The Reaction of Iodobenzene-*p*-sulphonyl Chloride (Pipsyl Chloride) with Certain Amino Acids and Peptides, and with Insulin

By J. C. FLETCHER

Wool Industries Research Association, Torridon, Headingley Lane, Leeds 6

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1. A system of separation using buffered Celite columns is described that enables the pipsyl derivatives of most of the common amino acids to be separated. 2. The reaction of pipsyl chloride with several amino acids not included in previous studies has been investigated. In particular, knowledge of the acid-soluble pipsyl derivatives of arginine, histidine, lysine, tyrosine and cysteic acid has been extended. 3. Reproducible factors have been obtained that enable corrections to be applied for the breakdown of pipsylamino acids on acid hydrolysis. 4. The reaction of pipsyl chloride with peptides has been studied under various conditions. 5. The extent of the reaction between pipsyl chloride and insulin depends on the nature of the solvent-buffer system, and under the best conditions so far found is about 75% complete. 6. In an Appendix, the separation of pipsylamino acids by thin-layer chromatography is described.

Isotopically labelled pipsyl* chloride was introduced as a sensitive reagent for amino groups in protein chemistry by Keston, Udenfriend & Cannan (1946). Its use for quantitative amino acid analysis (Velick & Udenfriend, 1951*a*), for the determination of the *N*-terminal residues of proteins (Udenfriend & Velick, 1951; Velick & Udenfriend, 1951*b*, 1953) and in the elucidation of amino acid sequences (Levy & Slobodian, 1952; Slobodian & Levy, 1953; Levy, 1957; Mechanic & Levy, 1959; Mechanic, Skupp, Safier & Kibrick, 1960) has been described, and it has recently been used for labelling the active centre of an enzyme (Kallos & Rizok, 1964) and for the estimation of free ϵ -amino groups in proteins (Oratz, Burks & Rothschild, 1966). In this Laboratory, pipsyl chloride has been used for the determination of the *N*-terminal residues of peptides encountered in amino acid sequence studies of a protein fraction from oxidized wool (Corfield, Fletcher & Robson, 1967), and its reaction with other protein fractions from wool is being investigated. Because previous work had suggested that complete reaction between pipsyl chloride and proteins is not easily achieved, the reaction between pipsyl chloride and insulin has been investigated. It was thought worth while to summarize the experience gained with pipsyl chloride, particularly in view of current interest in the application of another aromatic sulphonyl chloride, the fluorescent 1-dimethylaminonaphtha-

lene-5-sulphonyl chloride (Gray & Hartley, 1963). Accordingly, the present paper describes investigations of the reaction of pipsyl chloride with amino acids not included in the scheme of Velick & Udenfriend (1951*a*), with peptides and with insulin. The stability of a number of pipsylamino acids to acid hydrolysis is also reported.

MATERIALS AND METHODS

Materials

Chemicals. Amino acids and peptides were the purest available commercial samples, and were checked for homogeneity by paper chromatography. Insulin samples were (*a*) crystalline ox insulin (batch BC1 4245) supplied by Boots Pure Drug Co. Ltd. (Nottingham), and (*b*) I.U.P.A.C. crystalline ox insulin (batch 2189) supplied by British Drug Houses Ltd. (Poole, Dorset). Both samples, after being dried *in vacuo* over P_2O_5 for several weeks at room temperature, possessed N contents corresponding to approx. 6% of moisture (du Vigneaud & Miller, 1937).

Pipsyl chloride. This was prepared as described by Keston, Udenfriend & Cannan (1949). A solution of the product in cold benzene was passed through a short column of activated charcoal, which removed most of the colour. The solvent was evaporated until crystallization started, m.p. (uncorr.) 84° [Keston *et al.* (1949) quote m.p. 86°]. Unlabelled pipsyl chloride used for diluting [^{131}I] pipsyl chloride was purified by sublimation *in vacuo*.

Pipsylamino acids. The derivatives of the neutral and acidic amino acids were prepared as described by Keston *et al.* (1949).

[^{131}I]Pipsyl chloride. This was prepared by the procedure of Keston *et al.* (1949) from carrier-free [^{131}I]iodide obtained

* Abbreviations: pipsyl, iodobenzene-*p*-sulphonyl; in amino acid sequences $CySO_3H$ refers to cysteic acid.

from The Radiochemical Centre (Amersham, Bucks.) (cat. no. IBS-1). An activity of 15mc was used for each preparation, and 60-80% incorporation of ^{131}I was obtained. The weight of the [^{131}I]pipsyl chloride was adjusted in the range 50-200mg. according to the particular applications envisaged.

Standard solution of [^{131}I]pipsylglycine. To glycine (5mg.) in water (1ml.) in a glass-stoppered test tube were added [^{131}I]pipsyl chloride (5mg.) and NaHCO_3 (6mg.). The tube was immersed in boiling water and shaken for 10min. and the contents were acidified with 2N-HCl and extracted twice with ether (3ml.). The ethereal solution was transferred to a second stoppered tube and the pipsylglycine was extracted into 0.1N-NaOH (1ml.). After removal of the ether, the solution was acidified and the [^{131}I]pipsylglycine was collected by centrifuging and recrystallized twice from water (5ml.). The wet solid was dissolved and made up to 25ml. in 2% (w/v) NaHCO_3 solution, and a 5ml. portion of this solution was diluted to 50ml. The extinction of the resulting solution (about 0.4 unit) was measured at 248m μ in a 1 cm. quartz cell and the concentration of pipsylglycine was determined by reference to a standard extinction-concentration curve prepared with unlabelled pipsylglycine ($\epsilon 15.5 \times 10^3$).

Column chromatography

Celite 545. Celite 545 (Johns Manville and Co., New York, N.Y.; U.S.A.) was washed twice with 6N-HCl to remove iron and then with water until free from acid. It was dried at 120°.

Buffers. The following buffers were used: pH 5.2: 15.20g. of Na_2HPO_4 and 9.79g. of citric acid monohydrate/l.; pH 7.2: 49.28g. of Na_2HPO_4 and 5.44g. of citric acid monohydrate/l. (Britton, 1942).

Solvents. All solvents were laboratory-reagent grade. Chloroform, butan-1-ol and ethyl methyl ketone were distilled before use. Distillation does not free chloroform from ethanol, but the distilled solvent gave better results. Washing the chloroform with water and adding butan-1-ol as a stabilizer was unsatisfactory.

Preparation of columns. The dimensions of the columns used and the method of packing them were as described by Corfield, Fletcher & Robson (1956), and details of the columns are summarized in Table 1. The tubing used for the columns and the packing rod used were generally kept constant, but occasional lack of reproducibility in the running of the FR7.2 column may have been due to unappreciated variation of these factors (Matheson, 1965). The load was applied by evaporating a solution of pipsylamino acids in ethanol or ethanol-water from a microburette on to a disk of filter paper slightly smaller in diameter than the column. The column was allowed to run dry momentarily, the disk dropped on, and the load washed in with successive small volumes of the mobile phase.

Detection and measurement of [^{131}I]pipsylamino acids in the column effluent. In the earlier part of this work, the column effluent was divided by a drop-counting fraction collector into a number of fractions that were plated by hand on to aluminium trays for counting. Errors due to non-uniform plating were shown to be negligible, and the correction for incomplete transfer was reasonably constant and not greater than 2%. Later, an apparatus was designed that collected fractions directly in trays, evaporated off the solvent and presented the dry samples to an end-window Geiger-Müller counter (Corfield, Dilworth, Fletcher & Gibson, 1959). The ratio of count rate to mass of derivative was determined as follows. A portion of the standard solution of [^{131}I]pipsylglycine in 2% NaHCO_3 solution was acidified and the derivative was extracted quantitatively into ether. The ethereal solution was evaporated and the residue was dissolved in a small known volume of ethanol. Measured portions of this solution were evaporated on to filter-paper disks. Three or four standards were run on the G5.2 column, the first being put on at the start and the remainder in succession after the preceding peak had emerged. Standards were run on the FR7.2 column, from which pipsylglycine is not eluted, by packing a column of the same dimensions with pH 5.2 buffer. In all standard runs at least 10^4 total counts were recorded over each [^{131}I]pipsylglycine peak. Reproducibility was usually

Table 1. Details of chromatographic columns

Columns were packed as described by Martin (1949). The assembly for gradient elution is described by Corfield *et al.* (1956).

Designation	Dimensions (cm.)	pH of buffer	Eluting solvent	Fraction volume (ml.)	Approx. flow rate (ml./hr.)	Application
FR7.2	20 × 0.9	7.2	Ethyl methyl ketone-chloroform (1:1, v/v) followed by ethyl methyl ketone-chloroform (3:2, v/v)	0.9	10-12	Fast-running ether-soluble derivatives
G5.2	10 × 0.45	5.2	Chloroform-butan-1-ol gradient followed by butan-1-ol for pipsyl-cysteic acid	0.5	8	(a) Slow-running ether-soluble derivatives (b) Acid-soluble derivatives
G7.2	10 × 0.45	7.2	Chloroform-butan-1-ol gradient	0.5	8	Certain mixtures of ether-soluble derivatives

within the statistical counting error. Coincidence and decay corrections were applied to all counting data. The counters used, GEC type EHM2 and Mullard type MX123, were checked daily by counting a uranium oxide standard and arbitrary [^{131}I]pipsylglycine standards, and gave consistent operation over long periods. When freshly prepared and adjusted to a minimum weight of approx. 50 mg., [^{131}I]pipsyl chloride gave derivatives with specific activities up to 10^7 counts/min./ μmole when counted as described above.

Paper chromatography of pipsylamino acids

Paper chromatography was not used for quantitative purposes, but proved useful for the investigation of the products of the reaction between pipsyl chloride and the basic amino acids. Separations were made by the descending technique on Whatman no. 1 paper. A 5 hr. equilibration period was used. Unlabelled pipsylamino acids (0.1–0.2 μmole) were located as blue spots on a pale-blue background by exposing the dry chromatogram to chlorine gas for 5 min. in a vessel containing a dish of water and then dipping the paper into a saturated solution of *o*-tolidine in acetic acid–water (1:49, v/v) (Reindel & Hoppe, 1954). Iodobenzene-*p*-sulphonic acid, which contains no NH group, also responded to this reaction. The R_F values are given in Table 2.

Reactions with [^{131}I]pipsyl chloride on an analytical scale

(a) *Neutral and acidic amino acids.* A three-step reaction in bicarbonate buffer at 100° with intermediate extraction of the derivatives into ether was carried out as described by Keston *et al.* (1949). [^{131}I]Iodobenzene-*p*-sulphonic acid was removed from the ethereal solution by a five-funnel countercurrent extraction procedure (Udenfriend & Velick, 1951). After removal of the ether, the derivatives were dissolved in ethanol.

(b) *Peptides.* (i) The reaction with di- and tri-peptides was carried out as in (a), except that water-saturated ethyl acetate was used to extract the pipsyl-peptide (Levy & Slobodian, 1952). The pipsyl-peptide was hydrolysed in 6N-HCl for 16 hr., and the *N*-terminal pipsylamino acid was extracted from the diluted hydrolysate with ether. (ii) A one-step reaction with phosphate buffer was carried out as follows. Peptide (3–5 μmoles) in 0.25M- Na_2HPO_4 (0.4 ml.) was shaken at 100° with [^{131}I]pipsyl chloride (9 mg., 30 μmoles). When no more molten pipsyl chloride remained, the solution was cooled and acidified, and the pipsyl-peptide extracted with four 2 ml. portions of water-saturated ethyl acetate. The ethyl acetate was removed and the residue dissolved in ethanol. (iii) For the determination of the *N*-terminal residues of peptides, a solution of [^{131}I]pipsyl chloride (0.5 mg., 1.6 μmoles) in acetone (0.15 ml.) was added to the peptide dissolved in an equal volume of 0.04M- Na_2HPO_4 . After standing overnight at 37° , the reaction mixture was evaporated to dryness, 6N-HCl (0.2 ml.) was added and the tube was sealed and heated at 105° for 16 hr. The hydrolysate was diluted with water (0.8 ml.) and the ether-soluble pipsylamino acids were extracted with five 2 ml. portions of water-saturated ether. Iodobenzene-*p*-sulphonic acid was removed from the ethereal solution as described under (a). The hydro-

Table 2. R_F values of pipsylamino acids

An equilibration period of 5 hr. was adopted before descending development on Whatman no. 1 paper. Spots were made visible by exposing the paper to chlorine gas and dipping it into a saturated solution of *o*-tolidine in acetic acid–water (1:49, v/v) (Reindel & Hoppe, 1954).

Pipsyl derivative	R_F in butan-1-ol- N-NH_3 (5:3, v/v; upper phase)
Alanine	0.51
Arginine	0.43
Aspartic acid	0.04
Cystine (di-)	0.19
Cysteic acid	0.03
Glutamic acid	0.04
Glycine	0.41
Histidine	0.40
Isoleucine/leucine	0.70
Lysine (di-)	0.83
(N^ϵ -mono-)	0.39
Methionine	0.64
Methionine sulphone	0.39
Phenylalanine	0.60
Proline	0.42
Serine	0.32
Threonine	0.42
Tyrosine (di-)	0.69
(<i>O</i> -mono-)	0.46
Valine	0.65
Iodobenzene- <i>p</i> -sulphonic acid	0.52
Iodobenzene- <i>p</i> -sulphonamide	0.90

lysate, after ether extraction, was evaporated to dryness twice with the addition of water, and the residue was taken up in ethanol–water (1:1, v/v) to be examined for acid-soluble pipsylamino acids.

(c) *Insulin.* In most runs, insulin (approx. 1 μmole) in buffer solution (5 ml.) was added to [^{131}I]pipsyl chloride (30 or 60 μmoles) in ethanol or acetone (5 ml.), and the reaction allowed to proceed for 20 hr. at 37° . The reaction mixture was acidified with 6N-HCl and any pipsyl chloride remaining was extracted with ether (30 ml.). The ether was discarded and the aqueous phase was made 6N with respect to HCl and boiled under reflux for 16 hr. The ether-soluble pipsylamino acids were extracted with five 8 ml. portions of ether, and iodobenzene-*p*-sulphonic acid was removed from the ethereal solution as described under (a). Pipsylglycine and pipsylphenylalanine were separated on the G7.2 column. The hydrolysate, after extraction, was evaporated to dryness under reduced pressure, and the residue was transferred to a small tube with several portions of hot water. The solution was evaporated to dryness *in vacuo* and the residue dissolved in ethanol–water (1:1, v/v) or dimethylformamide–water (1:1, v/v).

RESULTS

Separation of pipsylamino acids on columns

Ether-soluble derivatives. The pipsyl derivatives of leucine plus isoleucine, phenylalanine, valine,

Fig. 1. Separation of acid-soluble pipsylamino acids on a G5-2 column (see Table 1). A chloroform-butan-1-ol gradient was used, changed at fraction no. 89 to butan-1-ol. Peaks: A, *O*-pipsyltyrosine; B, pipsylhistidine; C, *N* ϵ -pipsyl-lysine + pipsylarginine; D, iodobenzene-*p*-sulphonic acid; E, pipsylcysteic acid.

methionine and proline (fast-running group) were separated on the FR 7.2 column (Fig. 1 of Corfield *et al.* 1956). When mixtures of pure unlabelled derivatives were run on this column, no peak appeared in the solvent-front position. When ^{131}I -labelled reaction mixtures were run, a radioactivity peak was always present in this position. It was assumed to contain small amounts of *NN*-dipipsyl derivatives (Keston *et al.* 1949) and iodobenzene-*p*-sulphonamide. The more polar derivatives, those of alanine, glycine, threonine, serine, aspartic acid and glutamic acid, were separated on the G5-2 column (Fig. 4 of Corfield *et al.* 1956). The G7-2 column would not resolve all the derivatives of the fast-running group, but was useful for the separation of certain mixtures, e.g. pipsylphenylalanine and pipsylglycine from hydrolysates of pipsyl-insulin.

Other ether-soluble pipsylamino acids ran on these columns as follows. On the FR 7.2 column, *NO*-dipipsyltyrosine ran between the solvent front and the pipsyl-leucine-pipsylisoleucine peak. *N* α *N* ϵ -Dipipsyl-lysine ran with the pipsyl-leucines. On the G5-2 column, dipipsyl-lysine ran between the solvent front and pipsylalanine, and pipsylmethionine sulphone between pipsylthreonine and pipsylserine. An unidentified derivative of lysine ran with pipsylthreonine. *NN'*-Dipipsylcysteine ran as a rather broad peak overlapping pipsylserine.

Acid-soluble derivatives. *O*-Pipsyltyrosine, *N* α -pipsylhistidine, pipsylarginine plus *N* ϵ -pipsyl-lysine and pipsylcysteic acid were separated on the G5-2 column (Fig. 1). Iodobenzene-*p*-sulphonic acid formed by the hydrolysis of excess of pipsyl chloride remains almost entirely in the aqueous phase, and a large peak due to this substance was present on the chromatograms of reaction mixtures.

Recoveries of unlabelled pipsylamino acids from the columns. Recoveries calculated by using an ϵ value of 15.5×10^3 for all derivatives except pipsylproline (ϵ 16.6×10^3) were $100 \pm 4\%$ (mean $\pm$ s.d. of 35 results), except for pipsylmethionine, which gave variable recoveries in the range 80–100%.

Reaction between pipsyl chloride and the basic amino acids

The products of the reaction between pipsyl chloride and the basic amino acids in the presence of sodium hydrogen carbonate were first investigated qualitatively. The ether-soluble and acid-soluble reaction products were separated by paper chromatography in butan-1-ol-*N*-ammonia (5:3, v/v; upper phase) and detected by the chlorine-tolidine and Pauly, Sakaguchi and ninhydrin reactions (Block, Durrum & Zweig, 1955). Arginine and histidine each gave an *N* α -pipsyl derivative that partitioned between the ethereal and acid phases, together with a trace of an ether-soluble derivative that had a high R_f value (0.9) and that did not give the colour reaction characteristic of the amino acid side chain. The product of the reaction with lysine was soluble in ether, and gave two spots (chlorine-tolidine-positive, ninhydrin-negative) on the paper chromatogram with R_f values approx. 0.8 and 0.9. The faster-moving spot had the same R_f as iodobenzene-*p*-sulphonamide on both paper and thin-layer chromatograms (see the Appendix). The behaviour of the various derivatives in column chromatography was studied with [^{131}I]pipsylamino acids. The *N* α -pipsyl derivatives of arginine and histidine were separated on the G5-2 column (Fig. 2). The ether-soluble prod-

Table 3. *Reaction of pipsyl chloride with arginine, histidine and lysine*

In every reaction except (ii), where the amounts of amino acids were halved, the initial reaction mixture contained 1.3 μ moles of each amino acid dissolved in 0.6 ml. of 0.33 M-NaHCO₃. Pipsyl chloride (9 mg., 30 μ moles) was added and the mixture shaken at 100° until all the pipsyl chloride had reacted. The standard three-step reaction was carried out as described by Keston *et al.* (1949). When ether extraction after the intermediate steps was omitted, the volume of the solution was adjusted to 0.6 ml. and solid NaHCO₃ (15 mg.) and pipsyl chloride (9 mg.) were added. For further details see the Results section. It was assumed that 'derivative X' contained two pipsyl groups (see the Discussion section).

Reaction	Conditions	Fraction of amino acid converted into			
		N ^{α} -Pipsyl- arginine	N ^{α} -Pipsyl- histidine	Dipipsyl- lysine	Derivative X
(i)	One-step reaction with pipsyl chloride (7.6 mol.prop.)	0.82	0.62	0.62	Nil
		0.87	0.61	0.54	Nil
(ii)	As (i), but with 15.2 mol.prop.	0.74	0.74	0.75	0.03
(iii)	Standard three-step reaction with pipsyl chloride	0.80	0.65	0.73	0.28
(iv)	Two-step reaction: no ether extraction after first step	0.83	0.80	0.54	0.26
		0.83	0.77	0.35	0.24
				0.60	0.06
(v)	Three-step reaction: no ether extrac- tion after first two steps	0.87	0.62	0.29	0.55
(vi)	One-step reaction in borate buffer*	0.84	Dipipsylhistidine	N ^{α} -Pipsylhistidine	
		0.83†	0.41	0.32	
			Nil†	0.73†	

* Lysine was not present in this reaction.

† Products re-run after standing for 16 days in ethanol.

Fig. 2. Separation of the pipsyl derivatives of the basic amino acids on a G5-2 column. Peaks: A, N ^{α} N ^{ϵ} -dipipsyl-lysine; B, derivative obtained from lysine when the reaction with pipsyl chloride is carried out in more than one step (see the text); C, pipsylhistidine; D, pipsylarginine.

ucts of the reaction with lysine gave a peak on the solvent front of the FR 7.2 column (iodobenzene-*p*-sulphonamide runs in this position) and a larger peak in the position of pipsyl-leucine on the same column. These two peaks were also separated on the G5-2 column (Fig. 2). When the reaction with pipsyl chloride was carried out in more than one step, as described below, the ether-soluble products of the reaction included an unidentified substance that gave a peak at fraction no. 31 on the G5-2 column (Fig. 2).

Quantitative results. In the standard three-step

reaction procedure used for the neutral and acidic amino acids, the pipsylamino acids formed are extracted into ether after each step. Of the basic amino acids, lysine alone gives derivatives that can be completely extracted into ether: the monopipsyl derivatives of arginine and histidine remain largely in the aqueous phase and would thus be exposed to the action of fresh reagent in the succeeding steps. Several ways of carrying out the reaction were investigated, differing in (a) the number of steps, (b) the molar ratio of pipsyl chloride to amino acid, (c) the point in the procedure at which ether extraction was applied and (d) the replacement of bicarbonate as buffer salt by borate (Levy, 1957). The conditions are summarized and the results given in Table 3. The ethereal and aqueous phases were separately evaporated to dryness and the residues were taken up in equal volumes of ethanol and ethanol-water (1:1, v/v) respectively. Equal portions of these two solutions were evaporated on to separate filter-paper disks, which were then loaded on to a G5-2 column. (If both solutions were evaporated on to the same disk, the ether-soluble derivatives tailed badly.) The chromatogram of the products from reaction (vi) (borate buffer, arginine and histidine only) showed a new peak running close to the position of the dipipsyl-lysine peak: this was found to be an ether-soluble derivative of histidine. When the products from

(vi) were re-run after standing for 16 days, this peak was no longer present, and the N^α -pipsyl-histidine peak had increased to the extent to be expected if the new substance were a dipipsyl derivative that decomposed at room temperature to give the N^α -pipsyl derivative. Such instability is characteristic of N -acylimidazoles (Hoffman, 1953).

Attempted preparation of the derivatives of the basic amino acids. Arginine was allowed to react with 1 mol.prop. of pipsyl chloride in hot sodium hydrogen carbonate solution. On acidification, the reaction mixture yielded a glassy solid, which when examined by paper chromatography appeared to contain a mixture of N^α -pipsylarginine and iodobenzene- p -sulphonic acid. The nitrogen content of the material corresponded approximately to the proportions expected for a salt. A solution of histidine in aqueous sodium hydrogen carbonate solution was shaken at room temperature with a solution of 1 mol.prop. of pipsyl chloride in ether. On acidification of the aqueous phase, a mixture of N^α -pipsylhistidine and iodobenzene- p -sulphonic acid in the ratio 30:70 was obtained. Both these mixtures were triturated with chloroform to remove any hydrophobic material. The residues were freed from iodobenzene- p -sulphonic acid by dissolution in 0.25N-acetic acid-ethanol (4:1, v/v) and passage through a short column of Amberlite IR-4B equilibrated with the same solvent. In each case the material eluted from the column was homogeneous when examined by paper chromatography, but in neither case could the material be induced to crystallize. Pipsylarginine, however, gave a crystalline picrate.

$N^\alpha N^\epsilon$ -Dipipsyl-lysine. L-Lysine hydrochloride (2.8 m-moles) in 1.25M-sodium carbonate (15 ml.) containing sodium hydroxide (0.1 g.) was shaken for 24 hr. at room temperature with a solution of pipsyl chloride (5.6 m-moles) in ether (15 ml.). The suspension was filtered and the solid washed with water and stirred in 2N-hydrochloric acid for 2 hr. The oil produced was dried over phosphorus pentoxide, dissolved in methanol and filtered from a small quantity of white residue. Evaporation of the methanol gave an oil that crystallized from aqueous methanol (yield: 0.75 m-mole), m.p. 157° (uncorr.) (Found: C, 31.4; H, 3.2; I, 37.4; S, 9.5; $C_{18}H_{20}I_2N_2O_6S_2$ requires C, 31.9; H, 3.0; I, 37.4; S, 9.4%).

Stability of pipsylamino acids to acid hydrolysis

Ether-soluble pipsylamino acids. Stock solutions of mixtures of [^{131}I]pipsylamino acids in 0.1N-ammonia were prepared, and analysed by acidifying the solution, extracting the derivatives with ether and chromatographing them. Portions of the stock

solutions were evaporated to dryness in 50 ml. flasks, 6N-hydrochloric acid (10 ml.) was added and the solution was boiled under reflux for 16 hr. After the addition of an equal volume of water, the [^{131}I]pipsylamino acids were extracted with five 8 ml. portions of ether, [^{131}I]iodobenzene- p -sulphonic acid was removed by countercurrent extraction and the amounts of [^{131}I]pipsylamino acids remaining were determined by chromatography. For some of the pipsylamino acids, hydrolysis was also carried out in 6N-hydrochloric acid containing unlabelled pipsyl derivatives of insulin, wool keratin or α -keratose (Corfield, Robson & Skinner, 1958). There was no significant difference between means (not less than three replicates) for hydrolyses carried out in the presence and in the absence of pipsyl-protein.

Acid-soluble pipsylamino acids. Stock solutions of N^α -pipsylarginine and N^α -pipsylhistidine, prepared by the reaction of these amino acids with [^{131}I]pipsyl chloride, were extracted with ether to remove any dipipsyl or other derivatives that might decompose on hydrolysis to give the N^α -pipsyl derivatives. O -Pipsyltyrosine and N^ϵ -pipsyl-lysine were determined in a 16 hr. hydrolysate of [^{131}I]pipsyl- α -keratose, which contained approximately equal amounts of these two derivatives. After hydrolysis for a further 16 hr., the amounts of the derivatives remaining were determined.

The results, expressed as the fraction of the original amounts of pipsylamino acid that remained

Table 4. *Stability of pipsylamino acids to acid hydrolysis*

For three or more replicates, means $\pm$ s.d. (numbers of replicates in parentheses) are given.

Pipsylamino acid	Fraction of pipsyl-amino acid remaining after 16 hr. in refluxing 6N-HCl
Pipsyl-leucine	0.85 $\pm$ 0.04 (3)
Pipsyl-valine	0.83 $\pm$ 0.04 (3)
Pipsyl-alanine	0.70 $\pm$ 0.04 (7)
Pipsyl-glycine	0.56 $\pm$ 0.06 (9)
Pipsyl-threonine	0.66 $\pm$ 0.02 (7)
Pipsyl-serine	0.39 $\pm$ 0.04 (7)
Pipsyl-glutamic acid	0.77 $\pm$ 0.03 (6)
Pipsyl-aspartic acid	0.66 $\pm$ 0.05 (6)
O -Pipsyltyrosine	0.87 $\pm$ 0.05 (3)
N^ϵ -Pipsyl-lysine	0.79 $\pm$ 0.04 (3)
Pipsyl-phenylalanine	0.84, 0.82
Dipipsylcystine	0.09
N^α -Pipsylarginine	0.80, 0.76
N^α -Pipsylhistidine	0.82
Pipsylcystic acid	0.71
Pipsylproline	0.42, 0.23
Pipsylhydroxyproline	0.49

after hydrolysis, are shown in Table 4. For all the pipsylamino acids for which replicate results are quoted, except pipsylarginine and pipsylproline, at least two independent stock solutions of [¹³¹I]-pipsylamino acids or hydrolysates of [¹³¹I]pipsyl-proteins were used as starting materials in the determinations. No evidence of any radioactive degradation products other than [¹³¹I]iodobenzene-*p*-sulphonic acid and [¹³¹I]iodobenzene-*p*-sulphonamide was noted.

Reaction of pipsyl chloride with peptides

Determination of the extent of reaction of pipsyl chloride with several synthetic di- and tri-peptides under the conditions used by Levy & Slobodian (1952) gave results similar to those obtained by these authors (Table 5). The pipsyl-peptide, separated from the unchanged peptide by extraction into ethyl acetate, was hydrolysed to liberate the *N*-terminal pipsylamino acid, which was extracted from the hydrolysate with ether. In some cases the non-*N*-terminal amino acid remaining in the hydrolysate was estimated by quantitative conversion into pipsylamino acid. The results indicated that this procedure might be used for the partial characterization of some peptides on an ultra-micro scale, but it was abandoned as being too laborious for routine use.

It was found that yields comparable with those in the three-step procedure could be obtained in a one-step reaction in phosphate buffer, whether this was carried out at 100° or at 37° (Table 6). In this way the *N*-terminal residues of several peptides from a tryptic digest of a protein fraction from oxidized wool (Corfield *et al.* 1967) were determined. The extents of reaction, for those cases in which the amounts of peptide taken for reaction was known with sufficient accuracy, are given in Table 6. Owing to losses in transfer and division of samples, the errors in these results may be as high as 20%.

To make a partial check on the assumption that

Table 5. Reaction of pipsyl chloride with di- and tri-peptides

The peptide (approx. 3 μmoles) was treated with pipsyl chloride in a three-step reaction in bicarbonate solution as described by Levy & Slobodian (1952). The pipsyl derivative of the *N*-terminal amino acid was estimated after hydrolysis of the pipsyl-peptide. Corrections for breakdown of the derivatives during hydrolysis have been applied.

Peptide	Fraction converted into pipsyl-peptide	
	Present work	Levy & Slobodian (1952)
DL-Ala-Gly	0.86	0.84, 0.89
Gly-D-Val	0.70	
Gly-Gly	0.56	0.61, 0.57
L-Leu-Gly	0.72	
Gly-Gly-Gly	0.70	
L-Leu-Gly-Gly	0.64	

Table 6. Reaction of pipsyl chloride with peptides under various conditions

The results of the three-step reaction are quoted from Table 5. Details of the one-step reaction in phosphate buffer at 100° and the reaction in phosphate buffer at 37° are given in the Materials and Methods section. The oligopeptides are mostly peptides isolated from a tryptic digest of fraction U.S.3 of oxidized wool (Corfield *et al.* 1967). In the one-step reaction in phosphate buffer at 100°, the amount of pipsyl-peptide formed was measured directly. In all other cases, corrections for the breakdown of the *N*-terminal pipsylamino acid and *N*^ε-pipsyl-lysine during hydrolysis have been applied.

Peptide	Fraction converted into pipsyl-peptide		
	Three-step reaction in bicarbonate buffer, 100°	One-step reaction in phosphate buffer, 100°	One-step reaction in phosphate buffer, 37°
DL-Ala-Gly	0.86	0.56	0.48
Gly-Gly	0.56	0.92, 0.82	0.73
Leu-Val-Val-Glu-Asp-Ile-Asp-Ala-Lys (1T/12R)			0.48
CySO ₃ H-Glu-Asp-Ser-Ser-Lys (1T/4β1)			0.65 (0.78*)
Glu-Ala-Asp-CySO ₃ H-Glu-Ala-Ser-Gly-Arg (1T/4γ2)			0.90
Asp-Val-CySO ₃ H-Ala-Leu-Arg (1T/9γ5)			0.60
Glu-(Ala,CySO ₃ H,Glu,Val) (1T/4γ3C1)			0.32
Ala-Gly-Ser-CySO ₃ H-Gly-Arg (1T5γ1)			0.33
Ile-Leu-CySO ₃ H-Ala-Lys (1T/6γ4)			0.69
Leu-Asp-Ala-Pro-Thr-Val-Glu-Leu-Asp-Val-Glu-Ala-Val-Leu-Asp-Arg (1T/10R)			0.16
Thr-(Ala,Asp,Glu ₂ ,Gly,Lys,Val)-Arg			0.56 (0.50*)

* N^ε-Pipsyl-lysine (moles/mole of peptide).

the extent of decomposition observed for free pipsylamino acids during acid hydrolysis can be used to correct the recoveries of pipsylamino acids found after hydrolysis of pipsyl-peptides and pipsyl-proteins, known amounts of pipsylglycylglycine were hydrolysed for 16 hr. and the pipsylglycine in the hydrolysate was extracted and estimated. In duplicate experiments 0.58 and 0.68 mole of pipsylglycine/mole of pipsylglycylglycine were recovered. Both these results lie within two standard deviations from the mean value, 0.56, obtained for free pipsylglycine.

Reaction of pipsyl chloride with insulin

The conditions of reaction, and the amounts of pipsylglycine, pipsylphenylalanine, *O*-pipsyltyros-

ine and *N*^ε-pipsyl-lysine liberated from the pipsyl-insulin, are given in Table 7. The results are corrected for breakdown during hydrolysis and are expressed as moles/mole of insulin, corrected for residual water content.

DISCUSSION

The originators of the pipsyl method may have envisaged its use for the amino acid analysis of proteins on an ultra-micro scale, for which complete reaction with every amino acid and separation of all the derivatives would be necessary. The spectacular advances that have taken place since 1946 in the ease and sensitivity with which mixtures of free amino acids can be analysed have lessened interest (gas-liquid chromatography apart) in

Table 7. *Reaction of pipsyl chloride with insulin*

Except where otherwise stated, insulin (approx. 1 μ mole) was allowed to react with pipsyl chloride in 1:1 (v/v) aqueous buffer-organic solvent (10 ml.) for 20 hr. After hydrolysis of the pipsyl-insulin, the ether- and acid-soluble pipsylamino acids were estimated as described in the text. The results are corrected for the breakdown of the derivatives during hydrolysis.

Conditions	Pipsylamino acid (moles/mole of insulin)			
	Pipsylphenylalanine	Pipsylglycine	<i>N</i> ^ε -Pipsyllysine	<i>O</i> -Pipsyltyrosine
1. Ethanol-buffer mixtures				
(a) 0.05 M-NaHCO ₃ , 30 μ moles of reagent, 20°	0.36	0.46	0.35	0.89
(b) As in (a), except that further 30 μ moles of reagent added after 5 hr., 20°	0.37	0.54		
(c) 0.05 M-NaHCO ₃ , 60 μ moles of reagent, 20°	0.28	0.56	0.40	0.99
(d) Allowed to react as in (a), then pipsyl-insulin precipitated,* redissolved in buffer, and the reaction repeated	0.30	0.48		
(e) 0.05 M-NaHCO ₃ , 30 μ moles of reagent, 37°	0.41	0.61		
(f) 0.05 M-NaHCO ₃ , 30 μ moles of reagent, 55°	0.34	0.49	0.40	1.45
(g) 0.028 M-Na ₂ CO ₃ , 120 μ moles of reagent, 20°	0.55	0.35	0.68	1.84
(h) 0.021 M-Sodium sesquicarbonate, 0.2 mM-sodium EDTA, 60 μ moles of reagent, 37°	0.36	0.32	0.60	1.27
(i) Ethanol-0.25 M-NaHCO ₃ (2:1, v/v) (2 ml.), 60 μ moles of reagent; opalescent solution shaken for 5 hr. and left for 15 hr. at 20°	0.15	0.32	0.65	1.93
(j) As in (i), except that the reaction mixture was 8 M with respect to urea; the precipitated pipsyl-insulin was washed with water before hydrolysis	0.40	0.59	0.60	2.21
2. Dimethylformamide-0.05 M-NaHCO ₃ 60 μ moles of reagent, 37°	0.43	0.53		
3. Acetone-buffer mixtures				
(a) 0.05 M-NaHCO ₃ , 60 μ moles of reagent, 37°	0.64	0.48		
	0.58	0.47	0.58	2.13
	0.69†	0.49†	0.85†	2.74†
(b) 0.028 M-Na ₂ HPO ₄ , 60 μ moles of reagent, 37°	0.85	0.74	0.56	1.35
	0.71	0.72		
	0.69	0.68	0.62	2.10
	0.78†	0.84†	0.78†	2.36†
	0.72†	0.58†	0.73†	2.24†

* 4% of the protein N remained in solution.

† I.U.P.A.C. insulin.

amino acid analysis via amino acid derivatives. The system of separation of pipsylamino acids described here is not comprehensive, but it should generally be adequate for the determination of *N*-terminal residues. The least satisfactory feature is the inadequacy of the FR 7-2 column when the ether-soluble derivatives of tyrosine and lysine are present. The separation of these hydrophobic derivatives might be improved by the incorporation of urea in the stationary phase (Matheson, 1965) and by the use of a more elaborate gradient-elution system such as that used by Kesner, Muntwyler, Griffin & Abrams (1963) for the separation of DNP-amino acids. The present system also fails to separate *N* α -pipsylarginine and *N* ϵ -pipsyl-lysine. Oratz *et al.* (1966) have recently reported the presence of *N*^{im}-pipsylhistidine in the hydrolysates of pipsyl-proteins. No separate peak that might have corresponded to this derivative was found in the present work.

Previous findings that most of the acidic and neutral amino acids could be converted into pipsylamino acids in 95–100% yield were confirmed during the present work. Under none of the conditions studied was complete reaction obtained between pipsyl chloride and the basic amino acids. The yields of *N* α -pipsylarginine and *N* α -pipsylhistidine could not be increased above about 80%, and these derivatives resist further substitution when the reaction with pipsyl chloride is carried out in the presence of bicarbonate. The choice of optimum reaction conditions for histidine may be quite critical, as shown by its conversion into an unstable dipipsyl derivative when the reaction is carried out in borate buffer. The behaviour of lysine is more complex. In a single-step reaction it appears to be converted into *N* α *N* ϵ -dipipsyl-lysine, together with appreciable amounts of iodobenzene-*p*-sulphonamide. By the further action of pipsyl chloride, an ether-soluble substance is produced that runs at fraction no. 31 on the G5-2 column. The yields of this substance quoted in Table 3 have been calculated on the assumption that it contains two pipsyl groups. The identity of this substance can only be conjectured, but it may conceivably be formed via a tripipsyl derivative by the expulsion of an *N* α -pipsyl group, as suggested by Neadle & Pollitt (1965) for the decomposition of 1-dimethylaminonaphthalene-5-sulphonylamino acids in the presence of an excess of the corresponding sulphonyl chloride. Thus, in the reaction of pipsyl chloride with the basic amino acids, two- and three-step reactions do not increase the yields of pipsylarginine and pipsylhistidine, and have the disadvantage of producing a mixture of derivatives from lysine.

The stabilities of pipsylamino acids to acid hydrolysis are only moderate, the iodine atom having little tendency to decrease the basicity of

the sulphonamide and thus to decrease the ease of formation of the conjugate acid, through which the reaction is thought to proceed (Klamann & Hofbauer, 1953). As might be expected, the stabilities of pipsylamino acids are much less influenced by the presence of other substances in the hydrolysate than are the stabilities of DNP-amino acids. The amount of pipsylamino acid remaining after 16 hr. hydrolysis is reasonably reproducible, and it has been shown that equivalent amounts of free pipsylglycine and pipsylglycylglycine yield approximately the same amounts of pipsylglycine after 16 hr. hydrolysis.

For the di- and tri-peptides studied, a three-step reaction with pipsyl chloride with intermediate extraction of pipsyl-peptide gives substantial but incomplete conversion into the pipsyl-peptide. A one-step reaction in phosphate buffer at 37° gives similar yields, and, since the amount of peptide available for reaction, if it has been eluted from paper, is probably not known accurately, this more rapid and simple procedure is to be preferred. It should be possible to scale down the reaction with peptides a good deal further, so that, if a suitable scheme for the separation of pipsylamino acids and iodobenzene-*p*-sulphonic acid were devised, the latter need not be removed from the reaction mixture.

When the reaction between pipsyl chloride and insulin was carried out in ethanol-bicarbonate buffer at 20° the results were comparable with those obtained by Udenfriend & Velick (1951) for the terminal amino acids, i.e. approx. 0.4 mole of glycine and phenylalanine/mole of insulin, and this result was not improved by carrying out the reaction in two stages. A similar amount of *N* ϵ -pipsyl-lysine was found, whereas Udenfriend & Velick (1951) found this derivative in theoretical yield. Since these workers made no reference to *O*-pipsyltyrosine, and their determination was made by paper chromatography in a system in which the two derivatives have similar *R_F* values, it is possible that they may have estimated the sum of these two derivatives. A greater extent of reaction with the *N*-terminal residues was observed at 37°, but not at 55°. In all the reactions in ethanol-bicarbonate buffer, the extent of reaction with the *N*-terminal glycine residue was greater than that with the *N*-terminal phenylalanine residue. Such differences in the extent of reaction of the two *N*-terminal residues have been observed in the partial reaction of insulin with *O*-methylisourea (Evans & Saroff, 1957) and phenyl isocyanate (Andersen, 1954). Increased reaction with the side chains of lysine and tyrosine residues was obtained when the reaction mixture was made 8M with respect to urea, and also at the higher pH given by sodium carbonate. When ethanol was replaced by

acetone in the reaction medium, a marked improvement in the extent of reaction with *N*-terminal phenylalanine was obtained. The best results were obtained in acetone-phosphate buffer mixtures: the reaction with the *N*-terminal residues and lysine was about 75% complete and that with tyrosine was about 50%.

Recently Oratz *et al.* (1966), using the isotopic carrier method and a three-step reaction in tetramethylammonium borate buffer, have obtained complete reaction between pipsyl chloride and the ϵ -amino groups of lysine residues in several proteins, one of which was bovine insulin.

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