methionine in its protein is derived from that in the red-cell globin.

2. The conversion by *P. knowlesi* of [³⁵S]-methionine into [³⁵S]cystine has been shown to occur *in vitro*, and this provides a source of cystine sulphur for the parasite in addition to that already present in the haemoglobin and glutathione of the erythrocyte. Some estimates of the extent to which these sources contribute to the cystine requirement of the parasite have been made.

3. Under identical *in vitro* conditions it was found that the uptake of DL-[³⁵S]methionine by parasitized erythrocytes was markedly greater than that of DL-[³⁵S]cysteine.

4. The amounts of methionine and cystine present in the haemoglobin of a monkey erythrocyte are respectively 6.0 and 2.4 times that found in the protein of a mature parasite. The implications of this observation to the nutrition of the parasite have been discussed.

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Products of Hydrolysis of Dinitrophenylproline and N-Terminal Proline-peptides with Hydrochloric Acid

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(Received 17 November 1955)

During the examination of clupeine for the N-terminal amino acid residue (Scanes & Tozer, 1956), considerable difficulty was experienced in isolating ether-soluble dinitrophenyl (DNP) amino acid from the hydrochloric acid hydrolysate in the amount expected. A similar result was obtained by the alternative hydrolytic methods of Hanes, Hird & Isherwood (1952). As proline was sus-

pected as an N-terminal amino acid, the substitution of 12N for 6N hydrochloric acid, as recommended by Porter & Sanger (1948) for use with DNP-prolyl proteins, was tried, but without success. Porter & Sanger recovered 50% of the DNP-proline added to globin before hydrolysis, but other workers have reported much lower recoveries, namely, 20% from a collagen hydrolysate

(Bowes & Moss, 1953) and 15% from a gelatin hydrolysate (Courts, 1954) after hydrolysis with 10N and 12N acid respectively.

Salmine, which has been shown by various workers (Velick & Udenfriend, 1951; Landmann, Drake & Dillaha, 1953) to contain proline as the *N*-terminal amino acid, was used for reference purposes. In hydrolytic experiments carried out on DNP-salmine, DNP-proline and DNP-proline admixed with gelatin, we were able to recover only 5–10% of DNP-proline, even after as short a time as 4 hr. in 12N hydrochloric acid at 100°. The main products obtained were 2:4-dinitrophenol and two other DNP derivatives which did not correspond with any of the known DNP-amino acids when examined by column and paper chromatography. The small yields of DNP-proline obtained were rather disconcerting, since DNP-proline has been reported by so many workers as a major hydrolytic product, with up to 50% recovery. However, since the present work was completed, Phillips (1955), who identified proline as an *N*-terminal group of salmine by the examination of the spectral-absorption curve, noted the presence of DNP-proline for only up to 2 hr. in 6N acid hydrolysates, and Fraenkel-Conrat, Harris & Levy (1955) have reported the complete destruction of DNP-proline after hydrolysis with 10N hydrochloric acid at 105° for 2 hr., with the formation of dinitrophenol and two yellow artifacts.

Schramm & Braunitzer (1953) have isolated a number of DNP derivatives from hydrolysates of DNP-proline admixed with horse globin and from the DNP derivative of tobacco mosaic virus. These derivatives included 2:4-dinitrophenol as the major component after full hydrolysis, in addition to traces of prolyl peptides and an unknown yellow compound, although after partial hydrolysis for 2 hr. at 105° Schramm & Braunitzer found some DNP-proline in addition to DNP-prolyl peptides.

With the object of investigating the fate of DNP-proline when it is liberated in acid hydrolysates of DNP-proteins, we have carried out experiments on the hydrochloric acid hydrolysis of DNP-DL-proline and have isolated and identified the major hydrolytic products.

The DNP-hydrolytic products were examined by paper chromatography in four systems which were selected so that, by suitable choice of a combination of systems, the individual hydrolytic products of DNP-proline and the potential DNP-amino acids from a DNP-clupeine hydrolysate could be unequivocally identified. The preparative separation of the hydrolysis products was carried out by chromatography on columns of silicic acid–Celite (Johns Manville Co. Ltd., London, S.W. 1) (Green & Kay, 1952).

DNP-DL-proline was hydrolysed with 12N hydrochloric acid in sealed tubes for 1 hr. at 100°, and after a fourfold dilution of the solution the coloured products were extracted into ethyl acetate. After washing with water to remove hydrochloric acid and after removal of the solvent *in vacuo*, there remained an orange-yellow oil from which some crystalline material separated on standing overnight. Examination of the oily residue by paper chromatography showed the presence of 2:4-dinitrophenol, a small amount of unchanged DNP-proline, and two other coloured hydrolytic products *A* and *B*. After extraction with hot benzene a solid residue was obtained, which on recrystallization from benzene yielded a lemon-yellow crystalline solid, m.p. 142–142.5°. This was shown by paper chromatography to be product *A*, but a considerable proportion of *A* remained in the first benzene extract.

In subsequent experiments the separation of *A*, *B* and 2:4-dinitrophenol from each other was effected by chromatography on silicic acid–Celite columns with acetic acid–ethyl acetate–light petroleum (Green & Kay, 1952) as the developing solvent. The separation, which was not visually apparent on a preparative scale, was followed by paper chromatography of samples from the eluate fractions. 2:4-Dinitrophenol was least strongly adsorbed and was completely separated from *A*, but in some experiments an overlap of the *A* and *B* bands was experienced, and it was found necessary to concentrate and rechromatograph the intermediate fractions containing both *A* and *B*. After removal of the solvent from the pooled fractions, *A* was obtained as a yellow oily solid and *B*, admixed with residual DNP-proline, as a yellow oil.

Product *A* was readily recrystallized from hot benzene and was obtained as a pure crystalline yellow solid, m.p. 142–142.5°, with the formula $C_{11}H_7O_6N_2Cl$. The chlorine was not liberated by warming with ethanolic silver nitrate, but, when an attempt was made to convert the chloro compound into a phenolic ether, halide was liberated and a small amount of DNP-proline was isolated. Some DNP-proline was formed when *A* was distilled at 170° in a high vacuum. Product *A* dissolves in aqueous sodium bicarbonate, and potentiometric titration indicates the presence of a free carboxyl group. Thus assuming that the compound retains a dinitrophenyl residue the formula may be written as

As *A* contains one molecule of HCl more than the starting product, DNP-proline, these facts suggest that the proline ring has been broken on heating with hydrochloric acid with the formation of one of the DNP-aminochloroaleric acids I or II.

Product *A* gave an almost theoretical yield of proline after hydrolysis with either 6*N* or 12*N* hydrochloric acid at 100° for 16 hr. Reaction with ammonia yields ornithine and an unidentified ninhydrin-positive substance, whilst hydrolysis with baryta yields proline and another unidentified ninhydrin-positive substance.

Further purification of the *B* fraction to remove DNP-proline was effected by chromatography on a powdered cellulose column with the same solvent as was used in the paper chromatographic system *B*1 (see Experimental). The hydrolytic product *B* was then crystallized by slow evaporation of the solvent from a solution in a mixture of ether and light petroleum and was obtained as a crystalline yellow solid, m.p. 107.5–109°, with the formula $C_{11}H_{12}O_6N_2Cl$. Product *B* resembles *A* in that warming with ethanolic silver nitrate liberates no halide and potentiometric titration indicates the presence of a free carboxyl group. Product *B* also yields a small amount of DNP-proline when distilled in a high vacuum.

The hydrolytic reactions of *B* are practically identical with those of *A* at 100°: hydrolysis with hydrochloric acid, both 6*N* and 12*N*, gives an 80–90% yield of proline, hydrolysis with ammonia gives ornithine and an unidentified ninhydrin-positive substance, and hydrolysis with baryta yields proline and another unidentified ninhydrin-positive substance.

The absorption spectra of *A* and *B* were found to be very similar over the range 320–450 $m\mu$. and to agree with the absorption spectra of open-chain DNP-amino acids such as DNP-alanine rather than DNP-proline (cf. Schroeder, Honnen & Green, 1953).

The general properties of the hydrolytic products *A* and *B* described above suggested that these two

compounds were the isomeric DNP-amino-chloro-valeric acids (I) and (II) and this was proved by synthesis of these compounds as shown in Figs. 1 and 2 (FDNB represents 1-fluoro-2,4-dinitrobenzene).

Initially (III) was prepared from α -DNP- δ -benzoylornithine (Sanger, 1946), but owing to the difficulty of hydrolysis of this substance we were able to obtain only a very small amount of the required product. It was, however, prepared in good yield from DL-ornithine through the δ -benzyloxycarbonyl derivative (IV).

The direct routes from (III) and (VI) to (I) both yielded mixtures of DNP products which were shown by paper chromatography to include the required product (I). By the chlorination of (V) with thionyl chloride, followed by hydrolysis of the acid chloride thus formed, α -DNP-amino- δ -chloro-valeric acid (I) was obtained as a yellow crystalline solid which was shown by mixed melting point and by paper chromatography to be identical with the hydrolysis product *A*. Although, in general, good yields of (V) were obtained from α -amino- δ -hydroxyvaleric acid, during one attempted preparation of (V) the product obtained after crystallization was the δ -lactone (VI). In like manner the action of nitrous acid on (III) gave mainly (V), as shown by paper chromatography, but after crystallization only the δ -lactone (VI) was isolated.

The product (II), obtained by interaction of δ -DNP-DL-ornithine with nitrosyl chloride, behaved identically with hydrolysis product *B*, but attempts to isolate it in crystalline form were unsuccessful. The alternative route, however, in which the α -amino group of DL-ornithine was first replaced by chlorine (Hamilton, 1952) gave α -chloro- δ -DNP-amino-valeric acid (II) in good yield (Fig. 2). The identity of *B* with (II) was established by mixed melting

Fig. 1. Synthesis of α -DNP-amino- δ -chloro-valeric acid (I).

Fig. 2. Synthesis of α -chloro- δ -DNP-aminovaleric acid (II).

point and confirmed by paper and column chromatography.

This conversion of DNP-proline into the isomeric DNP-aminochlorovaleric acids represents an additional technique for the opening of the pyrrolidine ring (cf. Taylor & Baker, 1949). The reactions appear to be reversible, as both (I) and (II) yield some 5–10% of DNP-proline on heating with 12*N* hydrochloric acid at 100° for 1 hr.

It is suggested that the mechanism of the hydrolysis of DNP-proline with hydrochloric acid probably involves three simultaneous side reactions, two of which are reversible and result in the production of the DNP-aminochlorovaleric acids (I) and (II), whereas the third is an irreversible hydrolysis of DNP-proline to proline and 2:4-dinitrophenol, thus:

Support for this mechanism is afforded by the facts that both (I) and (II) give an almost quantitative yield of proline and considerable amounts of dinitrophenol after hydrolysis with hydrochloric acid at 100° for 16 hr. The extensive breakdown of a DNP-amino acid on acid hydrolysis into the component amino acid and dinitrophenol is not general, and according to Schramm & Braunitzer (1953) is restricted to DNP-proline, although Acher & Laurila (1953) have shown that hydrolysis of DNP-glycine and DNP-cystine also results in the formation of the corresponding amino acids. Thus the direct hydrolysis of the DNP-aminochloro-

valeric acids (I) and (II) would appear unlikely, and even if it did take place it should yield the corresponding aminochlorovaleric acids. It is improbable that these acids could be intermediates in the formation of proline from DNP-proline, as it has been shown by Albertson & Fillman (1949) that α -chloro- δ -aminovaleric acid does not undergo ring-closure to form proline under acid conditions, and we have found no trace of any amino acid other than proline in the aqueous fractions of the acid hydrolysates of (I) and (II).

Our work on the breakdown of DNP-proline is not in agreement with the results of Acher & Laurila (1953), who suggest that the hydrolysis of DNP-proline with hydrochloric acid is a first-order reaction resulting in the formation of proline and dinitrophenol. An examination of the hydrolysis of DNP-proline under conditions similar to those of Acher & Laurila, namely, a concentration of 100 $\mu\text{g.}$ of DNP-proline/ml. of 10*N* hydrochloric acid in a sealed, evacuated tube, but at 100° instead of 105°, showed a recovery of DNP-proline of less than 10% after 2 hr. instead of about 60% as found by Acher & Laurila. On the other hand, we recovered at least 50% as the mixed hydrolytic products (I) and (II), and it would therefore appear that the fractionation techniques used by Acher & Laurila did not separate DNP-proline from the breakdown products (I) and (II).

It seemed desirable to isolate the products of hydrolysis of DNP-L-proline with hydrochloric acid, as the characterization of these products could assist in a more rigid identification of *N*-terminal proline in natural compounds than can be obtained by paper and column chromatography.

Although we took such precautions as the exclusion of light and the use of anhydrous conditions

for purification (Rao & Sober, 1954), we were unable to isolate crystalline material, other than dinitrophenol, from the hydrolytic products of either DNP-L-proline or DNP-salmine. Failure to obtain DNP derivatives in crystalline form may be due to the generally lower melting points of derivatives of L-amino acids (Rao & Sober, 1954) or to partial racemization.

In the absence of crystalline hydrolytic products of DNP-L-proline, the identification of *N*-terminal proline after hydrolysis of the DNP-protein depends on identification by chromatography. As would be expected, the natural hydrolytic products from DNP-protein containing *N*-terminal proline behave in an identical manner on chromatograms to the synthetic products. In general, the following scheme, based on the reactions just described of the two DNP-aminochlorovaleric acids (I) and (II), should result in a positive identification of *N*-terminal proline in peptides or proteins.

The DNP-protein is hydrolysed at 100° with 12*N* hydrochloric acid for the minimum time (preferably 2–4 hr.) necessary to release most of the DNP-amino acid. The ether-soluble extract is then examined by chromatography on paper, with systems *B*1 or *A*2, whereby 5% of residual DNP-proline can be detected if the hydrolysis products are applied at a concentration equivalent to 50 µg. of original DNP-proline for each spot. If dinitrophenol is present in excessive amounts it is desirable to remove this substance, before chromatography, by high-vacuum sublimation of the ether-soluble extract at 100°.

If the presence of additional DNP-amino acids is indicated, or for further confirmation of the presence of *N*-terminal proline, the ether-soluble extract should be hydrolysed for 16 hr. at 100° with 6*N* hydrochloric acid. After extraction with ether, the presence of proline in the aqueous extract and of appreciable amounts of dinitrophenol in the ether extract would positively identify proline as an *N*-terminal group. On the other hand, the identification of proline but absence of dinitrophenol would indicate a DNP-peptide in which proline was not the *N*-terminal group. The remaining DNP-amino acids, if any, in the ether extract can be identified directly by paper chromatography and confirmed by alkaline hydrolysis to the corresponding amino acids.

In this manner salmine was shown to have proline as the *N*-terminal amino acid, but a clupeine component *Y* was found (Scanes & Tozer, 1956) to have both proline and alanine as *N*-terminal amino acids.

EXPERIMENTAL

All melting points are corrected. Micro-analyses for C, H, N and Cl are by F. E. Charlton, Porton, and by Weiler and Strauss, Oxford.

Table 1. R_F values of some DNP derivatives on paper chromatograms

For details of the solvent systems used see text.

DNP derivative	R_F value in solvent system			
	<i>A</i> 1	<i>BE</i> 2	<i>A</i> 2	<i>B</i> 1
DNP-alanine	0.25	0.73	0.23	0.30
α -DNP-amino- δ -hydroxyvaleric acid	0.19	0.79	0.17	0.42
α -DNP-amino- δ -valerolactone	0.75	0.10	0.17	0.0
DNP-arginine	0.24	0.68	0.37	0.35
DNP-glycine	0.14	0.64	0.12	0.24
α -DNP-ornithine	0.17	0.78	0.12	0.48
δ -DNP-ornithine	0.16	0.73	0.10	0.44
DNP-proline	0.25	0.76	0.22	0.42
DNP-serine	0.13	0.74	0.10	0.37
DNP-valine	0.41	0.73	0.50	0.34
Hydrolytic product <i>A</i>	0.36	0.66	0.52	0.26
Hydrolytic product <i>B</i>	0.21	0.67	0.30	0.26
2:4-Dinitrophenol	0.31	0.60	0.32	0.26

Paper chromatography. Four solvent systems, *A*1, *BE*2, *A*2 and *B*1, were used in this investigation, and by choice of a suitable combination of some or all of these systems it was possible to distinguish between any of the DNP derivatives listed in Table 1. *A*1 and *BE*2 were based on the systems of Blackburn & Lowther (1951) in which an organic solvent was equilibrated with phthalate buffer (50 ml. of 0.1*M* potassium hydrogen phthalate:45.45 ml. of 0.1*N*-NaOH), pH 6.0, and the top phase was used as developing solvent. System *A*1 consisted of *tert*-pentanol-buffer (4:1, v/v), and *BE*2 of 1 vol. of benzyl alcohol-ethanol (9:1, v/v) and 3.4 vol. of buffer. The developing solvent of system *A*2 (Bowes & Moss, 1953) was the top phase from a mixture of equal volumes of *n*-pentanol and 2*N*-NH₃ soln., but the developing solvent of system *B*1 (Rover & Fabre, 1953) was an aqueous buffer (pH 6.2) prepared from *m* trisodium citrate (100 ml.) and *N*-HCl (7.0 ml.). The paper was Whatman no. 1 filter paper and was pretreated with phthalate buffer (pH 6.0) for use with systems *A*1 and *BE*2. The DNP-derivative in ethyl methyl ketone (3 mg./ml.) was applied (5 µl.) to the paper with a micropipette, the solvent being removed in a current of air. The chromatograms were run at 20°, usually for 16 hr. with systems *A*1, *A*2 and *B*1. With system *BE*2 the paper, after application of the DNP derivatives, was equilibrated overnight in the chromatograph tank containing the *BE*2 aqueous phase before development of the chromatogram for a period of 3–4 hr.

The R_F values for the DNP compounds investigated during this work are given in Table 1. Variations in R_F values of individual DNP-amino acids were observed, especially with systems *BE*2 and *A*1, and hence control DNP-amino acids were always run with the unknown samples.

Conditions for hydrolysis of DNP-proline. The early work on hydrolytic conditions was designed to obtain a maximum recovery of DNP-proline from DNP-protamines containing proline as the *N*-terminal amino acid, but it became apparent that DNP-proline was not stable to any great extent under the conditions necessary to remove completely the DNP-prolyl group from the protein. Thus hydrolysis of DNP-DL-proline for 16 hr. with 12*N*-HCl

yielded less than 5% of DNP-proline and less than 10% of DNP products other than 2:4-dinitrophenol. Even under the milder conditions of hydrolysis with 96% acetic acid at 100° for 16 hr., only 50% of the original DNP-proline was recovered, whereas under these conditions DNP protein was not effectively hydrolysed.

The preliminary experiments indicated that optimum recovery of DNP compounds other than dinitrophenol was obtained by the use of 12N-HCl for hydrolysis of DNP-proline, and experiments to determine the optimum time were carried out for times varying from 0.25 to 16 hr. Some 10–20% DNP-proline was recovered after 45 min. at 100°, but only 5–10% was detected after 4 hr., by which time considerable amounts of 2:4-dinitrophenol and proline were present in the hydrolysate. For the preparative isolation of the DNP-proline hydrolytic products *A* and *B*, a hydrolysis time of 1 hr. was selected as giving the optimum yield of *A* and *B* combined with the minimum amount of residual DNP-proline. For DNP-salmine, on the other hand, a hydrolysis time of 4 hr. was preferred, as this gave a reasonable yield of hydrolytic products and the minimum of DNP-prolyl peptides.

Isolation and purification of the products of acid hydrolysis of DNP-DL-proline

DNP-DL-Proline (1 g. divided into four lots) was hydrolysed with a total of 400 ml. of 12N-HCl in sealed tubes at 100°. The contents were agitated to effect solution and heating was continued for a further 1 hr. After cooling, the bulked solution was diluted with 3 vol. of water and extracted with solvent. Ether was used in the early experiments, but subsequently ethyl acetate was preferred because of the more favourable partition of the coloured hydrolysis products between the solvent and either dilute acid or water. Two extractions with ethyl acetate (2 × 600 ml.) were carried out and the bulked extract was washed with water. The solvent was then removed *in vacuo*, leaving a light orange-yellow oil which on standing gave a small quantity of yellow crystalline solid. In one experiment the oil was separated from the solid by extraction with hot benzene (15 ml.) and the solid recrystallized from hot benzene, when pure hydrolysis product *A* (114 mg.), m.p. 142–142.5°, was obtained. In general, however, the hydrolytic products were separated by chromatography on a silicic acid–Celite column (Green & Kay, 1952).

Moist silicic acid (500 g.) (British Drug Houses Ltd.) was heated at 200° for 2 hr., cooled in a desiccator, ground and passed through an 85-mesh sieve. The yield (110–250 g.) varied unpredictably from batch to batch and some batches were very unsatisfactory for chromatography of DNP products. The acid was thoroughly shaken with one-half its weight of sieved Celite 545 and 250 g. of the mixture was slurried with acetone and poured into a tube of 58 mm. diameter. A positive pressure (8 cm. Hg) was applied, and the column, which was at least 15 cm. in height, was pre-washed with dry ether (250 ml.) followed by light petroleum, b.p. 60–70° (250 ml.), and finally with the developing solution (250 ml.). The latter consisted of acetic acid (3 vol.) and ethyl acetate (12 vol.) made up to 100 vol. with light petroleum, b.p. 60–70°. The hydrolytic products, dissolved in the minimum volume (about 4 ml.) of a mixture of acetone and light petroleum b.p. 60–70° (1:1, v/v), were applied to the column and the developing solvent (4.5 l.) was added. 2:4-Dinitrophenol was eluted before the yellow

bands had reached the bottom of the column. Complete separation into bands of *A* and *B*, which was observed with columns of small diameter, was not obtained with the 58 mm. diameter columns. Elution was continued until all the yellow material had been removed, the eluate being collected in 25 ml. fractions with an automatic fraction collector. Samples of each fraction, after evaporation to dryness, were examined by paper chromatography in system *A*1, and by the use of these results the eluate fractions were combined to give pooled fractions containing *A* and *B* respectively. In a typical experiment product *A* was obtained in fractions 1–27 and *B* in fractions 36–86, the intermediate fractions containing a small overlap of *A* and *B*.

Solvent was removed from the pooled fractions by distillation *in vacuo*, the last traces of acetic acid being eliminated by redistillation of the residue *in vacuo* with added benzene (20 ml.). Product *A* was readily recrystallized from boiling benzene, and in a typical experiment 275 mg. of pure crystalline *A* was obtained.

The unchanged DNP-proline, which was present in the pooled fraction containing *B*, was separated from *B* by chromatography on a cellulose column. The mixture, dissolved in ethyl acetate, was applied to a column (58 mm. diam.) of Whatman cellulose powder and the chromatogram developed with 2 l. of the buffer (pH 6.2) used in the paper-chromatography system *B*1. DNP-proline appeared in the early eluate fractions, and product *B* in subsequent fractions, and the separation, which was complete, was followed by paper chromatography of samples of the eluate fractions by the use of system *B*1. The fractions containing *B* were pooled, acidified with 6N-HCl and extracted with ethyl acetate. The ethyl acetate solution was washed with water and the solvent removed by distillation *in vacuo*. Crystallization was achieved by dissolving the residue in ether (50 ml.), adding light petroleum (b.p. 60–80°) (50 ml.), and allowing the solvent to evaporate slowly in a current of dry filtered air, when yellow crystals of *B* separated (140 mg.). The product was purified by two further crystallizations, yielding yellow crystals (83 mg.) of *B*, m.p. 107.5–109°.

General properties of hydrolytic products A and B

Product *A* crystallized from benzene in yellow prisms, m.p. 142–142.5°. Its ultraviolet absorption spectrum in 1% NaHCO₃ showed maxima at 265 mμ. (ε 9700) and 361 mμ. (ε 17 800) and a minimum at 300 mμ. (ε 3100). (Found: C, 42.05; H, 3.8; N, 13.2; Cl, 11.3; equiv. by titration, 322. C₁₁H₁₅O₆N₃Cl requires C, 41.6; H, 3.8; N, 13.2; Cl, 11.2%; equiv. as monobasic acid, 318.) When heated in a high vacuum at 170–180° it distilled to yield a yellow oil which was shown by paper chromatography, with systems *A*1 and *B*1, to consist mainly of unchanged *A* with about 20% of DNP-proline.

Product *A* is sparingly soluble in water but dissolves readily in NaHCO₃. Potentiometric titration in aqueous acetone solution with 0.05N-NaOH, which had been standardized under identical conditions against pure DNP-alanine, indicated the presence of a free carboxyl group. It forms an insoluble silver salt, but warming with acidified ethanolic AgNO₃ does not liberate halide. On heating with phenol and 2N-NaOH at 100° for 1 hr., halide was liberated and alkali-soluble material was obtained which gave a solid on acidification. Recrystallization of the latter from

a mixture of ether and light petroleum gave yellow crystals of impure DNP-proline, m.p. 175–177°, not depressed on admixture with authentic DNP-DL-proline. Although some *A* was recovered after heating with 12*N*-HCl at 100° for 16 hr., a quantitative yield of proline was obtained after heating with 6*N*-HCl for 16 hr. Ornithine and unidentified ninhydrin-positive material were obtained after heating with aqueous NH₃ (sp.gr. 0.88) at 100° for 4 hr., and proline and unidentified ninhydrin-positive material were obtained after heating with saturated Ba(OH)₂ at 100° for 4 hr.

Compound *B* was obtained in the form of yellow prisms, m.p. 107.5–109°. Its ultraviolet absorption spectrum in 1% NaHCO₃ showed maxima at 265 mμ. (ε 8400) and 362 mμ. (ε 18 000) and a minimum at 300 mμ. (ε 2100). (Found: C, 41.2; H, 3.9; N, 13.1; Cl, 11.1; equiv. by titration, 314. C₁₁H₁₃O₆N₂Cl requires C, 41.6; H, 3.8; N, 13.2; Cl, 11.2%; equiv. as monobasic acid 318.)

Like product *A*, product *B* yields some DNP-proline on heating in high vacuum and no halide when heated for a short time with acidified ethanolic AgNO₃. The presence of a free carboxyl group was shown by potentiometric titration. Product *B* also yielded products similar to *A* after hydrolysis, namely, proline (in high yield) after 16 hr. with 6*N*- and 12*N*-HCl at 100°, ornithine and unidentified ninhydrin-positive material after 4 hr. with aqueous NH₃ (sp.gr. 0.88) at 100°, and proline and unidentified ninhydrin-positive material after 4 hr. with saturated (BaOH)₂ solution at 100°.

Chromatographic behaviour of A and B. In system *A*1, product *A* is well separated from *B* and from DNP-proline, but *B* is not completely separated from DNP-proline. Systems *B*1 and *BE*2, in which *A* and *B* run together, are of value in separating DNP-proline from these products. In system *A*2, 2,4-dinitrophenol and *B* run together but DNP-proline and *A* are well separated, and as DNP-proline runs slowly this system is well suited for the detection of relatively small amounts (1–2 μg.) of DNP-proline in the presence of the breakdown products. Excessive amounts of dinitrophenol may cause interference, and should be removed by high-vacuum sublimation at 100°.

Syntheses

α-2:4-Dinitrophenyl-δ-benzoyloxycarbonyl-DL-ornithine. δ-Benzoyloxycarbonyl-DL-ornithine (IV) was obtained by slight modifications of the method used by Syngé (1948) for the preparation of δ-benzoyloxycarbonyl-L-ornithine. The reaction product (IV) from DL-ornithine hydrobromide (1.07 g.) on recrystallization from water gave colourless plates (0.8 g.), m.p. 251–252°; Harris & Work (1950) report m.p. 256°.

δ-Benzoyloxycarbonylornithine (IV) (0.53 g.) was mixed with NaHCO₃ (0.57 g.) in water (8 ml.), a solution of FDNB (0.57 ml.) in ethanol (16 ml.) added and the mixture shaken for 2 hr. The ethanol was distilled *in vacuo* and the residue diluted with water (10 ml.) and extracted with ether (2 × 20 ml.). A small amount of insoluble material was removed from the aqueous solution by filtration and the DNP derivative precipitated as an oil by the addition of 6*N*-HCl (4 ml.). As the oil did not solidify on standing at 0° it was extracted with ether (25 ml.) and after washing with 0.5*N*-HCl and with water the ethereal solution was dried (Na₂SO₄) overnight. The ether was removed *in vacuo*, considerable frothing taking place towards the end of the

distillation. The amorphous yellow solid (0.8 g.) was used directly for the next stage.

α-2:4-Dinitrophenylornithine (III). The crude α-2:4-dinitrophenyl-δ-benzoyloxycarbonylornithine (0.3 g.) was heated at 60–70° with 12*N*-HCl (5.5 ml.) for 1 hr. After removal of HCl *in vacuo* the residue was dissolved in water (20 ml.) and extracted with ether, but no trace of ether-soluble starting material was observed. The aqueous solution was evaporated to dryness *in vacuo*, and after addition of water the evaporation was repeated several times. The residue was dissolved in boiling water (30 ml.), filtered and neutralized with pyridine (0.1 ml.). On standing, yellow needles (160 mg.) separated, m.p. 220–221° (decomp.). The mixed m.p. with an authentic sample of α-2:4-dinitrophenylornithine (m.p. 220–222°), prepared according to the method of Sanger (1946), was not depressed.

Reactions of α-2:4-dinitrophenylornithine with NOCl and HNO₃. A solution of NOCl, prepared by grinding AgNO₃ (0.5 g.) with ice-cold 6*N*-HCl (5 ml.), was added to α-2:4-dinitrophenylornithine (50 mg.) in peroxide-free dioxan (5 ml.), and the clear solution heated at 65°. Heating was continued for 5 hr., by which time some coloured ether-extractable material had been formed. The solution was cooled, diluted with water (5 ml.) and extracted with ether (3 × 30 ml.). The ether extract was washed with water (30 ml.) and the ether removed *in vacuo*. The oily residue, which could not be crystallized, was found by paper chromatography to contain five yellow-coloured components, one of which corresponded with the required δ-chloro derivative (I).

Because of the difficulty in replacing the δ-amino group directly by Cl, the action of nitrous acid was investigated, and under the normal analytical conditions an almost quantitative release of N₂ was obtained.

α-2:4-Dinitrophenylornithine (19 mg.) in acetic acid (1 ml.) was shaken with saturated NaNO₂ until evolution of gas had ceased (1 hr.). The solution was distilled to dryness *in vacuo* and the residue extracted with a mixture of ethyl acetate (10 ml.) and water (10 ml.). The ethyl acetate solution, which contained all the yellow-coloured material, was distilled *in vacuo*. Paper chromatography of the solid residue (with system *A*1) showed 2 spots, the stronger one corresponding to α-2:4-dinitrophenylamino-δ-hydroxyvaleric acid (V). Crystallization from a mixture of ethyl acetate and light petroleum (b.p. 40–60°) gave yellow crystals, m.p. 179–181° (decomp.), not depressed on admixture with α-2:4-dinitrophenylamino-δ-valerolactone (VI) (see below).

α-2:4-Dinitrophenylamino-δ-hydroxyvaleric acid (V). α-Amino-δ-hydroxyvaleric acid (0.8 g.), prepared from γ-butyrolactone by the method of Pleninger (1950), was mixed with NaHCO₃ (1.06 g.) in water (20 ml.). A solution of FDNB (1.22 g.) in ethanol (40 ml.) was then added and the mixture shaken for 2 hr. The ethanol was removed *in vacuo* and the residue diluted with water (40 ml.) and extracted with ether (2 × 40 ml.). The aqueous solution was acidified with 2*N*-H₂SO₄ (4.5 ml.), stood at 0–5° (1 hr.), and the precipitate filtered, washed with cold water (10 ml.) and dried. Crystallization of the crude material (1.6 g.) from a mixture of ethyl acetate and light petroleum, b.p. 60–80°, gave pure α-2:4-dinitrophenylamino-δ-hydroxyvaleric acid as yellow prisms, m.p. 141.5–142°. (Found: C, 44.2; H, 4.0; N, 13.6. C₁₁H₁₃O₇N₂ requires C, 44.1; H, 4.35; N, 14.0%.)

α-2:4-Dinitrophenylamino-δ-valerolactone (VI). In one preparation of the δ-hydroxyvaleric acid (V) (see above) the

material separated as an oil on attempted crystallization from a mixture of ethyl acetate and light petroleum. After removal of the solvent *in vacuo* the residue was recrystallized from a mixture of ethyl acetate and light petroleum and gave pure α -2:4-dinitrophenylamino- δ -valerolactone as yellow prisms (1.3 g.), m.p. 186–188°. (Found: C, 47.0; H, 3.95; N, 15.0. $C_{11}H_{11}O_6N_3$ requires C, 47.0; H, 3.9; N, 14.95%.)

The lactone (VI), which was insoluble in Na_2CO_3 , was partially reconverted into the δ -hydroxy acid (V) by shaking an ethyl acetate solution with 0.1N-NaOH; identification of the δ -hydroxy acid was made by mixed m.p. and by paper chromatography, with systems A1, BE2 and B1.

α -2:4-Dinitrophenylamino- δ -chlorovaleric acid (I). α -2:4-Dinitrophenylamino- δ -hydroxyvaleric acid (130 mg.) in pyridine (0.1 ml.) was cooled in an ice bath and $SOCl_2$ (0.15 ml.) added from a micropipette during 10 min. After heating at 50° for 90 min. the reaction mixture was cooled in an ice bath, ice (3 g.) was added, and the mixture of oil and solid was stirred while it warmed to room temp. After acidification with 6N-HCl (1.5 ml.) the dark-red solution was extracted with ethyl acetate (2 $\times$ 5 ml.). The ethyl acetate solution was washed with 2N-HCl (2 $\times$ 5 ml.) and with water (2 $\times$ 10 ml.) and extracted with 1% Na_2CO_3 (2 $\times$ 10 ml.), when much of the red colour remained in the ethyl acetate. The Na_2CO_3 extract was washed with ethyl acetate (2 $\times$ 10 ml.), acidified with 2N-HCl (10 ml.), and re-extracted with ethyl acetate (2 $\times$ 10 ml.). The ethyl acetate extract was washed with 2N-HCl (10 ml.) and water (2 $\times$ 10 ml.) and dried (Na_2SO_4) overnight. After removal of solvent *in vacuo* the residue was shown by paper chromatography (with systems A1, A2 and B1) to be the required acid (I) and to be almost free of other DNP compounds. In a preliminary experiment, where the reaction mixture was not cooled during the $SOCl_2$ addition, the reaction product was shown to contain DNP-proline as well as the required acid (I). Crystallization of the residue from benzene gave a crystalline solid (21 mg.) which on recrystallization gave α -2:4-dinitrophenylamino- δ -chlorovaleric acid (I) as yellow prisms, m.p. 140.5–141.5°, not depressed on admixture with the acid hydrolysis product A from DNP-DL-proline.

Alternatively, the chlorination of the δ -hydroxy acid (V) was effected with PCl_5 in acetyl chloride at 0°, the product being decomposed with ice and ether extracted from acid solution. Examination by paper chromatography in systems A1, A2 and B1 showed the presence of the required chloro compound (I) and of DNP-proline, but the products were not isolated as crystalline solids.

α -Chloro- δ -2:4-dinitrophenylaminovaleric acid (II). α -Chloro- δ -aminovaleric acid (100 mg.), prepared from DL-ornithine by the method of Hamilton (1952), was mixed with $NaHCO_3$ (140 mg.) in water (2 ml.). A solution of FDNB (190 mg.) in ethanol (4 ml.) was then added and the mixture shaken for 2 hr. The ethanol was removed *in vacuo* and the residue diluted with water (4 ml.) and extracted with ether (2 $\times$ 5 ml.). The aqueous solution was acidified with 2N-HCl (1 ml.) and stood at 0° for 48 hr. The yellow product, which separated as an oil, was extracted into ether (2 $\times$ 15 ml.) and after drying (Na_2SO_4) the ether was removed *in vacuo*. The residual oil solidified after standing in the dark for some days at 20°. It was crystallized by solution in hot ethyl acetate followed by careful addition of light petroleum, b.p. 40–60°, until a slight

turbidity was produced. This was allowed to clear before re-warming and addition of more light petroleum. After standing overnight the mixture gave pure α -chloro- δ -2:4-dinitrophenylaminovaleric acid (II) as yellow prisms, m.p. 106–108°, not depressed on admixture with product B obtained by hydrolysis of DNP-DL-proline with HCl. (Found: C, 41.9; H, 4.1; N, 13.1. $C_{11}H_{12}O_6N_3Cl$ requires C, 41.6; H, 3.8; N, 13.2%.)

SUMMARY

1. The stability of DNP-proline has been examined under conditions of hydrolysis with hydrochloric acid such as are used in the identification of *N*-terminal residues of proteins.

2. Two hitherto undescribed hydrolytic products of DNP-DL-proline have been isolated and details of their general properties are given.

3. These products have been shown by synthesis to be α -2:4-dinitrophenylamino- δ -chlorovaleric acid and α -chloro- δ -2:4-dinitrophenylaminovaleric acid.

4. A scheme for the identification of *N*-terminal proline is suggested in the light of these findings.

We wish to thank Mr L. H. Kent for his advice and interest during the course of this investigation and Mr R. Strange for amino-N determinations.

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