-leucylglycine (0.65 g) was dissolved in glacial acetic acid saturated with hydrogen chloride (5 ml). Almost immediately crystals began to appear and they were filtered off after a few minutes (0.21 g). Paper chromatography in pyridine-amyl alcohol¹² and in acetic acid-butanol¹³ showed identity with glycine.

To the mother liquor ten vols. of dry ethyl ether were added. Needleshaped crystals were collected (0.47 g). Recrystallization from glacial acetic acid – hydrogen chloride and ethyl ether. M. p. 128–31°. For U.V.-absorption curve see Fig. 2. (Found: N 9.64. $C_{13}H_{17}ON_2ClS$ requires: N 9.83).

The product was taken up in a small volume of glacial acetic acid-sulfuric acid (1 M) and ethyl ether added to crystallization. The crystals melted at 133° C. (Found: C 44.4; H 5.30; N 8.03; S 18.2. $C_{13}H_{18}O_5N_2S_2$ requires: C 45.0; H 5.25; N 8.08; S 18.5).

Sodium salt of phenyl thiocarbamyl-L-leucine. L-Leucine (2.7 g) was dissolved in pyridine-water (3:2, 50 ml) and 2 N sodium hydroxide added to pH 9. Phenyl isothiocyanate (5 ml) was added and pH was kept at 9 during the reaction by the continuous addition of sodium hydroxide. After one hour the reaction mixture was extracted several times with equal volumes of benzene and finally with ethyl ether and the extracts discarded. A small insoluble residue was filtered off and pH was again adjusted to 9 with sodium hydroxide. The solution was concentrated *in vacuo* to a thick syrup. This was repeatedly

* Since the 5-thiazolinone slowly reacts with ethanol all measurements were made immediately after dissolution.

extracted first with acetone and then with ethyl ether and finally dried *in vacuo* (4.95 g). (Found: N 9.15. $C_{13}H_{17}O_2N_2SNa \cdot 1 H_2O$ requires: N 9.15).

2-Anilino-4-isobutyl-5-thiazolinone. The sodium salt of PTC-L-leucine (2.3 g) was suspended in ethylene chloride (10 ml). Phosphorus oxychloride (0.6 ml) was added slowly under cooling in ice and the reaction mixture then left for two hours at room temperature. The precipitated salts were removed and the solvent then evaporated in a current of dry air. To the oily residue several vols. of dry ethyl ether were added and a stream of hydrogen chloride passed through the solution for a short while. Crystals appeared which were filtered off and recrystallized three times in quick succession from glacial acetic acid-sulfuric acid (1 M) and ethyl ether (0.8 g). M. p. 133°. R_F -value in solvent A and U.V.-absorption curve identical with that of the initial product obtained from the degradation of PTC-leucylglycine (see above). (Found: C 44.5; H 5.30; N 8.02; S 18.3. $C_{13}H_{16}O_2N_2S_2$ requires: C 45.0; H 5.25; N 8.08; S 18.5).

Attempted conversion of 5-isobutyl-3-phenyl-2-thiohydantoin into 2-anilino-4-isobutyl-5-thiazolinone. 5-isobutyl-3-phenyl-2-thiohydantoin (0.25 g) was dissolved in dry nitromethane (10 ml) and the solution saturated in the cold with dry hydrogen chloride. After 15 min. the solvent was removed *in vacuo*. Paper chromatography in solvents A and B and U.V.-absorption showed that the original product had been recovered unchanged.

Conversion of 2-anilino-4-isobutyl-5-thiazolinone into 5-isobutyl-3-phenyl-2-thiohydantoin by heat. Dry samples of 2-anilino-4-isobutyl-5-thiazolinone hydrochloride were heated at 115° C for varying times. The products were then dissolved in abs. ethanol and the U.V.-absorption measured (Fig. 3).

A solution of 2-anilino-4-isobutyl-5-thiazolinone hydrochloride (1 mg/ml) in glacial acetic acid was heated at 93° C. At intervals samples were diluted with abs. ethanol (1:100) and the U.V.-absorption measured (Fig. 4).

Conversion of 2-anilino-4-isobutyl-5-thiazolinone into 5-isobutyl-3-phenyl-2-thiohydantoin in aqueous acid. Solutions of 2-anilino-4-isobutyl-5-thiazolinone hydrochloride in acetone (10 mg/ml) were diluted with 4 N hydrochloric acid (1:10). Paper chromatograms in solvent A were run on samples taken after various times along with authentic samples of PTC-leucine and of PTH-leucine*. Already after 1 min. the spot corresponding to the 5-thiazolinone had almost completely disappeared and been substituted by a strong spot corresponding to PTC-leucine. After 5 min. a spot corresponding to PTH-leucine began to appear. The latter spot grew at the expense of the other spots and it was the only one to persist after 90 min.

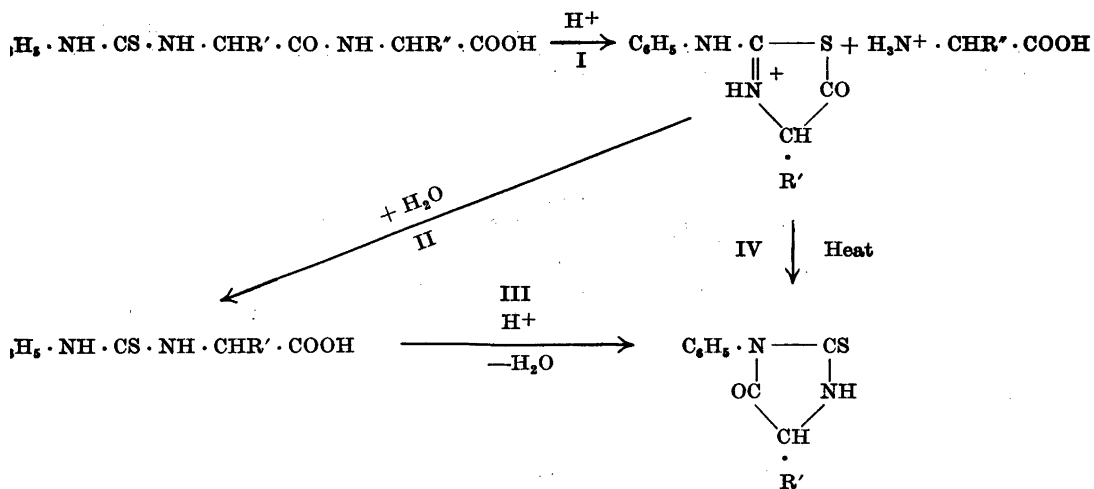
Solutions of 2-anilino-4-isobutyl-5-thiazolinone hydrochloride and PTC-leucine (approx. 10 mg/ml) were made up in dry acetone. These solutions were diluted (1:1 000) with water previously adjusted to pH 3 and equilibrated at 30° and 50° C. The change in the U.V.-absorption at 243 $m\mu$ was then followed. The results were recalculated for equimolar concentrations (Fig. 5).

Cleavage of phenyl thiocarbonyl-DL-leucylglycine in aqueous acid. Solutions of PTC-DL-leucylglycine (15 mg/ml) in acetone were diluted with 4 N hydrochloric acid (1:10). Paper chromatograms in solvent A were run on samples withdrawn after various times along with authentic samples of PTC-leucylglycine, 2-anilino-4-isobutyl-5-thiazolinone, PTC-leucine and PTH-leucine (Fig. 6)*. After 1 min. a strong spot corresponding to the 5-thiazolinone and a weak spot corresponding to PTC-leucine were visible, after 15 min. PTH-leucine began to appear and this was the only substance present after 80 min.

DISCUSSION

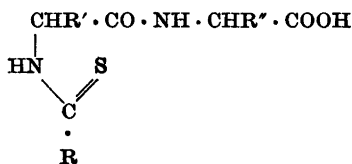
The reaction sequences observed can be summarized in the following way:

* The 5-thiazolinone tends to react with the paper at the point of application and it was therefore necessary to develop the chromatogram without delay.



This reaction mechanism helps to clarify several hitherto obscure points. Thus the considerable difference in the rate of cleavage of PTC-peptides in nonaqueous^{4,7} and in aqueous solution^{5,6} is only apparent since the reactions observed were not the same. In the former case only reaction I was followed whereas in the latter instance measurements were made on the reaction sequence I—II—III of which reaction III is by far the slowest (Fig. 5). Furthermore, the frequently noted appearance of multiple spots on paper chromatograms as the result of a single step in the degradation has most likely often been due to the presence of intermediates. For the same reason the extent of cleavage of the peptide may have appeared low if calculated from the yield of thiohydantoin.

A unified aspect can be laid on several of the reactions for the degradation of peptides from the N-terminal. The reactive N-substituted peptide derivatives can be formulated:



where R represents various substituents such as C₆H₅NH-^{3,4}, HS-¹⁴ or alkoxy groups¹⁵ and the cleavage of the peptide bond ensues on the establishment of the —CO—S— linkage.

It is interesting to note that in aqueous medium the —CO—S-linkage has only a transient existence since the thiocarbamyl configuration is rapidly re-established through hydrolysis after fission of the peptide bond. One may therefore be justified in designating the role of the thioketonic grouping (or its enolic form) as catalytic. Apparently, the nature of the neighboring R group greatly influences this catalytic activity.

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