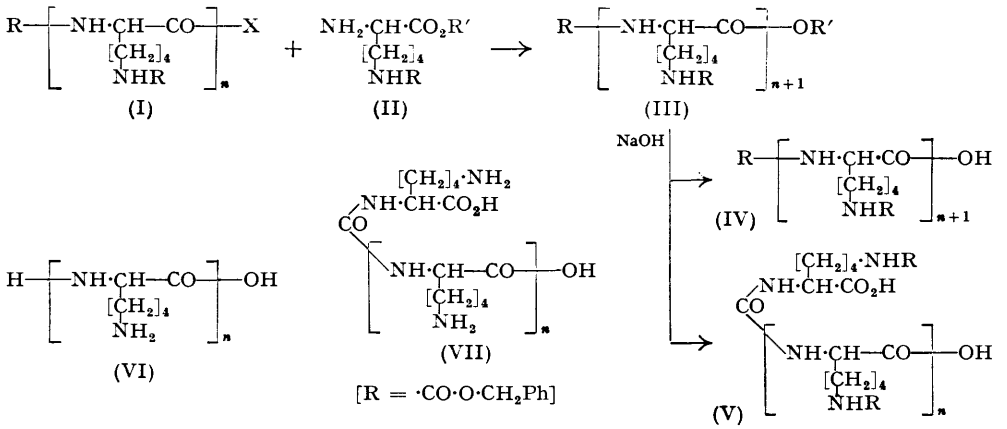
95. *Some Peptides of Lysine.*

By S. G. Waley and J. Watson.

Syntheses of di-, tri-, tetra-, and penta-lysine * (VI; $n = 2$, 3, 4, and 5) have been achieved, mainly by Wieland and Sehring's method (*Annalen*, 1950, **569**, 122). Difficulties were encountered in the separation of ureas (V) formed as by-products in the hydrolysis of the carbobenzyloxypeptide esters. Separation of these peptides of lysine by paper chromatography and their subsequent estimation by the ninhydrin colour reaction have been studied. The titration curves of the peptides are also described.

Enzymic hydrolysis of polylysine gives dilysine * (VI; $n = 2$) and trilysine (VI; $n = 3$) as final products. Higher peptides are also formed during the reaction (Waley and Watson, unpublished work). To aid identification of these substances, their synthesis was undertaken and some of their properties were studied.

The syntheses were of the type shown in the scheme. Since both the terminal α- and the ε-amino-groups in (III) are protected by the same group the α-amino-group cannot be preferentially liberated and thus the peptide chain can only be built up by the addition of monomeric units. As well as the older methods using acid chlorides (restricted to the synthesis of dipeptides) and azides (Fruton, *Adv. Protein Chem.*, 1949, **5**, 1), there are many recent methods (Wieland, *Angew. Chemie*, 1951, **63**, 7) employing different acylating agents. We have used the mixed anhydrides with benzoic acid (Wieland and Sehring, *Annalen*, 1950, **569**, 122) extensively in our work.



For the preparation of dilysine, *NN'*-dicarbobenzyloxylysine chloride (I; $n = 1$, X = Cl) (Bergmann, Zervas, and Ross, *J. Biol. Chem.*, 1935, **111**, 245) was washed with ice-water (cf. Synge, *Biochem. J.*, 1948, **42**, 99) and coupled with N^{ϵ}-carbobenzyloxylysine methyl ester (II; R' = Me), and the dipeptide ester (III; $n = 1$, R' = Me) hydrolysed with alkali to the acid (IV; $n = 1$). Hydrogenation over palladised charcoal gave dilysine (VI; $n = 2$), characterised as the Reineckate and flavianate. The ester (III; $n = 1$, R' = Me) was also prepared in rather better yield from the mixed anhydride (I; $n = 1$, X = O·COPh). The preparation of dilysine from Fischer and Susuki's "lysine anhydride" (*Ber.*, 1905, **38**, 4173) has been reported by Greenstein (*J. Biol. Chem.*, 1933,

* Since L-lysine was used throughout this work, the designation of the optical form is omitted; also, lysyl-lysine is written as dilysine, and the higher peptides are similarly described.

101, 603), but it has since been shown by Adamson (*J.*, 1943, 39) that the product obtained by Fischer and Susuki from lysine methyl ester is a mixture.

For the next stage the dipeptide hydrazide (I; $n = 2$, X = $NH{\cdot}NH_2$) was converted into the azide and condensed with (II; R′ = Me) to give the tripeptide ester (III; $n = 2$, R′ = Me). Alkaline hydrolysis gave material containing the urea (V; $n = 2$). The urea was separated by its greater solubility in weakly alkaline solutions; the purified acid (IV; $n = 2$) was then reduced to trilysine (VI; $n = 3$). The formation of ureas by the action of alkali on carbalkyloxy-peptides was first observed by Fischer (*Ber.*, 1902, **35**, 1095), correctly interpreted by Wessely and Kemm (*Z. physiol. Chem.*, 1928, **174**, 306; cf. Goldschmidt and Wick, *Annalen*, 1952, **575**, 217), and used as a degradative method by Wessely, Schlögl, and Korger (*Nature*, 1952, **169**, 708). To avoid urea formation, the benzyl ester (II; R′ = CH_2Ph) was used in the Wieland synthesis to prepare the tripeptide benzyl ester (III; $n = 2$, R′ = CH_2Ph), which on reduction gave trilysine. The use of benzyl esters was not necessary in the preparation of dilysine, since urea formation was not observed in this case. After these peptides had been synthesised Brand and his co-workers (*J. Amer. Chem. Soc.*, 1951, **73**, 4025, 4027) described their preparation by the azide method.

For the synthesis of tetralysine, the tripeptide acid (IV; $n = 2$) is required, and this can only be prepared by saponification of the ester. After separation from the urea, the acid (IV; $n = 2$) was converted into the mixed anhydride of benzoic acid and coupled with the amino-ester (II; R′ = CH_2Ph) to give the tetrapeptide benzyl ester (III; $n = 3$, R′ = CH_2Ph). Hydrogenation gave tetralysine (VI; $n = 4$). The tetrapeptide methyl ester (III; $n = 3$, R′ = Me), required for the synthesis of pentalysine, was prepared in the same way as the benzyl ester. Saponification gave the acid (IV; $n = 3$) contaminated with material of lower equivalent weight. The contaminant, probably the urea (V; $n = 3$), has not been obtained pure. The acid (IV; $n = 3$) was converted by the Wieland method into the pentapeptide benzyl ester (III; $n = 4$, R′ = CH_2Ph), and thence into pentalysine (VI; $n = 5$).

Of the methods used above for lengthening the peptide chain, the Wieland synthesis is preferred to the azide method as it is easier and gives purer products.

The lysine peptides (VI) were obtained as crystalline hygroscopic hydrochlorides and their purity established chromatographically and by potentiometric titration.

None of the previously described solvents satisfactorily separated the lysine peptides on paper chromatograms, but a mixture of *n*-butanol, acetic acid, pyridine, and water proved suitable, and was used throughout this work. The four peptides of lysine were well separated both from each other and from lysine, but not from the corresponding ureas (VII). The peptides and the ureas were best distinguished by potentiometric titration, since only the peptides possessed a group buffering at pH 7—8, and the shape of the titration curve showed whether the peptide was contaminated with the urea.

Pardee (*J. Biol. Chem.*, 1951, **190**, 757) has shown that a simple relation can be deduced between the R_F values of peptides and those of the constituent amino-acids. When all the residues are of one kind this takes the form

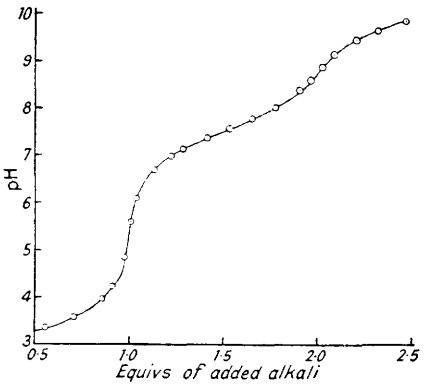
$$\ln(1/R_F - 1) - n\ln(1/R_F' - 1) = [(n-1)A + B]/\boldsymbol{R}T$$

where R_F refers to a peptide containing n amino-acid residues, R_F' refers to the parent amino-acid, A and B are constants, $\boldsymbol{R}$ is the gas constant, and T the absolute temperature. This equation holds accurately for peptides di- to hepta-lysine; the R_F values for hexa- and hepta-lysine were obtained from chromatograms of an acid hydrolysate of polylysine. The constants A and B were −450 cal./mole and 0 respectively, which are comparable with the values found in other solvents by Pardee. The constant B represents the difference between the work required to transfer the terminal groups of the peptide and those of the amino-acid from one phase to the other. Thus in our case this difference is negligible.

The estimation of these lysine peptides after separation by paper chromatography was also investigated. The method used, which depends on the ninhydrin colour reaction (Boissonnas, *Helv. Chim. Acta*, 1950, **33**, 1975; Fowden, *Biochem. J.*, 1951, **48**, 327) has hitherto only been applied to the estimation of amino-acids. We have successfully estimated di- and tri-lysine following Fowden's procedure with only minor modifications.

Although the optical density was a linear function of the amount of peptide present, the gradient differed in different chromatograms so that calibration solutions had to be run each time.

The titration curves of the peptides were determined at 25·8° in a solution 0·083M with respect to potassium chloride, a glass electrode being used; a typical curve is shown in the Figure. End points at *ca.* pH 5·5 and pH 8·5, and buffering at *ca.* pH 7·5 are clearly discernible; the last is due to the α-amino-group. The amount of titrant consumed between the end-points is a measure of the amount of α-amino-nitrogen, which may be compared with the total amount of nitrogen found by analysis. Another measure of the amount of α-amino-nitrogen is provided by the gradient of the curve where buffering is a maximum, since it may readily be shown that, when the buffering power of the solvent is negligible, the concentration of the buffering group is 1·737β, where β is the buffer capacity.



Titration of dilysine, 4·77 × 10^{-3}M. *Theoretical line calc. with* pK_1 = 3·1, pK_2 = 7·56, pK_3 = 10·35, *and* pK_4 = 10·85.

The agreement between both these measures of the α-amino-nitrogen and that calculated from the total nitrogen was satisfactory in all cases. The titration curve of the urea

Peptide	Dilysine	Trilysine	Tetralysine	Pentalysine
p*K* of α-NH_2-group	7·56	7·26	7·15	7·07

(VII; $n = 2$), however, showed no buffering between pH 5 and pH 9. The p*K*'s of the α-amino-groups are given in the Table. There appears to be a linear relation between p*K* and the reciprocal of the number of lysine residues in the peptide.

Experimental

Dicarbobenzyloxylysine (I; $n = 1$, X = OH) and ε-carbobenzyloxylysine methyl ester hydrochloride (II; R′ = Me) were prepared by the method of Bergmann, Zervas, and Ross (*loc. cit.*); paper chromatography showed the latter to be contaminated with lysine methyl ester hydrochloride. Before use, the ε-carbobenzyloxylysine methyl ester hydrochloride was decomposed with aqueous potassium hydrogen carbonate and extracted with ethyl acetate; the lysine methyl ester remained in the aqueous layer. A similar procedure was applied to ε-carbobenzyloxylysine benzyl ester hydrochloride (II; R′ = CH_2Ph) (Erlanger and Brand, *J. Amer. Chem. Soc.*, 1951, **73**, 4025).

Tri-(N-*carbobenzyloxy*)*dilysine Methyl Ester* (III; $n = 1$, R′ = Me).—(*a*) Powdered phosphorus pentachloride (14 g.) was added to dicarbobenzyloxylysine (26 g.) in dry ether (300 c.c.) at 0°. After 2 hours, the filtered solution was shaken twice with ice-water, and added to ε-carbobenzyloxylysine methyl ester (18 g.) in ethyl acetate (450 c.c.) and 1·4N-potassium hydrogen carbonate (150 c.c.). The mixture was stirred for 2 hours, and the dipeptide ester (24·3 g., 56%; m. p. 121—122°) collected and washed with water and then ether. Recrystallisation from ethyl acetate raised the m. p. to 123° (Erlanger and Brand, *loc. cit.*, give m. p. 115—117°), $[\alpha]_D^{20}$ −8·1° (*c*, 4·45 in EtOH) (Found: C, 64·5; H, 6·7; N, 8·3. Calc. for $C_{37}H_{46}O_9N_4$: C, 64·4; H, 6·7; N, 8·1%).

(*b*) Benzoyl chloride (5·16 c.c.) was added to dicarbobenzyloxylysine (18·4 g.) and 1-ethylpiperidine (5·91 c.c.) in benzonitrile (50 c.c.) at 0°. After 10 minutes at room temperature this solution was added to ε-carbobenzyloxylysine methyl ester (13·06 g.) in ethyl acetate (200 c.c.), ether (200 c.c.), and 1·4N-potassium hydrogen carbonate (78 c.c.). The mixture was stirred

vigorously until much solid had separated and then shaken at intervals during 1 hour. The dipeptide ester (19 g., 62%; m. p. 121—123°) was collected and washed with water and ether.

Tri-(N-carbobenzyloxy)dilysine (IV; $n = 1$).—N-Sodium hydroxide (50 c.c.), tricarbobenzyloxydilysine methyl ester (26 g.), and acetone (250 c.c.) were shaken for 1 hour and neutralised with 2·47N-hydrochloric acid (20·25 c.c.), and the acetone was distilled off. The dipeptide (22 g., 86%; m. p. 145—147°) was collected; recrystallisation from ethyl acetate did not raise the m. p. (Erlanger and Brand, *loc. cit.* give m. p. 145°); $[\alpha]_D^{20}$ was −1·5° (*c*, 4·98 in AcOH) (Found: C, 63·8; H, 6·6; N, 8·3%; equiv., 682. Calc. for $C_{36}H_{44}O_9N_4$: C, 63·9; H, 6·5; N, 8·3%; equiv., 690).

Dilysine (VI; $n = 2$).—Tricarbobenzyloxydilysine (1·98 g.) in methanol (30 c.c.) and 0·98N-hydrochloric acid (6·0 c.c.) was reduced in hydrogen in the presence of 10% palladised charcoal (2 g.) for 2½ hours. The filtered mixture was evaporated, and the residue crystallised by trituration with methanol (yield 0·444 g.). It gave a negative biuret reaction and had R_F 0·17. *Dilysine di-Reineckate* crystallised from water in pink plates (Found: C, 25·0; H, 4·9; Cr, 10·9. $C_{20}H_{38}O_3N_{16}S_8Cr_2,3H_2O$ requires C, 24·9; H, 4·6; Cr, 10·8%). The *diflavianate* crystallised from 80% ethanol in yellow prisms, m. p. 209° (decomp.) (Found: C, 40·1; H, 4·4; S, 6·8. $C_{32}H_{38}O_{19}N_8S_2,3H_2O$ requires C, 40·2; H, 4·6; S, 6·7%).

Tri-(N-carbobenzyloxy)dilysine Hydrazide (I; $n = 2$, X = NH·NH_2).—Anhydrous hydrazine (15 c.c.) was added to tricarbobenzyloxydilysine methyl ester (30 g.) in methanol (175 c.c.). After several days the solid was collected, washed with water, and recrystallised from aqueous ethanol (yield 21·7 g., 72%; m. p. 186—187°). Further recrystallisation raised the m. p. to 190—191° (Erlanger and Brand, *loc. cit.* give m. p. 187°) (Found: C, 62·7; H, 6·85; N, 12·0. Calc. for $C_{36}H_{46}O_8N_6$: C, 62·6; H, 6·7; N, 12·2%).

Tetra-(N-carbobenzyloxy)trilysine Methyl Ester (III; $n = 2$, R′ = Me).—(*a*) Tricarbobenzyloxydilysine hydrazide was converted into the azide and condensed with ε-carbobenzyloxylysine methyl ester essentially as described by Erlanger and Brand. Their product, however, melted at 142—145°, ours at 160—162°; $[\alpha]_D^{20}$ was −10·4° (*c*, 5·0 in AcOH) (Found: C, 64·2; H, 7·1; N, 9·15. Calc. for $C_{51}H_{64}O_{12}N_6$: C, 64·3; H, 6·7; N, 8·8%).

(*b*) Tricarbobenzyloxydilysine (11·1 g.) in tetrahydrofuran (30 c.c.) and 1-ethylpiperidine (2·22 c.c.) was treated at 0° with benzoyl chloride (1·88 c.c.), and the mixture added to ε-carbobenzyloxylysine methyl ester (7·1 g.) in ethyl acetate (90 c.c.) and 1·4N-potassium hydrogen carbonate (30 c.c.). After 45 minutes' vigorous stirring at 0° the product was collected and recrystallised from methanol (yield 9·1 g., 58%; m. p. 164—165·5°) (Found: C, 63·6; H, 6·9; N, 9·4. Calc. for $C_{51}H_{64}O_{12}N_6$: C, 64·3; H, 6·7; N, 8·8%). The condensation can also be carried out in benzonitrile but the product is less pure.

Tetra-(N-carbobenzyloxy)trilysine Benzyl Ester (III; $n = 2$, R′ = CH_2Ph).—Tricarbobenzyloxydilysine was converted into the mixed anhydride and condensed with ε-carbobenzyloxylysine benzyl ester, as described for tricarbobenzyloxydilysine methyl ester. The product (50%) had m. p. 157—158° after recrystallisation from 95% ethanol. The analytical sample melted at 161—162° (Brand, Erlanger, Polatnick, Sachs, and Kirschenbaum, *J. Amer. Chem. Soc.*, 1951, **73**, 4026, give m. p. 153—154°) (Found: C, 66·6; H, 6·9; N, 8·4. Calc. for $C_{57}H_{68}O_{12}N_6$: C, 66·6; H, 6·6; N, 8·2%).

Tetra-(N-carbobenzyloxy)trilysine (IV; $n = 2$).—Tetracarbobenzyloxytrilysine methyl ester (21·1 g.), suspended in acetone (150 c.c.) and 0·6N-sodium hydroxide (50 c.c.), was stirred for 1½ hours, then filtered, and the filtrate diluted with water (700 c.c.) and saturated with carbon dioxide. The gelatinous precipitate was collected (filtrate A), washed with water, dissolved in warm acetone (500 c.c.) and 3N-hydrochloric acid (10 c.c.), and filtered, and acetone removed from the filtrate by distillation. *Tetracarbobenzyloxytrilysine* was collected and recrystallised from ethyl methyl ketone–ether (charcoal) (yield, 10 g., 52%; m. p. 173—174°) (Found: C, 63·9; H, 6·4; N, 9·2%; equiv., 946, 966. $C_{50}H_{62}O_{12}N_6$ requires C, 64·0; H, 6·6; N, 9·0%; equiv., 939).

When the filtrate A was acidified the *urea* (V; $n = 2$) was precipitated; it was purified by extraction from ethyl acetate with 1·4N-potassium hydrogen carbonate and acidification. Recrystallisation from ethyl methyl ketone–ether gave a sample, m. p. 153—155° (decomp.) (0·74 g. of m. p. 150—152°) (Found: C, 60·8; H, 7·0; N, 9·8%; equiv., 441. $C_{43}H_{56}O_{12}N_6$ requires C, 60·9; H, 6·6; N, 9·9%; equiv., 424).

Trilysine (VI; $n = 3$).—Tetracarbobenzyloxytrilysine benzyl ester was reduced in the presence of palladised charcoal and the product isolated as described by Brand *et al.* (*loc. cit.*); it had R_F 0·12 and gave a negative biuret reaction.

$N^{\alpha'}$-(*Lysine*-$N^{\alpha''}$-*carbonyl*)*dilysine* (VII; $n = 2$).—The carbobenzyloxyurea (V; $n = 2$) (0·48 g.) in 85% acetic acid (20 c.c.) and 0·98N-hydrochloric acid (0·58 c.c.) was reduced in the

presence of 10% palladised charcoal (1 g.) for 2½ hours. After evaporation of the filtered solution, the product crystallised under methanol–ethanol; its R_F was 0·12.

Penta-(N-carbobenzyloxy)tetralysine Methyl Ester (III; $n = 3$, R′ = Me).—The *tetrapeptide methyl ester* was prepared in 62% yield from tetracarbobenzyloxytrilysine as described for tetracarbobenzyloxytrilysine methyl ester [method (*b*)]. After recrystallisation from methanol it melted at 156—157° (Found: C, 64·4; H, 6·9; N, 9·3. $C_{65}H_{82}O_{15}N_8$ requires C, 64·3; H, 6·8; N, 9·2%).

Penta-(N-carbobenzyloxy)tetralysine Benzyl Ester (III; $n = 3$, R′ = CH_2Ph), similarly prepared in 48% yield and recrystallised from 95% ethanol, had m. p. 170—172° (Found: C, 66·25; H, 7·1; N, 9·25. $C_{71}H_{86}O_{15}N_8$ requires C, 66·1; H, 6·7; N, 8·7%).

Penta-(N-carbobenzyloxy)tetralysine (IV; $n = 3$).—Pentacarbobenzyloxytetralysine methyl ester (3·3 g.) was suspended in hot acetone (50 c.c.) and 1·02N-sodium hydroxide (4 c.c.) and stirred for 30 minutes. The mixture was again warmed, treated with water (4 c.c.), warmed again after 20 minutes, and treated with 1·02N-sodium hydroxide (0·4 c.c.). After a further 20 minutes the mixture was diluted with water (100 c.c.) and acetone (60 c.c.) and saturated with carbon dioxide. The precipitate was dissolved in warm acetone (200 c.c.) and 3N-hydrochloric acid (1·1 c.c.), diluted with water, and freed from acetone by distillation. The *tetrapeptide*, recrystallised from ethyl methyl ketone (0·64 g. of m. p. 157—159°) had m. p. 162—163° (Found: C, 63·2; H, 7·0; N, 9·7%; equiv., 1204. $C_{64}H_{80}O_{15}N_8$ requires C, 64·0; H, 6·7; N, 9·3%; equiv. 1201).

Tetralysine (VI; $n = 4$).—Pentacarbobenzyloxytetralysine benzyl ester was reduced in the same way as tetracarbobenzyloxytrilysine benzyl ester; the tetralysine, precipitated from methanol by ethanol, had R_F 0·09 and gave a positive biuret reaction.

Hexa-(N-carbobenzyloxy)pentalysine Benzyl Ester (III; $n = 4$, R′ = CH_2Ph).—The viscous solution of pentacarbobenzyloxytetralysine (0·592 g.) in benzonitrile (4 c.c.) and 1-ethylpiperidine (0·055 g.) was stirred with benzoyl chloride (0·07 g.) for 15 minutes, and to it were added ε-carbobenzyloxylysine benzyl ester (0·21 g.) in ethyl acetate (6 c.c.) and 1·4N-potassium hydrogen carbonate (1·5 c.c.). After 2 hours' shaking, the *pentapeptide benzyl ester* was collected, washed with water and ether, and recrystallised thrice from ethanol (yield, 0·252 g. of m. p. 175—177°), m. p. 177—178° (Found: C, 65·5; H, 6·6; N, 9·5. $C_{85}H_{104}O_{18}N_{10}$ requires C, 65·8; H, 6·7; N, 9·0%).

Pentalysine (VI; $n = 5$).—The foregoing ester was reduced in the same way as tetracarbobenzyloxytrilysine benzyl ester; pentalysine was precipitated from methanol by ethanol–ether; it gave a positive biuret reaction and had R_F 0·06.

Paper Chromatography.—(a) *Qualitative.* The descending technique was used. The solvent was *n*-butanol–acetic acid–water–pyridine (30 : 6 : 24 : 20; homogeneous). Whatman No. 1 or No. 4 papers were employed. R_F values for the individual peptides given above are calculated on the basis of R_F for lysine = 0·24.

The R_F values for the peptides were obtained from a hydrolysis of polylysine (20 mg.) in constant-boiling hydrobromic acid (1 c.c.), kept for 8 days at 25°. A portion (0·02 c.c.) of the solution was evaporated and then transferred to a sheet of Whatman No. 4 paper, together with solutions of lysine, and di-, tri-, tetra-, and penta-lysine. These substances were thus shown to be present in the acid hydrolysate; there were also well-defined spots above the pentalysine, probably due to hexa- and hepta-lysine.

(b) *Quantitative.* A solution of dilysine acetate in 50% aqueous *iso*propanol containing 1·22 mg. of nitrogen per c.c. was serially diluted with the same solvent to 2/3, 4/9, 8/27, and 16/81 of its original strength. Each solution (0·0054 c.c.) was placed on Whatman No. 1 paper, and the chromatogram developed overnight with the above-mentioned solvent. The positions of the spot on the dried paper were located, and the ninhydrin reaction carried out, as described by Fowden (*loc. cit.*). Each solution was then diluted with 8·0 c.c. of 50% *n*-propanol and its optical density determined in a 1-cm. cell with a Hilger Spekker absorptiometer with a yellow filter (606). Trilysine could be estimated similarly.

The equivalent weights of acids were determined by titration in methanol–benzene by Fritz and Lisicki's method (*Analyt. Chem.*, 1951, **23**, 589). Analyses were performed by Mr. G. Ingram.

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