136. *Peptides. Part IV.* Selective Removal of the C-Terminal Residue as a Thiohydantoin. The Use of Diphenyl Phosphor*isothiocyanatidate.†

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Diphenyl phosphoro*iso*thiocyanatidate (I) is conveniently prepared from diphenyl phosphorochloridate and potassium thiocyanate. In acetonitrile or dimethylformamide solution, with the triethylamine salts of *N*-acylated peptides it gives 1-acyl-2-thiohydantoins (V) in high yield. As shown already by others, these substances are readily hydrolysed by alkali to a thiohydantoin (VI) and the acylated peptide or amino-acid lacking the terminal residue.

PART III of this series * described a method for selective removal from a peptide chain of the amino-acid bearing a free carboxyl group, the " *C*-terminal residue " (Sanger, *Adv. Protein Chem.*, 1952, **7**, 1). This procedure appears superior to older ones; yet it is not wholly satisfactory, for the degraded peptide derivative is sometimes seriously contaminated with the starting material. Of the earlier methods the most promising is that of Schlack and Kumpf (*Z. physiol. Chem.*, 1926, **154**, 125), which involves conversion of an *N*-acylpeptide into an acylthiohydantoin (V) and subsequent alkaline cleavage of this intermediate to (VI) and (VII). A recent study of this method (Waley and Watson, *J.*, 1951, 2394) confirms its general soundness and suggests that the principal difficulty lies in the first step, treatment of the original *N*-acylpeptide with acetic anhydride and ammonium thiocyanate at 100°. We now describe an alternative technique, which gives high yields of acylthiohydantoins (V) under mild conditions and therefore adds considerably to the value of Schlack and Kumpf's method.

The preparation of acylthiohydantoins by means of acetic anhydride and ammonium thiocyanate is due to T. B. Johnson and his collaborators (*J. Amer. Chem. Soc.*, 1913, **35**, 1130, 1136; and earlier papers), who considered that the function of these reagents was to produce a mixed anhydride, an acyl *iso*thiocyanate (IV), which would then isomerise to the acylthiohydantoin. Corby, Kenner, and Todd (*J.*, 1952, 1234) recently showed that mixed anhydrides are frequently accessible through " exchange reactions " between anhydrides and anions, owing to the tendency for production of the least reactive anhydride by elimination of the stable ion corresponding to the strongest acid. Thiocyanic acid is relatively strong in aqueous solution (Suzuki and Hagisawa, *Chem. Abs.*, 1949, **43**, 2074; Gorman and Connell, *J. Amer. Chem. Soc.*, 1947, **69**, 2063) but significantly weaker in alcohol (Murray-Rust and Hartley, *Proc. Roy. Soc.*, 1930, *A*, **126**, 84). We therefore expected its anion to enter into exchange reactions. Tetraphenyl pyrophosphate proved to be an anhydride sufficiently reactive to undergo exchange with potassium thiocyanate,

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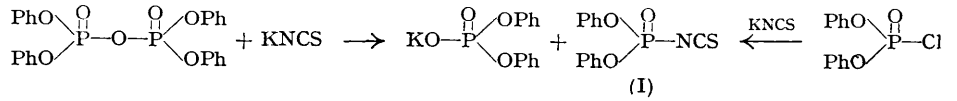
for a mixture of their acetonitrile solutions rapidly deposited potassium diphenyl phosphate, leaving diphenyl phospho*iso*thiocyanatidate (I) in solution. This new mixed anhydride

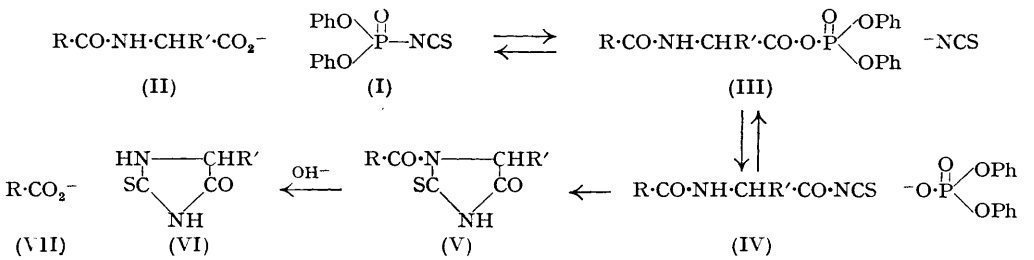


(I)

is obtained more directly from diphenyl phosphorochloridate (chlorophosphonate) and potassium thiocyanate. Its structure is confirmed by comparison of its refractivity (75·83) with that of diphenyl phosphorochloridate (66·07); the difference between these values (9·76) agrees well with that expected from Vogel's results (*J.*, 1948, 1842) for the *iso*thiocyanato-structure (9·77) but not with that corresponding to the thiocyanato-structure (7·56). Still stronger evidence is afforded by the addition of *cyclo*hexylamine to give *N*-diphenoxyphosphinyl-*N'-cyclo*hexylthiourea, $C_6H_{11}\cdot NH\cdot CS\cdot NH\cdot PO(OPh)_2$. This reaction is analogous to the behaviour of acyl *iso*thiocyanates, for instance, benzoyl *iso*thiocyanate (Dixon and Taylor, *J.*, 1908, **93**, 684), but, in contrast to these cases, none of the simple amide, diphenyl phosphoramidate, is obtained by hydrolysis with dilute acid or alkali. This circumstance is fortunate for the application of (I) to the preparation of neutral substances, in particular acylthiohydantoins.

When the mixed anhydride (I) is brought into contact with the triethylammonium salt of an *N*-acylpeptide, further exchange reactions take place. The anion (II) of this relatively weak oxyacid can displace the *iso*thiocyanate ion, forming an acyl phosphate (III), which in turn is attacked by the *iso*thiocyanate ion giving the desired acyl *iso*thiocyanate (IV). The net effect is total elimination of the diphenyl phosphate anion. As the subsequent isomerisation of the acyl *iso*thiocyanate (IV) into the acylthiohydantoin (V) is effectively irreversible the whole process proceeds to completion.

5-Benzyl-2-thio-1-toluene-*p*-sulphonylglycylhydantoin (V; $R = C_6H_4Me\cdot SO_2\cdot NH\cdot CH_2$, $R' = CH_2Ph$) is obtained from the triethylamine salt of toluene-*p*-sulphonylglycyl-DL-phenylalanine in quantitative yield in 110 hours (after 14 and 24 hours, 55 and 84% respectively). The slowness of the process contrasts with the rapidity of exchange between tetraphenyl pyrophosphate and the anions of dibenzyl phosphate (Mason and Todd, *J.*, 1951, 2267) and thiocyanic acid (see above), but is not surprising in view of the number of possible side equilibria. The alternative, less convenient, technique



(VII) (VI) (V) (IV)

of adding tetraphenyl pyrophosphate to a mixture of potassium thiocyanate and the triethylammonium salt in acetonitrile appears to permit a rather faster reaction; the thiohydantoin (V; $R = C_6H_4Me\cdot SO_2\cdot NH\cdot CH_2$, $R' = CH_2Ph$) is obtained in 96% yield after only 24 hours. By the former method a number of simple acylthiohydantoins were prepared without difficulty, except that in the cases with a terminal glycine residue (*i.e.*, $R' = H$) the solution became coloured, presumably owing to minor condensation reactions of the reactive methylene group in (V; $R' = H$).

Waley and Watson (*J.*, 1951, 2394) showed that 1-carbobenzyloxyglycyl-5-methyl-2-thiohydantoin is hydrolysed by 0·1N-sodium hydroxide at 0° in 20 minutes. In testing the generality of this cleavage we found it convenient to follow the reaction spectroscopically, essentially according to the procedure of Kjaer and Eriksen (*Acta Chem. Scand.*, 1952, **6**, 448). As hydrolysis with 0·01N-alkali at room temperature proceeds, the ratio between the optical densities at 262 and 278 mμ of samples, withdrawn and diluted with

acid, increases. It does not, however, reach the expected constant figure, and falls gradually again after rapidly reaching a maximum. The plausible explanation, that the thiohydantoin ring in both (V) and (VI) is attacked by alkali, is supported by the observation that the ultra-violet absorption of 5-*iso*butyl-2-thiohydantoin at 260 mμ diminishes gradually in 0·01N-alkali. It is therefore not surprising that, in our experience, hydrolysis by two equivalents of 0·01N-sodium hydroxide during 30 minutes at room temperature gives satisfactory, but by no means quantitative, yields of the desired products. In the case of (V; R = $C_6H_4Me{\cdot}SO_2{\cdot}NH{\cdot}CH_2$, R′ = CH_2Ph) the reaction is unusually slow, doubtless owing to the influence of the ionised sulphonamide group, but the hydrolysis products are obtained in good yield.

Sheehan and Frank (*J. Amer. Chem. Soc.*, 1950, **72**, 1312) have demonstrated the ready synthesis of the peptide linkage through anhydrides of the type (III), which are unfortunately not readily accessible. It seemed possible that the exchange reaction between a tetraester of pyrophosphoric acid and the anion of an *N*-acylpeptide or amino-acid might provide a convenient method of their preparation. But experiments on the condensation of toluene-*p*-sulphonylalanine with *cyclo*hexylamine were unpromising, whether the pyrophosphate applied was tetrabenzyl, tetraphenyl, or tetra-*p*-nitrophenyl (Khorana and Todd, unpublished work). Presumably in the basic reaction medium the acyl phosphates (III) are rapidly brought into equilibrium with the two symmetrical anhydrides, a reaction observed by Sheehan and Frank (*loc. cit.*). This equilibrium is unimportant in the preparation of acylthiohydantoins owing to the irreversibility of the final cyclisation step.

Experimental

M.p.s are corrected.

*Diphenyl Phosphoro*iso*thiocyanatidate* (I).—(a) *From diphenyl phosphorochloridate.* Diphenyl phosphorochloridate (chlorophosphonate) (Brigl and Müller, *Ber.*, 1939, **72**, 2121) had n_D^{20} 1·5502. From d_4^{20} 1·2960 (Anschütz and Emery, *Annalen*, 1889, **253**, 120), $[R_L]_D$ is 66·07. The addition of this substance (50·1 g.) to a solution of potassium thiocyanate (19·3 g.) in acetonitrile (200 c.c.) caused immediate deposition of a white solid, accompanied by a slight rise in temperature. The mixture was shaken during 3 hours, kept for 3 hours more, and diluted with dry benzene (300 c.c.). The solid was removed and the filtrate evaporated in a vacuum to a clear yellow liquid which, purified by short-path distillation at 105°/0·1 mm., had n_D^{20} 1·5829 (43·5 g.). This *ester* was used in further work, but a portion purified by fractionation [b. p. 210°(bath)/0·1 mm.] had n_D^{20} 1·5851, d_4^{14} 1·2877, $[R_L]_D$ 75·83 (Found: C, 54·0; H, 3·5; N, 4·7. $C_{13}H_{10}O_3NPS$ requires C, 53·6; H, 3·5; N, 4·8%).

(b) *From tetraphenyl pyrophosphate.* When prepared on 0·3-mole scale according to Corby, Kenner, and Todd (*J.*, 1952, 1238) tetraphenyl pyrophosphate crystallised (m. p. 42—47°). When its acetonitrile solution (9·75 c.c. containing 4·21 mmoles) was added to a solution of potassium thiocyanate (0·408 g., 4·21 mmoles) in acetonitrile (10 c.c.), a gelatinous solid separated within a few minutes and was removed after 3 hours. Short-path distillation of the evaporated filtrate at 90°/0·05 mm. gave diphenyl phosphor*iso*thiocyanatidate as a pale yellow mobile liquid, n_D^{20} 1·5811 (Found: C, 54·3; H, 3·0; N, 5·4%).

N-*Diphenoxyphosphinyl*-N′-cyclo*hexylthiourea.*—*cyclo*Hexylamine (1·5 c.c.) was added to a solution of diphenyl phosphoro*iso*thiocyanatidate in acetonitrile (5 c.c. containing 2·38 mmoles), whereupon heat was evolved and crystals were rapidly deposited. Next day the neutral fraction was isolated from the evaporated mixture by partition, in the usual manner with thorough back-extractions, between ethyl acetate and 3N-hydrochloric acid, followed by aqueous sodium hydrogen carbonate solution and water. It consisted of a pale yellow syrup (0·956 g., 2·45 mmoles), from which the *thiourea* (0·673 g.) was obtained as colourless prisms (from aqueous ethanol), m. p. 129·5—130·5° (Found, in material dried at 70°: C, 58·6; H, 6·0; N, 7·2. $C_{19}H_{23}O_3N_2PS$ requires C, 58·4; H, 5·9; N, 7·2%).

*Hydrolysis of Diphenyl Phosphoro*iso*thiocyanatidate.*—(a) *By acid.* Diphenyl phosphoro*iso*thiocyanatidate (0·338 g.) was dissolved in dioxan (20 c.c.) and N-sulphuric acid (15 c.c.). After 24 hours the solution was neutralised with aqueous sodium carbonate solution and evaporated to dryness at 50°/1 mm. Partition of the residue between ethyl acetate, aqueous sodium hydrogen carbonate solution, and water, yielded only 0·017 g. of neutral fraction.

(b) *By alkali.* A mixture of the phosphoro*iso*thiocyanatidate (0·286 g.), saturated aqueous

sodium hydrogen carbonate solution (10 c.c.), water (25 c.c.), and dioxan (25 c.c.) likewise gave only 0·018 g. of neutral fraction after 24 hours.

5-Benzyl-2-thio-1-toluene-p-sulphonylglycylhydantoin (V; R = $C_6H_4Me{\cdot}SO_2NH{\cdot}CH_2$, R′ = CH_2Ph.—(a) *By use of acetic anhydride.* Acetic anhydride (4 c.c.), acetic acid (0·5 c.c.), ammonium thiocyanate (0·5 g.) and toluene-*p*-sulphonylglycyl-DL-phenylalanine (1 g.; unpublished preparation by Mr. D. W. Clayton in this laboratory) were heated during 30 minutes at 100°. The deep red-brown solution was then poured into water (100 c.c.), treated with excess of sodium hydrogen carbonate, and extracted with ethyl acetate (4 × 50 c.c.). Evaporation of the ethyl acetate left a brown gum (1·34 g.), from which the *acylthiohydantoin* (0·26 g.; m. p. 72°) was obtained by crystallisation from benzene (150 c.c.). Recrystallisation from benzene (15 c.c.) afforded colourless plates, m. p. 72° with previous softening (Found, in material dried at 50° over paraffin wax: C, 59·0; H, 5·2; N, 8·8. Calc. for $C_{19}H_{19}O_4N_3S_2,C_6H_6$: C, 60·6; H, 5·1; N, 8·5%), whereas recrystallisation from aqueous ethanol gave colourless prisms, m. p. 166—169° (Found, in material dried at 95°: C, 54·6; H, 4·8; N, 9·9. $C_{19}H_{19}O_4N_3S_2$ requires C, 54·7; H, 4·6; N, 10·1%).

(b) *By use of diphenyl phosphoroisothiocyanatidate.* (i) Toluene-*p*-sulphonylglycyl-DL-phenylalanine (0·376 g., 1 mmole) was kept during 110 hours in acetonitrile (15 c.c.) with triethylamine (0·16 c.c., 1·16 mmole) and an acetonitrile solution of the phosphoro*iso*thiocyanatidate (2·31 c.c., containing 1·1 mmoles). The solvent was evaporated under reduced pressure and the residue separated into neutral and acidic fractions by partition between ethyl acetate (50 c.c.) and N-hydrochloric acid (30 c.c.), which was rejected after four further extractions, followed by extraction of the ethyl acetate with aqueous sodium hydrogen carbonate solution (3 × 15 c.c.) and water (2 × 15 c.c.). After back-extractions the acylthiohydantoin was recovered, by evaporation of the ethyl acetate, as a pale yellow foam (0·422 g., 1·01 mmole); crystallised from benzene (20 c.c.), it had m. p. 72—74° (0·441 g., 0·89 mmole). Thorough extraction with ethyl acetate of the acidified aqueous layers gave diphenyl hydrogen phosphate (0·279 g., 1·11 mmole) as a colourless gum crystallising slowly in rosettes.

(ii) Reaction for 24 hours gave as neutral fraction 0·350 g. (0·84 mmole) and 0·356 g. in the acid fraction (1·11 mmoles of diphenyl hydrogen phosphate and 0·19 mmole of starting material). Reaction for 14 hours gave 0·55 and 0·54 mmole, respectively.

(c) *By use of tetraphenyl pyrophosphate.* A solution of tetraphenyl pyrophosphate in acetonitrile (5·2 c.c., containing 2·25 mmoles) was added to a solution of toluene-*p*-sulphonylglycyl-DL-phenylalanine (0·752 g., 2 mmoles), triethylamine (0·275 c.c., 2 mmoles), and potassium thiocyanate (0·234 g., 2·4 mmoles) in acetonitrile (25 c.c.). After 3 hours deposition of potassium diphenyl phosphate began and this was collected after 24 hours (0·501 g., 1·74 mmoles). The dissolved material was separated in the usual way into diphenyl hydrogen phosphate (0·706 g., 2·82 mmoles) and the thiohydantoin, a pale yellow foam (0·795 g., 1·91 mmoles), m. p. 72—74° (0·955 g., 1·93 mmoles).

General Method for the Preparation of 1-Acyl-5-alkyl-2-thiohydantoins (V).—To a solution of the *N*-acylpeptide (2 mmoles) in anhydrous acetonitrile (15 c.c.) were added triethylamine (0·3 c.c., 2·2 mmoles) and then diphenyl phosphoro*iso*thiocyanatidate (0·640 g., 2·2 mmoles). The solution was kept at room temperature in a sealed flask during 2 days and then evaporated in a vacuum. The solution of the residue in ethyl acetate (25 c.c.) was twice washed with 2N-hydrochloric acid (30 c.c. total) and then mixed with water (25 c.c.). Aqueous sodium hydrogen carbonate solution was then added in small portions to the vigorously shaken mixture until the pH of the aqueous phase reached 7. The product was then recovered by evaporation of the dried ethyl acetate layer and recrystallised for analysis from acetone by addition of light petroleum (b. p. 40—60°). For results see Table 1.

Spectrophotometric Study of the Alkaline Hydrolysis of 1-Acyl-2-thiohydantoins.—As expected from published data, the absorption of 1-benzoylglycyl-5-*iso*butyl-2-thiohydantoin in 0·001N-hydrochloric acid had $\varepsilon_{max.}$ 19,530 at 278 mμ and $\varepsilon_{max.}$ 28,700 at 236 mμ, whereas that of 5-*iso*butyl-2-thiohydantoin in the same medium showed $\varepsilon_{max.}$ 17,400 at 262 mμ and $\varepsilon_{max.}$ 10,100 at 228 mμ. A solution of the latter compound in 0·01N-sodium hydroxide was kept at 18° and aliquots were removed and acidified with hydrochloric acid to 0·001N before measurement of the optical density at 262 mμ (1-cm. cuvettes, Beckman DU instrument), with results as in Table 2. The former compound (18·4 mg., 0·05 mmole) was rapidly dissolved in 0·01N-sodium hydroxide (10 c.c.). Aliquots (2 c.c.) were removed, acidified with 0·01N-hydrochloric acid (7 c.c.), diluted with aqueous alcohol (40%) to 50 c.c. and then ten times further diluted with 0·001N-hydrochloric acid for optical density measurements (Table 3). The same technique was employed for the leucyl compound (Table 4).

For the compound of Table 5 the amount of substance was reduced so that three equivalents of alkali were present. The compound had maxima at 280 (ε 15,700) and 234 mμ (ε 22,700).

Products of Alkaline Hydrolysis of 1-*Benzoylglycyl*-5-iso*butyl*-2-*thiohydantoin*.—The acylthiohydantoin (0·167 g.) was kept with 0·01N-sodium hydroxide (100 c.c., 2 equivs.) for 30 minutes. The solution was then brought to pH 6 with dilute hydrochloric acid, concentrated in a vacuum to small bulk, and thoroughly extracted with ether. Evaporation of the dried ethereal extract yielded crude 5-*iso*butyl-2-thiohydantoin (0·091 g.; theor., 0·086 g.); recrystallisation from benzene removed a little insoluble amorphous material and gave the product, m. p. 165—168° (Schlack and Kumpf record m. p. 170—171°), depressed to 152—155° on admixture with starting material. The aqueous layer was acidified with N-hydrochloric acid (1 c.c.) and evaporated to dryness in a vacuum. Hippuric acid (0·091 g.; theor., 0·090 g.) was recovered from the residue by extraction with ethyl acetate but was contaminated with brown impurities of sulphurous odour.

Products of Alkaline Hydrolysis of 1-*Benzoylglycyl*-5-iso*propyl*-2-*thiohydantoin*.—The acylthiohydantoin (0·177 g.) was dissolved (shaking) in 0·001N-sodium hydroxide (111 c.c., 2 equivs.) and kept at 20° during 15 minutes. Isolation of the neutral and the acid fraction as in the preceding experiment gave 5-*iso*propyl-2-thiohydantoin (0·095 g., theor., 0·085 g.) contaminated with a little starting material and hippuric acid (0·092 g.; theor., 0·099 g.).

Products of Alkaline Hydrolysis of 5-*Benzyl*-2-*thio*-1-*toluene*-p-*sulphonylglycylhydantoin*.—A solution of the acylthiohydantoin (0·094 g., 0·23 mmole) in 0·1N-sodium hydroxide (11 c.c.)

TABLE 1.

2-Thiohydantoin	M. p.	Yield [a] %	Formula	Found, %: C	H	N	Required, %: C	H	N
1-Benzoylglycyl	210—215° [b]	95	$C_{12}H_{11}O_3N_3S$	51·9	4·4	14·9	52·0	4·0	15·2
1-Benzoylglycyl-5-*iso*propyl	186—189	90	$C_{15}H_{17}O_3N_3S$	56·1	5·0	13·3	56·4	5·4	13·2
1-Benzoylglycyl-5-*iso*butyl	185—186	87 [c]	$C_{16}H_{19}O_3N_3S$	57·5	5·4	12·5	57·6	5·7	12·6
1-Benzoyl-DL-alanyl	187—188	100 [d]	$C_{13}H_{13}O_3N_3S$	53·9	5·0	14·3	53·6	4·5	14·4
1-Benzoyl-DL-leucyl	171—172 [e]	93	$C_{16}H_{19}O_3N_3S$	—	—	13·0	—	—	12·6

[a] Of crude material. [b] With decomp. The solution was never homogeneous and the product largely crystallised during the reaction. Schlack and Kumpf (*Z. physiol. Chem.*, 1926, **154**, 125) record m. p. 204—205° (decomp.). [c] A similar experiment with dimethylformamide as solvent instead of acetonitrile gave over 90% yield. [d] The reaction mixture became orange-yellow and the crystalline material was recovered in poor yield from the brown gum. [e] Schlack and Kumpf (*loc. cit.*) record m. p. 172—173°.

TABLE 2. *Stability of* 5-iso*butyl*-2-*thiohydantoin in* 0·01N-*alkali*.

Time (min.)	35	60	132	270
D (262 mμ)	0·389	0·356	0·325	0·290

TABLE 3. *Alkaline hydrolysis of* 1-*benzoylglycyl*-5-iso*butyl*-2-*thiohydantoin*.

Time (min.)	0	5	10	20	30	60
D (262 mμ)	0·180	0·306	0·309	0·327	0·320	0·297
D (278 mμ)	0·338	0·218	0·180	0·181	0·176	0·170
Density ratio	0·53	1·40	1·72	1·81	1·82	1·75

TABLE 4. *Alkaline hydrolysis of* 1-*benzoyl*-DL-*leucyl*-2-*thiohydantoin*.

Time (min.)	7	23	90
D (260 mμ)	0·305	0·345	0·322
D (278 mμ)	0·072	0·085	0·076
Density ratio	4·24	4·06	4·11

TABLE 5. *Alkaline hydrolysis of* 5-*benzyl*-2-*thio*-1-*toluene*-p-*sulphonylglycylhydantoin*.

Time (min.)	5	20	60	120	1050
D (260 mμ)	0·318	0·375	0·380	0·350	0·237
D (280 mμ)	0·310	0·236	0·210	0·238	0·205
Density ratio	1·08	1·67	1·92	1·55	1·22

was kept at 16° for 1 hour before acidification with dilute hydrochloric acid. The acid was neutralised with an excess of solid sodium hydrogen carbonate, and the neutral fraction extracted by ethyl acetate (5 × 30 c.c.). Acidification to pH 1 and a second ethyl acetate extraction of the aqueous phase then gave the acid fraction (0·052 g.; theor., 0·052 g.), which on recrystallisation from water had m. p. 147—148°, undepressed by authentic toluene-*p*-sulphonyl-

glycine (m. p. 148°). Recrystallisation of the neutral fraction (0·048 g.; theor., 0·046 g.) from aqueous ethanol gave a yellow powder, m. p. 175—182° (Johnson and O'Brien, *J. Biol. Chem.*, 1912, **12**, 211, record m. p. 185° for 5-benzyl-2-thiohydantoin). Paper chromatography detected only glycine and only phenylalanine in hydrolysates of the acid (concentrated hydrochloric acid during 15 hours at 120°) and neutral (saturated aqueous barium hydroxide during 40 hours at 140°) fraction respectively.

When the acylthiohydantoin (0·208 g., 0·5 mmole) was kept during 20 minutes at 0° with 0·1N-sodium hydroxide (20 c.c.), hydrolysis was only 35% complete. The acylthiohydantoin (0·167 g., 0·4 mmole) was 20% hydrolysed in 24 hours at 16° by triethylamine (0·11 c.c., 0·8 mmole) in 50% aqueous ethanol (16 c.c.).

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