60. THE 3:5-DINITROBENZOYL DERIVATIVES OF THE AMINO-ACIDS AND THEIR USE IN SEPARATING THE ISOMERS OF LEUCINE AND VALINE

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In recent papers, Saunders [1934; 1938] has described the preparation of the 3:5-dinitrobenzoyl derivatives of certain amino-acids and has suggested their use for purposes of identification. This possibility has been confirmed by the present author; however, certain discrepancies have been observed between published data and present experience and these observations are embodied in this communication.

In the first place, the reaction between 3:5-dinitrobenzoyl chloride and amino-acids has been found to be much slower than stated by Saunders. Reaction times of the order of 30 min. have been found, compared with quoted times of 2 min. for similar quantities of material. It was also observed that some 3:5-dinitrobenzoic acid is always obtained during benzoylation, even when molecular proportions of reactants are employed, and this can be very troublesome. It is suggested by Saunders that practically no 3:5-dinitrobenzoic acid is formed during the reaction.

It has also been found that the melting points of some of the derivatives differ considerably from those quoted by Saunders, and such differences are noted in the experimental section of the paper. Also in the cases of valine and leucine more than one derivative has been isolated. The melting points of the derivatives which the author has been able to prepare are quoted in Table 1. These melting points have been consistently obtained in this laboratory and are believed to be correct.

Table 1. *3:5-Dinitrobenzoyl derivatives of amino-acids*

Derivative of:	M.P. °C
Glycine	182·2, shrinks 181·4
<i>l</i> (+)-Alanine	177, softens 176·6
<i>l</i> (-)-Threonine	181, softens 180
<i>l</i> (-)-Serine	183, shrinks 112
<i>l</i> (-)-Cystine	Derivative not suitable
<i>dl</i> -Valine	211·4, softens 210·8
<i>l</i> (+)-Valine	207
<i>nor</i> Valine (?)	182
<i>l</i> (-)-Leucine	188, softens 187
<i>l</i> (+)- <i>iso</i> Leucine	170·4, softens 169·2
<i>nor</i> Leucine (?)	163
<i>dl</i> -Phenylalanine	161
<i>l</i> (-)-Tyrosine	No derivative obtainable
<i>l</i> (-)-Aspartic acid	184·8, softens 184·4
Na-salt of aspartic acid derivative	218
<i>l</i> (+)	} -Glutamic acid 104
<i>d</i> (-)	
<i>dl</i>	
<i>r</i> -Glutamic acid	204
Synthetic β -hydroxyglutamic acid	229·5, darkens 228
3:5-Dinitrobenzoic acid	207·8
3:5-Dinitrobenzoyl chloride	74

A derivative has been isolated from aspartic acid, whereas Saunders isolated no derivative, but deduced from a formaldehyde titration that 50 % of the aspartic acid remained unchanged. Experience has shown that this derivative is very easily hydrolysed and may be decomposed if it is kept in a solution more acid than pH 2 for any length of time.

The crystalline forms of these derivatives are described in the experimental section, and photographs of most of them have been taken to ensure a more reliable record.

As many of the derivatives have similar melting points, determination of melting point alone is not sufficient to characterize them. Their melting points are, however, very sensitive to the presence of impurities, particularly 3:5-dinitrobenzoic acid, and it is convenient to prepare standard preparations of all these derivatives so that mixed melting points can be taken.

A very important observation has been made in that the various isomeric valines and leucines can be separated by the fractional precipitation of their 3:5-dinitrobenzoyl derivatives at different pH. No such separation could be effected by fractional crystallization. The great value of this separation lies in the fact that no means of estimating the proportion of the different isomers of valine and leucine in a mixture has hitherto been available. It was during the course of such separation that derivatives were isolated from valine and leucine which differed from the normal in melting point, but were yet pure. It is suggested that they are the derivatives of *norvaline* and *norleucine*. This is subject to confirmation. They are indicated by a query in Table 1.

It has been found very difficult to free any derivative from traces of 3:5-dinitrobenzoic acid once it has been contaminated. In every case, therefore, precipitation of the 3:5-dinitrobenzoyl derivative has been effected gradually by adding standard acid to the alkaline solution in stages, filtering off each precipitate as it forms, and recording the pH by means of indicators. The derivatives of dicarboxylic amino-acids are insoluble in benzene; this enables any free 3:5-dinitrobenzoic acid to be separated by continuous extraction in a Jena sintered glass apparatus as used for rubber analysis. This treatment was not suitable for the derivatives of monobasic monoamino-acids.

EXPERIMENTAL

3:5-Dinitrobenzoyl chloride hydrolyses very rapidly on exposure to air. It is best to obtain a freshly crystallized specimen and put it up in 1 g. portions in dry tubes immediately; such a specimen has been kept in good condition for more than a year. When pure it crystallizes in needles and melts at 74°.

3:5-Dinitrobenzoic acid which is always obtained as a by-product crystallizes in thick rectangular plates, M.P. 207·8°.

In all cases described below 1 g. of 3:5-dinitrobenzoyl chloride was used, and was added slowly, with mechanical shaking between each addition, to the calculated amount of amino-acid in 5 ml. water, containing the calculated quantity of 2*N* NaOH (2 mol.). Excess of acid chloride was not used owing to the difficulty of separating free 3:5-dinitrobenzoic acid from the amino-acid derivative.

Derivatives of individual amino-acids

Glycine. On acidifying the reaction mixture with *N* HCl to pH 3·3 a fine crystalline precipitate was obtained; M.P. 158–160°. Crude yield 1·06 g. (91 % of theory). It was found difficult to separate from traces of 3:5-acid by crystallization, but after crystallizing twice from 10 % acetic acid and once from water, it gave long fine needles, M.P. 182·2°, shrinking at 181·4°.

Loss in wt. on drying at 110° = 6.5 % (theory for $C_5H_7O_7N_3 \cdot H_2O$, 6.27 %). Saunders [1934] quotes M.P. 179° .

l(+)- and dl-alanine. On acidifying the solution after benzylation of *dl*-alanine (B.D.H.) to pH 2.6, and leaving in the refrigerator overnight, 0.729 g. of derivative, M.P. 176° , softening at 172° was obtained (63 % yield). After crystallization from 10 ml. 10 % acetic acid it yielded 0.61 g., M.P. 177° sharp, containing no water of crystallization.

A sample of *l(+)-alanine* also gave a derivative with the same melting point (58 % yield); mixed M.P. with the *dl*-derivative, 174° , softening at 172° . The above data agree with those quoted in the literature.

Valine. Great discrepancies have been observed here between the figures quoted in the literature and those actually obtained. The derivative has been prepared from three samples of valine, first from a specimen of *dl*-valine, and then from two samples of *l(+)-valine* which had been presented to the author.

dl-Valine (B.D.H.) gave, on bringing the reaction mixture to pH 2.8, a crude product M.P. 184° (68.5 % yield). This, after purification, crystallized in glistening flat rectangular platelets, M.P. 211.4° , softening at 210.8° . (Found: C, 46.45; H, 4.27; N, 13.42 %. $C_{12}H_{13}O_7N_3$ requires C, 46.35; H, 4.18; N, 13.50 %.) This derivative contains no water of crystallization, whereas Saunders found for the derivative of *l(+)-valine* M.P. $157-158^{\circ}$, and also 1 mol. of water of crystallization.

l(+)-Valine. As both samples of natural valine proved to be mixtures and as the results differed with each sample, it will be easier to record the findings diagrammatically.

In all, taking into account various minor fractions, there were isolated: 0.508 g. *l*(+)-isoleucine derivative, M.P. 170.2°, equivalent to 0.205 g. *l*(+)-isoleucine or 40 % of the initial material; 0.196 g. valine derivative, M.P. 181.5°, equivalent to 0.074 g. of valine or 14.5 % of the starting material.

As the total yield of derivatives (54.5 %) is of the same order as the yield normally obtained in an acylation with 3:5-dinitrobenzoyl chloride, it seems reasonable to suppose that this sample of so-called valine was in reality a mixture of isoleucine and valine in the proportion of approximately 74 % isoleucine and 26 % valine.

It is important to note that the M.P. of this valine derivative is far removed from that obtained with *dl*-valine (B.D.H.) and also from that obtained by Saunders [1934] who gives M.P. 157–158°.

Analysis of these two derivatives by Schoeller gave the following results: isoleucine derivative: found: C, 47.87; H, 4.40; N, 13.04 %; calc. C, 48.00; H, 4.62; N, 12.92 %; valine derivative: found: C, 46.57; H, 4.36 %, N, 13.35 %; calc. C, 46.35; H, 4.36; N, 13.50 %.

Valine 2. From this sample there have been isolated (see diagram): 0.31 g. of isoleucine derivative, M.P. 170°, equivalent to 0.115 g. of free isoleucine or 7.5 % of the initial weight of substance; 0.64 g. of a valine derivative, M.P. 182°, equivalent to 0.26 g. of free valine, or 17 % of the starting material (this derivative is identical with that isolated from valine 1); 1.22 g. of another valine derivative, M.P. 207°, equivalent to 0.458 g. of free valine or 30 % of the starting material; this valine derivative is apparently identical with that obtained from synthetic *dl*-valine (B.D.H.) as no lowering of M.P. occurs on mixing. As this synthetic valine is certainly that variety with a *gem*-dimethyl group, it is possible to fix the structure of the valine giving the 3:5-dinitrobenzoyl derivative, M.P. 207°. The structure of the valine giving the 3:5-dinitrobenzoyl derivative, M.P. 182°, remains to be determined. It is suggested provisionally that this is *nor*valine.

The three derivatives isolated from valine 2 were analysed by Schoeller: 3:5-dinitrobenzoyl derivative, M.P. 182°: C, 46.52; H, 4.39; N, 13.31 %; 3:5-dinitrobenzoyl derivative, M.P. 207°: C, 46.48; H, 4.23; N, 13.50; valine derivative requires C, 46.35; H, 4.18; N, 13.50 %; 3:5-dinitrobenzoyl derivative, M.P. 170°: C, 47.78; H, 4.42; N, 13.02 %; isoleucine derivative requires C, 48.00; H, 4.62; N, 12.92 %. The 3:5-dinitrobenzoyl derivative, M.P. 182°, crystallizes in very long but narrow rectangular rods, while the derivative with M.P. 207° crystallizes in large thin hexagonal plates which often have an irregular outline. The derivative from *dl*-valine, M.P. 211.4°, crystallizes in the same form as the derivative with M.P. 207°, which confirms their identity. Photographs have been taken of these crystals.

It is interesting to compare the yields of 3:5-dinitrobenzoyl derivatives from these two samples of valine:

	3:5-Dinitrobenzoyl derivatives isolated			
	M.P. 207° %	M.P. 182° %	M.P. 170° %	Total %
Valine 1	0	14.5	40	54.5
Valine 2	30	17	7.5	54.5

The yield in either case is 54.5 % expressed as amino-acid isolated in terms of starting material. This yield is of the same order as is usually obtained in acylating pure amino-acids, so that if no other amino-acids are assumed to be

was pure. Until methods for the isolation of amino-acids have been perfected it would seem idle to give tables showing the percentage composition of proteins.

The melting point quoted by Saunders for the valine derivative is 157–158°, and he states that it crystallizes with 1 H₂O. The two derivatives isolated in the present work are anhydrous, and neither melting point is in the vicinity of that quoted.

l(-)-Leucine. For this preparation a sample of *l*(-)-leucine was obtained from Schuchardt's, and was of guaranteed purity.

This *l*(-)-leucine derivative had M.P. 188°, softening at 187.2°, on a standard thermometer; it contained no water of crystallization. These data agree with those quoted by Saunders.

The yield obtained was 61 % of theory; there was also obtained 3.4 % of *l*(+)-isoleucine derivative, M.P. 169°. Thus this amino-acid, in spite of its label, was not pure and consisted of a mixture of 95 % *l*(-)-leucine and 5 % *l*(+)-isoleucine. A sample of leucine isolated from the hydrolysis of horsehair was also used to prepare the derivative as this was a specimen thought to be of great purity.

From 1.132 g. of this there were isolated 1.32 g. leucine derivative, M.P. 188°, equivalent to 0.532 g. leucine or 47.2 % of the original material. This derivative crystallizes in very thin rectangular plates with one or both ends cut off obliquely.

There was also isolated another derivative, M.P. 163°, also crystallizing in very thin rectangular plates, but with all ends perfectly square, in amount corresponding to 0.097 g. leucine or 8.55 % of the starting material. (Found: C, 47.75; H, 4.60; N, 13.02 %. Theory: C, 48.00; H, 4.61; N, 12.91 %.)

The total yield of leucine derivatives from this specimen of leucine is thus 55.7 % of the initial leucine which is of the same order as is usually obtained.

One may calculate on this basis that the composition of this sample of leucine was approximately:

	%
<i>l</i> (-)-Leucine	84.8
<i>nor</i> Leucine (?)	15.2
	<hr/> 100.0

It is assumed provisionally that this derivative, which analyses for a leucine derivative but does not correspond with that either of leucine or *isoleucine*, is that of *nor*leucine.

l(+)-*iso*Leucine. A sample of pure *l*(+)-*isoleucine* from Schuchardt was used for the preparation of this derivative.

This derivative crystallizes in long narrow rectangular plates. A derivative was also prepared from the *isoleucine* isolated from gliadin by Town [1929], and this also had M.P. 170°.

Serine. A sample of pure serine isolated by the author from gliadin was used in this preparation. 0.457 g. serine was acylated, but as the derivative did not precipitate until the solution was acidified to pH 1, it was contaminated with free 3:5-dinitrobenzoic acid which was difficult to remove by crystallization from 25 % acetic acid (10 ml.). The weight of crude derivative was 0.964 g. and its M.P. 157°, softening at 140°. It was extracted continuously with benzene in a Jena sintered glass extraction thimble for 45 min. The benzene on evaporation yielded 0.32 g. 3:5-dinitrobenzoic acid. The residue insoluble in benzene, 0.61 g., M.P. 183°, was crystallized from water and then had M.P. 183°, softening at 181° and shrinking at 112°, crystallizing in needles. Yield 46.8 %. This serine derivative was dried at 110°: loss in weight, 5.45 %; calc. for $C_{10}H_9N_3O_8$, H_2O , 5.66 %. (Found: C, 37.99; H, 3.77; N, 13.22 %; calc. for hydrated serine derivative: C, 37.90; H, 3.37; N, 13.24 %. The anhydrous derivative has M.P. 183°. Saunders quotes the melting point of serine derivative as 94-95°.

l(-)-*Threonine*. A pure sample of this amino-acid isolated by the author from gliadin was used for the preparation of the derivative. The same difficulty was encountered as in the preparation of the serine derivative, that the solution had to be acidified to pH 2 before any precipitate was formed. On leaving in the ice-chest for some hours a precipitate of 0.07 g. 3:5-dinitrobenzoic acid formed. The solution had now to be left for over a week.

117 mg. of threonine were benzoylated, and 79 mg. of a derivative, M.P. 135°, slowly separated. It was extracted continuously with benzene and gave 34 mg. residue, which after crystallizing from 5 ml. 10 % acetic acid had M.P. 181°, softening at 180°. It crystallized in long thin rectangular rods. Yield 11.2 %. (Found: C, 41.83; H, 3.60; N, 13.30 %; calc. for $C_{11}H_{11}N_3O_8$: C, 42.18; H, 3.51; N, 13.42 %.)

The yields of derivatives of both these hydroxy-acids are low, presumably owing to their greater solubilities.

Cystine. The derivative is not really suitable for the identification of cystine, and although the author's experience differs rather from the description of Saunders, its description is unwarranted.

Phenylalanine. *dl*-Phenylalanine (B.D.H.), 0.715 g., was acylated, and after bringing to pH 7, an oil was precipitated. Sufficient methyl alcohol was added just to dissolve the oil and the solution was left for 24 hr.; 0.90 g. crystals separated, M.P. 147°, softening at 142°. The filtrate was brought to pH 4 and treated in the same way; a second crop was obtained, 0.22 g., M.P. 110°, softening at 105°. Only 3:5-dinitrobenzoic acid was obtained on further acidification.

The mixed crops were crystallized from 30 ml. 60 % methyl alcohol and gave 0.78 g., M.P. 160°, softening at 107°. Yield 50.3 %. On recrystallization from 25 ml. 50 % acetic acid, the derivative had M.P. 161°, softening at 160°, and crystallized in thin rectangular rods; wt. 0.57 g. The substance has no water of crystallization. (Found: C, 53.64; H, 3.90; N, 11.60 %; calc. for $C_{16}H_{13}N_3O_7$: C, 53.50; H, 3.62; N, 11.70 %.)

Saunders quotes M.P. 93°, and states that the compound crystallizes with 0.5 mol. H_2O .

Glutamic acid. Natural glutamic acid hydrochloride (0.797 g.) isolated by the author from gliadin was used and, after acylation, the solution was acidified to pH 2. A slight oily precipitate separated which rapidly solidified and, after 12 hr. in the ice-chest, 1.073 g. of derivative were obtained, M.P. 130°, softening at 102°. The derivative is quite insoluble in benzene, and after extracting continuously for 1 hr. with boiling benzene the residue (0.570 g.) had M.P. 155°, softening at 153°. The benzene on evaporation gave 0.448 g. of 3:5-dinitrobenzoic acid.

The residue insoluble in benzene was crystallized from 8 ml. 10 % acetic acid and yielded 0.46 g. of a derivative, M.P. 104°, softening at 102°, crystallizing in very long fine needles. It has a water content of 5.47, 5.45 %; $C_{12}H_{11}N_3O_9$, H_2O requires 5.04 %. The anhydrous derivative has M.P. 182°, softening at 179°. The yield of derivative is 38.4 % of theory. Saunders quotes M.P. 98–99° and finds that it crystallizes with 0.5 ml. H_2O .

From the mother liquors from the separation of glutamic acid as its hydrochloride from a protein hydrolysate, the author has isolated a glutamic acid which yields a 3:5-dinitrobenzoyl derivative, M.P. 204°, and crystallizes in large but very thin hexagonal plates which tend to overlay one another. It crystallizes in the anhydrous condition. This is believed to be the derivative of *r*-glutamic acid; it is certainly not that of *dl*-glutamic acid which gives the derivative M.P. 104° [Town, 1941].

Aspartic acid. Natural aspartic acid (0.58 g.) was acylated and acidified to pH 2, when 0.27 g. of 3:5-dinitrobenzoic acid separated. The filtrate was left for 12 hr. in the ice-chest and 0.36 g. of derivative obtained, m.p. 180°, softening at 177°. This was crystallized from 3 ml. 10 % acetic acid in which it was easily soluble: 0.13 g., m.p. 184.8°, softening at 184.4°, shrinking slightly at 100°. From the mother liquor there was also isolated 0.017 g. of the glutamic acid derivative, m.p. 204°, identical with that isolated from the dicarboxylate fraction of gliadin [Town, 1941]. This has been obtained in small quantity from all the specimens of natural aspartic acid (three) which the author has examined.

The aspartic acid derivative readily crystallizes from water, in which, however, it is rather soluble; it is readily decomposed in solutions more acid than pH 2. The substance crystallizes with 1 H₂O: loss in weight at 110°, 5.65 %, calc. for C₁₁H₉N₃O₉, H₂O 5.22 %. The anhydrous material has C, 40.1; H, 2.90; N, 12.87 %; calc. for C₁₁H₈N₃O₈, C, 40.40; H, 2.75; N, 12.84 %. The yield of derivative is only 25 %, this low figure probably being due to its solubility and to its ready decomposition in acid solution.

In a repetition of this experiment, the solution after benzylation was only acidified to pH 4, when a glistening white precipitate formed consisting of a mass of fine rectangular plates, 0.692 g., m.p. 220°. This was very soluble in water, and originally must have been salted out by the other sodium salts present. It dissolved in 4 ml. H₂O on warming, and separated in shining flat plates on cooling, wt. 0.32 g. (Found: C, corrected for C in ash 37.4; H, 3.09; N, 11.71; Na, 6.66 %; the mono Na salt of 3:5-dinitrobenzoylaspartic acid, C₁₁H₈N₃O₈Na, requires C, 37.80; H, 2.30; N, 12.02; Na, 6.60 %.) No other 3:5-dinitrobenzoyl derivative has been found to give a sodium salt which can be salted out in this manner.

β-Hydroxyglutamic acid. A sample of synthetic β-hydroxyglutamic acid (0.232 g.) kindly presented by Prof. Harington was used in this experiment.

After benzylation, the solution was acidified to pH 1 and left for 12 hr. in the ice-chest. The derivative was filtered, wt. 0.426 g., and extracted continuously with benzene. The benzene extract gave 0.30 g. 3:5-dinitrobenzoic acid, and the residue, 0.120 g., m.p. 220–221°, was dissolved in 10 ml. absolute methyl alcohol, filtered from a trace of ferric iron impurity, freed from methyl alcohol and crystallized from 12 ml. 25 % acetic acid. It crystallized in long fine needles, wt. 0.116 g., m.p. darkening at 228° and melting with decomposition at 229.5°. Yield 27 % of theory. (Found: C, 40.39; H, 3.12; N, 11.70 %; calc. for C₁₂H₁₁N₃O₁₀, C, 40.40; H, 3.08; N, 11.78 %.)

SUMMARY

It has been confirmed that 3:5-dinitrobenzoyl chloride is a very valuable reagent for the identification of amino-acids. A table of melting points of those derivatives which the author has been able to prepare is given.

The melting points found for some derivatives, notably those of phenyl-alanine, serine and valine, differ considerably from those quoted by Saunders.

There have been isolated two derivatives from the specimens of valine available, and three derivatives from the samples of leucine at the author's disposal. These are thought to be the derivatives of valine and *nor*valine, and of leucine, *isoleucine* and *norleucine* respectively. The identity of the *nor*valine and *norleucine* derivatives is subject to confirmation.

It has been found possible to separate the components in mixtures of the various isomers of valine and leucine by fractional precipitation of their deri-

vatives at differing *pH*. This is important, as no means of separating mixtures of such isomers has hitherto been available.

A derivative has been isolated from aspartic acid, of which the Na salt is readily salted out at *pH* 4. The aspartic acid derivative is easily hydrolysed in solutions more acid than *pH* 2.

No derivative could be isolated from tyrosine.

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