The Synthesis of L-Amino Acids and their Derivatives Labelled with ^{3}H

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Received on March 26, 1971

SUMMARY

Methods have been devised for the synthesis of a range of L*-amino acids and of certain of their derivatives and of* D*-serine labelled with ^{3}H at their α-carbon atoms by a racemisation method followed by enzymic resolution. Conditions have been found which allow good recovery of materials of high specific activity for a given outlay of isotope with the added advantage that experimental technique has been maintained to the simplest level possible The stereochemical purity of the labelled amino acids synthesized is in excess of 99.99 %.*

The ^{3}H-labelling method for determining C-terminal residues in peptides developed by Matsuo [1] has been briefly investigated.

INTRODUCTION.

To meet the requirements of a routine general method for the production of a range of labelled amino acids an investigation was undertaken of a promising procedure, namely racemisation of acetylamino acid in tritiated solvent. This procedure seemed to fulfil the desirable conditions that a cheap, easily-handled form of isotope was used, i.e. tritiated water, and that experimental procedure was simple. However, as will be evident from a survey of the previous literature, no consideration seems to have been given by any author to maximising either the yield or the specific activity of the recovered compound.

Crawhall and Smyth [2] described the racemisation technique for labelling valine at the α-position. DL-Valine was refluxed with a mixture prepared from tritiated water and excess of acetic anhydride under anhydrous conditions for 90 min, and labelled DL-valine was recovered by acidic hydrolysis of the crude acetylvaline. No attempt was being made by these authors to prepare

highly active valine, but their results do show a good radiochemical yield. They further demonstrated the rapid opening of the ring of the oxazolone intermediate under aqueous conditions which stabilises the label originally at the C-4 position of the oxazolone (the α-carbon atom of the generated amino acid).

The racemisation technique, using hot acetic anhydride and tritiated (or deuterated) acetic acid has also been used for labelling DL-alanine [3], DL-lysine [4], DL-glutamic acid [5] and DL-lysine, DL-arginine and DL-histidine [6]. In this last instance the acetylamino acids (lysine was converted to the $N^{\alpha},N^{\varepsilon}$-dichloroacetyl derivative) were resolved with renal acylase to afford the pure L-isomers, and tritium was shown to be located only at the α-position in L-lysine and L-arginine and located 92% at the α-position in L-histidine by the use of L-amino acid oxidase.

In all the publications mentioned above, the molar ratio of amino acid to reaction solvent was arbitrary and the reaction conditions were somewhat harsh. Neither point was considered in discussion by the various authors.

An interesting publication by Hoberman and D'Adamo [7] described the preparation of deuterated D-lactic acid from D-alanine derived from a renal acylase digestion of *N*-acetyl-DL-alanine-α-^{2}H. The point of real interest is that racemisation labelling of *N*-acetylalanine was claimed to have been affected by the extremely mild conditions of du Vigneaud and Meyer [8] employing acetic anhydride and sodium acetate in deuterated aqueous solution at room temperature. Unfortunately, no more experimental detail of the labelling than this was given.

The racemisation method is suitable for study for the following reasons :

(i) ease of labelling technique,

(ii) ready accessibility of compounds for labelling and avoidance of elaborate synthetic procedures,

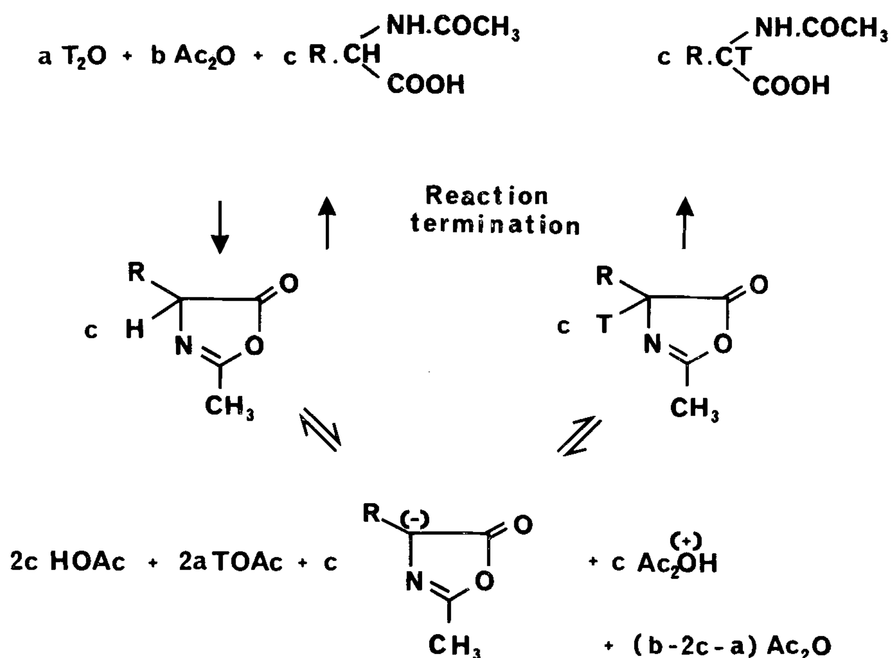
(iii) incorporation of the isotope at a specific stable site,

(iv) general applicability to all amino acids.

It can be objected that racemisation is, in itself, a disadvantage. However, the acetyl derivatives are suitable substrates for direct enzymic resolution to recover pure L-amino acids without the need for intermediate chemical operations.

Theoretical and Practical Considerations.

The mechanism of racemisation of an acetylamino acid in a mixture of acetic anhydride and tritiated water is represented by the over-simplified equation



From this relationship it follows that

(i) the ratio by which the label is diluted in the reaction medium

$$y' = \frac{2a}{2a + 3c}$$

(ii) the total recovery of label (radiochemical yield) is

$$y'' = \frac{c}{2a + 3c}$$

in an ideal case where, on opening the oxazolone ring under aqueous conditions at the end of the reaction, no loss of label occurs from the α-carbon atom of the acetylamino acid. The amount of acetic anhydride used is immaterial provided that excess is present, i.e. $b > 2c + a$, although this relationship need not be strictly observed as only a small amount of the generated oxazolone will exist as carbanion. Probably a sufficient condition is that $b > c + a$. Figure 1 is a graphical representation of the two variables y' and y'' for different values of c/a as set out in Table 1.

The choice of the value of c/a has to seek a balance between recovering, for a given expenditure of isotope, a small amount of highly labelled product

TABLE 1. The percentage specific activity of the reaction medium (y′) and the percentage radiochemical yield (y″) of recovered α-^{3}H amino acid as a function of the proportion (c/a) of the acetylamino acid taken relative to the tritiated water.

c/a	0	1/3	1/2	2/3	1	2	3	6	∞
y′	100	67	57	50	40	25	18	10	0
y″	0	11	14	17	20	25	27	30	33

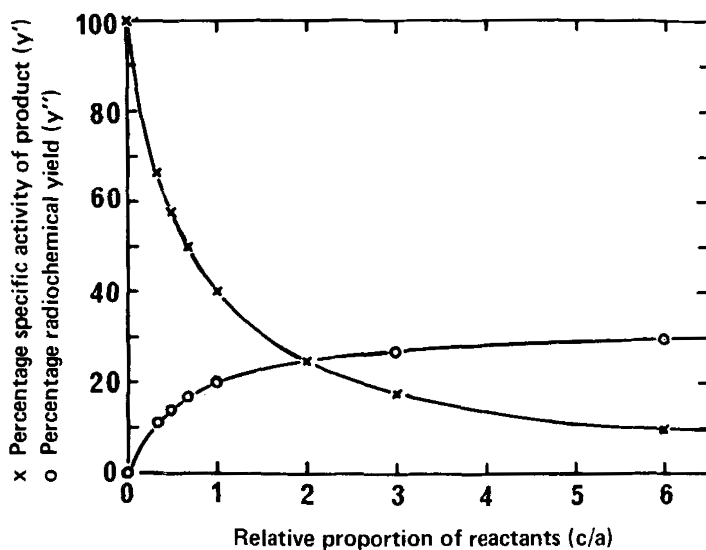


FIG. 1. Graphical representation of Table 1.

or a larger amount of less active material. The value c/a = 1/2 was chosen as the ratio for practical investigation.

For practical simplicity, if 50 μl (2.78 mmole) of tritiated water and 0.5 ml (5.3 mmole) of acetic anhydride are dispensed, the amount of acetylamino acid (c) that can be used is determined by the condition

$$c + a < b < 2c + a$$

Substituting $$c + 2.78 < 5.3 < 2c + 2.78$$

or $$2.52 > c > 1.26$$

Now, the amount of tritiated water taken is 2.78 mmole, so for c/a = 1/2, c = 1.39 mmole. This value satisfies the condition derived above.

An average molecular weight for an acetylamino acid is about 190, so 1.39 mmole is about 265 mg. This amount of material should be soluble in 0.5 ml of hot acetic acid-acetic anhydride. For optimum radiochemical yield in the du Vigneaud and Meyer [8] racemisation method, 1.39 mmole of acetylamino acid would need to be dissolved in 50 μl of water, which is most unlikely to be possible.

The simple treatment given above will only hold for acetylamino acids with inert side chains. Introduction of functional side-chains will increase the dilution of the isotope in the reaction medium. Blocking groups will offset this effect, e.g. use of *N,O*-diacetyltyrosine, *N*-acetyl-*O*-benzyl serine, N^{ε}-substituted-N^{α}-acetyllysine, etc. This approach could also be advantageous in that blocking groups would not have to be added after labelling with concomitant loss of active material, so the choice of blocking groups must be governed by the synthetic work in which the labelled amino acids is to be used. At the beginning of this work, the feasibility of enzymically resolving such compounds was largely an unknown factor.

EXPERIMENTAL.

Materials. — L- and DL-amino acids were purchased from Cambrian Chemicals Ltd., London, S.E.16, *O*-benzyl-DL-serine (Fluka) from Ralph Emanuel Ltd., Alperton, Middx., renal acylase I, α-amylase (fungal, crude, type IV-A), L-amino acid oxidase (from *Crotalus adamanteus*) and D-amino acid oxidase (hog kidney) from Sigma Chemical Co., St. Louis Mo., USA and tritiated water (~5 Ci/ml) from the Radiochemical Centre, Amersham, Bucks.

Analytical methods. — Compounds were examined on thin layers (0.25 mm) of MN-Silica-gel S-HR (Macherey-Nagel and Co.) using the following solvent systems for development : A, butan-1-ol-acetic acid (glacial)-water (10 : 1 : 3, by vol) [9]; B, phenol-water (3 : 1 w/w) [10]; C, propan-2-ol-formic acid-water (20 : 1 : 5, by vol) [9]; D, ethanol-aq. NH_3 (sp. gr. 0.88) (7 : 3, by vol) [11]. Radioactive compounds were detected using a Panax Thin-Layer Radiochromatogram Scanner System E.0111/XPD-05 and assayed with a Packard Model 3003 Liquid Scintillation Spectrometer in 10 ml of scintillator [containing 30 g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen and 340 g of naphthalene in a mixture of 2.5 litre of toluene and 1.7 litre of methylCellosolve] using hexadecane-α,β-$^{3}H_2$ as internal standard.

Compounds for labelling.

Known *N*-acetyl derivatives of L- and DL-amino acids were prepared by standard methods with the exception of the following which have not been described.

N-*Acetyl*-DL-*glutamic acid γ-benzyl ester.* — L-Glutamic acid γ-benzyl ester (5 g), suspended in a mixture of acetic anhydride (5 ml) and glacial acetic acid (5 ml), was heated under reflux until solution had occurred (~1 min). Water (2 ml) was added and the solvent was removed by rotary evaporation. The residue was crystallized from ethyl acetate/petroleum ether (b.p. 60-80°) and recrystallised from the same solvent mixture to give the racemic *N-acetyl derivative* (5.0 g, 85%). It had m.p. 115°, $[\alpha]_D = 0 \pm 2°$ (*c* 4.0, ethanol) (Found : C, 60.1; H, 6.21; N, 5.22. $C_{14}H_{17}NO_5$ requires C, 60.2; H, 6.09; N, 5.02%). The compound was recovered unchanged after heating under reflux with acetic anhydride in acetic acid for 10 min.

N-*Acetyl*-L-*glutamic acid γ-benzyl ester.* — L-Glutamic acid γ-benzyl ester (4.75 g) was suspended in water (20 ml) and titrated to pH 8.5 with 4*N*-NaOH on a pH-stat under vigorous stirring. Acetic anhydride (1.9 ml) was added in portions (0.1 ml) such that the pH-stat maintained the solution within the range pH 7.0-10.5. After 1 h further stirring, 6 *N*-H_2SO_4 equivalent to the total alkali consumed was added. The solution was evaporated to dryness and ethanol (2 × 20 ml) was distilled from the residue to remove the last traces of water. The residue was extracted with hot ethyl acetate (3 × 20 ml) and the combined extracts were evaporated to dryness. The residue, after two recrystallisations from ethyl acetate gave the *N-acetyl derivative* (4.1 g, 73%). It had m.p. 124-6°, $[\alpha]_D = +13 \pm 2°$ (*c* 2.0, ethyl acetate) (Found : C, 59,9; H, 5.95; N, 5.07).

N-*Acetyl*-N^{im}-*benzyl*-L-*histidine.* — N^{im}-Benzyl-L-histidine (7.5 g) was dissolved in 4 *N*-NaOH solution (7.5 ml) and three separate portions of acetic anhydride (1 ml) and 4 *N*-NaOH solution (2.5 ml) were added at interval of 5 min while the reaction mixture was stirred vigorously under ice cooling. After addition of a further portion (1 ml) of acetic anhydride, stirring was continued at room temperature for 1 h, 2 *N*-H_2SO_4 (30 ml) was added and the mixture was evaporated to dryness. The residue was extracted with portions of hot ethanol (total volume 200 ml) and the combined extracts were evaporated to dryness. The residue, after two recrystallisations from ethanol gave 4.7 g (53%) of the *N^{α}-acetyl derivative.* It had m.p. 204° $[\alpha]_D = +41 \pm 1°$ (*c* 5.0, water) (Found : C, 62.5; H, 6.09; N, 14.7. $C_{15}H_{17}N_3O_3$ requires C, 62.7; H, 5.92; N, 14.6%).

N^{α}-*Acetyl*-N^{im}-*benzyl*-DL-*histidine.* — N^{im}-Benzyl-L-histidine (4.9 g) was dissolved in 4 *N*-NaOH solution (4.9 ml) and treated with acetic anhydride (8 ml) in portions while the reaction mixture was stirred vigorously under ice cooling. After 1 h further stirring, 2 *N*-H_2SO_4 (9.8 ml) was added and the product was worked up as described above. The material was recrystallised twice from moist ethanol to give the *N^{α}-acetyl derivative* (2.9 g, 51%). It had m.p. 191-2° (decomp.), $[\alpha]_D = 0 \pm 2°$ (*c* 5.0, water) (Found : C, 62.7; H, 5.89; N, 14.6%).

N^{α}-*Acetyl*-N^{δ}-*benzyloxycarbonyl*-DL-*ornithine.* — N^{δ}-Benzyloxycarbonyl-DL-ornithine was converted to the N^{α}-acetyl derivative as described for the

corresponding L-lysine compound by Neuberger and Sanger [12] and isolated by precipitation as the *N,N*-dicyclohexylamine salt from acetone solution. The salt (17.2 g) was dissolved in water (200 ml) and the solution was adjusted to pH 1 with dilute HCl solution. Dicyclohexylamine hydrochloride was removed by vacuum filtration and the filtrate was extracted with ethyl acetate (3 × 150 ml). The combined extracts were washed with brine (3 × 150 ml), dried (Na_2SO_4) and evaporated below 40° to give an oil which was crystallised from acetone by addition of petroleum ether (b.p. 60-80°) with chilling and trituration. Recrystallisation from the same solvent system gave the *N$^{\alpha}$-acetyl derivative* (9.2 g, 84% : overall yield from DL-ornithine hydrochloride 20%). It had m.p. 100-103° (Found : C, 58.2; H, 6.41; N, 9.39. $C_{15}H_{20}N_2O_5$ requires C, 58.4; H, 6.49; N, 9.09%).

N$^{\alpha}$-*Acetyl*-N$^{\delta}$-*benzyloxycarbonyl*-L-*ornithine.* — This was prepared as described for the corresponding L-lysine compound by Neuberger and Sanger [12] and purified *via* the *N,N*-dicyclohexylamine salt as described above for the racemic compound. The compound was obtained as an oil which crystallised on storage to give the *N$^{\alpha}$-acetyl dérivative.* It had m.p. 106°, $[\alpha]_D = +4.4 \pm 1.0°$ (*c* 5.0, dimethylformamide) (Found : C, 58.6; H, 6.62; N, 9.01).

Investigation of conditions necessary for labelling representative N-*acetylamino acids.*

All racemisation labelling experiments were first conducted without isotope on optically active *N*-acetylamino acids. In all cases where optically active starting material was available, racemisation was complete under the stated experimental conditions as judged by the optical rotation of the reaction mixture and by isolation of crystalline racemic material.

N-Acetylamino acids (1.38 mmole) were treated with a mixture of acetic anhydride (0.5 ml) and tritiated water (50 μl, ~250 mCi) under various conditions as given in Table 2. Water (2 ml) was added to destroy the excess of acetic anhydride and the mixture was evaporated to dryness. Water (2 × 5 ml) was evaporated from the residue to remove labile tritium. The residue was dissolved in the minimum amount of a suitable solvent and crystalline material was recovered by evaporation in an air stream. Amino acids were recovered either by acidic or enzymic hydrolysis as described later. Results are summarised in Table 2.

Tritium location analysis of a sample of N-*acetyl*-DL-*methionine.*

The sample (300 mg, 12.7 mCi/mmole) was heated under reflux with aqueous 2 *N*-HCl (10 ml) for 1 h and cooled. Samples (0.20 ml) were withdrawn and added to (*a*) 458 mg DL-methionine dissolved in dilute aqueous HCl (5 ml), and (*b*) 635 mg acetic acid dissolved in a slight excess of NaOH (5 ml).

TABLE 2. The effect of racemisation conditions on the specific activity and the purity of recovered amino acid derivatives.

Expt. No.	Acetylamino acid	Racemisation conditions [e]	Recovered *N*-acetyl derivative		Specific activity of the recovered amino acid (mCi/mmole)	% efficiency of labelling at the α-position
			% Radio-chemical purity	Specific activity (mCi/mmole)		
1	Arginine [a]	Reflux	60	12.9	6.1	50
2	Glutamic acid	Reflux	100	19.3	11.4	51
3	γ-Benzyl glutamate	100°, 3 min	100	—	24.4	95
4	Glycine [b]	100°, 3 min	100	—	31,1	?
5		Reflux	72	—	27.8	?
6	Histidine [c]	Reflux	100	30.7	9.2	51
7	N^{im}-Bzl-histidine	100°, 3 min	100 [f]	35.6	19.2	75
8	ε-Acetyllysine	Reflux	100 [g]	23.9	11.4	51
9		Reflux	100	24.3	10.6	47
10		Reflux, 5 min	100	31.0	7.7	34
11	ε-Z-lysine	100°, 3 min	100	—	19.0	84
12		Reflux	100	31.9	23.8	105
13	Methionine	100°, 3 min	100	41	20.8	79
14		Reflux	94	55.6	16.8	64
15		Reflux, 5 min	92	50.8	—	—
16	δ-Z-ornithine	100°, 3 min	100	—	21.0	93
17		Reflux	100	28.7	24.1	107
18	Phenylalanine	100°, 3 min	100	—	14.6	55
19		Reflux	99.4	41.5	15.6	59

TABLE 2. *(continued)*

Expt. No.	Acetylamino acid	Racemisation conditions [e]	Recovered *N*-acetyl derivative		Specific activity of the recovered amino acid (mCi/mmole)	% efficiency of labelling at the α-position
			% Radio-chemical purity	Specific activity (mCi/mmole)		
20	Proline	100°, 3 min	100	—	7.5	29
21		Reflux	100	33.8	21.6	84
22		Reflux, 45 min	100	85.8	20.9	81
23	*O*-Benzylserine	100°, 1 min	99.0	—	3.9	15
24		100°, 3 min	96.3	—	10.6	40
25		100°, 5 min	89 [h]	15.3	9.8	38
26		Reflux	66 [h]	18.7	—	—
27	Tryptophan [b]	100°, 3 min	99	16.4	13.4	60
28		Reflux	97	32.8	16.4	73
29	*O*-Acetyltyrosine [d]	100°, 3 min	100	—	15.9	62
30		Reflux	100	32.0	19.8	77
31	Valine	100°, 3 min	100	—	15.8	62
32		Reflux	100	47.7	14.4	56

[a] Dihydrate. Racemised in 0.79 ml Ac_2O.
[b] >0.50 ml Ac_2O used for solubility reasons.
[c] Monohydrate. Racemised in 0.65 ml Ac_2O.
[d] Saponified before resolution.
[e] «Reflux» means brought to boiling point, heat removed and reaction terminated with water.
[f] Crystalline product recovered in 15% yield.
[g] 50 μl H_2O added to terminate reaction.
[h] Non-crystalline product.

The remainder of the acid hydrolysate was evaporated to dryness several times with addition of water to remove the excess of HCl. The residue was taken up in water (2 ml), adjusted to pH 7 (LiOH), ethanol (8 ml) added and the solution was stored at 4° for 2 h. DL-Methionine was collected by vacuum filtration. It had a specific activity of 8.7 mCi/mmole.

The sample (*a*) was adjusted to pH 7 (LiOH) and ethanol (4 volumes) added. After storage at 4° for 2 h, DL-methionine was collected by vacuum filtration. It had a specific activity of 0.12 mCi/mmole.

The sample (*b*) was adjusted to pH 4 (HCl) and *S*-benzylthiouronium chloride (2 g) was added. The solution was stored at 4° for 2 h and the *S*-benzylthiouronium acetate collected by vacuum filtration. It had a specific activity of 15.2 μCi/mmole; corresponding to an activity of 3.75 mCi/mmole for the acetic acid present in the hydrolysis mixture.

Effect of isotope on the reaction rate of labelled N-*acetyl*-DL-*methionine with renal acylase I.*

The activity of renal acylase I was determined by the spectrophotometric method of Mitz and Schlueter [13] using *N*-acetyl-DL-methionine as substrate. Determination of the activity towards labelled substrate was by thin-layer chromatography (TLC) in solvent system A and use of the Panax scanner system with correction for the amount of label present in the acetyl group of the substrate. The activity of the preparation at 37° was 1,600 ± 160 μmole *N*-acetyl-L-methionine/h/mg enzyme and 1,900 ± 160 μmole labelled *N*-acetyl-L-methionine/h/mg enzyme.

Recommended conditions for the enzymic hydrolysis of N-*acetylamino acids.*

Labelled *N*-acetylamino acid (1.38 mmole) was dissolved in water (5 ml) and the solution was adjusted to pH 7.5 with LiOH soln. Resolution was effected at 37° using either renal acylase I or mould acylase (an aqueous extract of crude α-amylase). The quantities of mould acylase necessary were determined by rate studies on the individual substrates using TLC and the Panax scanner system. The quantities of renal acylase needed were calculated from the data prepared by Greenstein [14].

The conditions of resolution and the yields and analyses of the recovered L-amino acids are summarised in Table 3.

[α-^3H]-D-*Serine*. — *N*-Acetyl-*O*-benzyl-DL-serine (1.38 mmole) was labelled, worked up and resolved with renal acylase I in the normal manner (Table 3, expt. 9). The resolution mixture was deproteinised by boiling with a little charcoal and filtered. The filtrate and washings were adjusted to pH 1 (HCl) and extracted with ethyl acetate (3 × 5 ml). Both phases were back-washed. The organic phase was evaporated to dryness, dissolved in water and redigested for 24 h with a further portion (10 mg) of renal acylase (Table 3,

TABLE 3. Conditions for the enzymic resolution of *N*-acetylamino acids (1.38 mmole) and the yields and stereochemical purity of the recovered amino acids.

Expt. No.	Amino acid recovered	Acylase [e] used	Digestion time (h)	% theoretical amount of acid recovered	% Radio-chemical purity	Weight (mg) of oxidase used per mg of amino acid	% Stereo-chemical purity
1	L-Glutamic acid γ-benzyl ester	Mould 130 mg	28	55	100	1.6	>99.99
2	Glycine	— [a]	—	83	100	—	—
3	N^{im}-Benzyl-L-histidine	Mould 110 mg	112	6.3	98.8	2.1	>99.99
4	N^{ε}-Z-L-lysine	Mould 200 mg	16	75.5	99.0	2.3	>99.99
5	L-Methionine	Renal 0.5 mg	16	64	100	0.9	>99.8
6	N^{δ}-Z-L-ornithine	Mould 300 mg	42	67	99.5	3.0	>99.99
7	L-Phenylalanine	Renal 8 mg	40	64	99.7	0.9	>99.99
8	DL-Proline [b]	—	—	100	100	—	—
9	*O*-Benzyl-L-serine	Renal 10 mg	60	43	99.1	1.1	>99.99
10	D-Serine [c]	Renal 2×10 mg	60 + 24	37	100	11	>99.8
11	L-Tryptophan	Mould 10 mg	66	59	98.4	0.8	>99.99
12	L-Tyrosine [d]	Mould 200 mg	16	77	100	0.5	>99.99
13	L-Valine	Renal 10 mg	16	72.5	100	1.3	>99.99

[a] Recovered after acidic hydrolysis.
[b] Converted to DL-proline amide acetate in 61% yield.
[c] From acidic hydrolysis of *N*-acetyl-*O*-benzyl-D-serine.
[d] Saponified before resolution (2 *N*-NaOH, 1 h).
[e] Mould acylase was obtained by extraction of crude α-amylase with water and removal of solids by centrifugation.

expt. 10). 1% of the total counts appeared as *O*-benzyl-L-serine. After deproteinisation with a little charcoal, the filtered mixture was applied to a column (5 ml) of Dowex 50 (H^+ form) resin and eluted with five bed volumes of 6% (by vol) acetic acid. The eluate was examined by TLC (Solvent A) and scanning for radioactivity to confirm the absence of *O*-benzyl-L-serine and then evaporated to dryness. The residue was heated under reflux for 30 min with aqueous 6 *N*-HCl, cooled and extracted with ether (10 ml). The aqueous phase was evaporated to dryness several times to remove excess of HCl, dissolved in a little water and adjusted to pH 7 (LiOH). After evaporation, crystallisation from 80% ethanol gave 28 mg (37%) of product.

Stereochemical purity (Parikh *et al.* [15]). — L-Amino acids (~20 μmole) were dissolved (or suspended) in 0.05 M-tris buffer (pH 7.2, 0.5 ml) and the appropriate volume of L-amino acid oxidase (*C. adamanteus*) solution was added. The mixtures were incubated for 16 h at 37° in a Dubnoff shaker under an atmosphere of O_2. Portions (2 μl) of solution were withdrawn both before addition of the enzyme and after incubation and developed on adjacent strips of TLC plates (solvent A) and examined with the Panax scanner system. In all cases radioactivity was completely absent after digestion from the position corresponding to the amino acid. In those cases where radiochemical impurities remained, these were identifiable as *N*-acetyl-D-amino acid (see Table 3). The desired sensitivity of analysis (>99.99%) was obtained by examining the appropriate volume of digest and relating this to the initial count obtained before digestion. Results are summarised in Table 3.

D-Serine was examined similarly but with hog kidney D-amino acid oxidase (Table 3).

L-Methionine and D-serine were also analysed by the procedure of Manning and Moore [16]. The amino acid (~20 μmole) was treated as described by Manning and Moore and column chromatographic analysis showed the L-methionine to be contaminated with a little sulphoxide but D-methionine was completely absent at a level where 1 part in 500 of diastereoisomer would have been detected. D-Serine was also pure to this degree. Racemic amino acids were similarly treated and analysed for reference purposes.

Investigations of the method of Matsuo [1] *for labelling C-terminal amino acids in peptides.*

A mixture of acetic anhydride (2.0 ml) and tritiated water (100 μl, ~500 mCi) containing Synacthen * (6.8 mg) was heated to boiling and water (2 ml) was added to stop the reaction. The peptide was worked up in the usual manner and hydrolysed with aqueous 6 *N*-HCl for 16 h at 115° in an evacuated tube. The hydrolysed sample was prepared for analysis by solution

* Synthetic β^{1-24}-corticotrophin (supplied by CIBA, Basle).

in water (1 ml) and addition of leucine-^{14}C (5 µl, 10 µCi/ml) as an internal standard. A sample (0.3 ml) of hydrolysate was applied to the 60 cm column of a Beckman amino acid analyser and eluted in the normal manner. Fractions (20 sec) were collected manually in scintillation vials for 3 h. Scintillation fluid (15 ml) was added to each fraction for assay of radioactivity. A further sample (0.3 ml) of hydrolysate was similarly assayed for labelled amino acids using the 25 cm column of the analyser (for basic amino acids). For amino acid estimation, samples (20 µl) of the peptide hydrolysate were analysed in the normal manner.

The procedure was repeated with Hypertensin ** (0.5 mg) and the reagents at one-half the scale described above.

Results are summarised in Tables 4 and 5.

TABLE 4. Analysis of the amino acids recovered from an acid hydrolysate of Synacthen labelled with ^{3}H by the method of MATSUO *et al.* [1]. Details are given in the text.

Amino acid	Activity in 0.3 ml sample (nCi)	Quantity in 0.3 ml sample (nmole)	Specific activity (µCi/mmole)
Ser	2.52	910	2.8
Glu	558	524	1,060
Pro	551	1,620	340
Gly	87.6	1,030	80
Val	2.9	1,530	2
Met	272	492	550
Tyr	132	719	180
Phe	2.4	360	7
His	4,130	426	9,700
Lys	0	2,020	0
Arg	0	1,410	0
Orn	0	26	0
Trp	0	223	0

DISCUSSION.

Preparation of the N^{α}-acetylamino acids resulted in the synthesis of several new compounds. *N*-Acetyl-DL-glutamic acid γ-benzyl ester was prepared from L-glutamic acid γ-benzyl ester by acetylation with racemisation in hot acetic acid-acetic anhydride. On further treatment with these reagents the compound was recovered unchanged confirming that racemisation had occurred during synthesis. The literature contains no reference to the racemic

** Synthetic angiotensin II (supplied by CIBA, Basle).

TABLE 5. Analysis of the amino acids recovered from an acid hydrolysate of Hypertensin labelled with ^3H by the method of MATSUO *et al.* [1]. Details are given in the text.

Amino acid	Activity in 0.3 ml sample (nCi)	Quantity in 0.3 ml sample (nmole)	Specific activity (mCi/mmole)
Asp (from Asn)	36.4	120	0.30
Pro	20.4	130	0.16
Val	0	250	0
Tyr	0	93	0
Phe	2,680	120	22.3
His	1,200	100	12.0
Arg	0	116	0
Orn	0	16	0

compound but two [17, 18] to the L-isomer. As both authors prepared their materials by our method but under more vigorous conditions it follows that they were handling racemic material. Neither report included an optical rotation value for the claimed L-glutamic acid derivative. A Japanese patent [17] claims m.p. 114°, compared with our 115°, and Shalitin and Bernhard [18] report m.p. 105-7°. As their material was not crystallised but merely solidified by petroleum ether treatment, this low m.p. may reflect the degree of impurity of their preparation. We have also prepared the L-compound by acetylation of γ-benzyl L-glutamate above pH 7, preventing racemisation, and below pH 10.5, preventing saponification of the benzyl ester. It has a m.p. of 126° which supports the arguments advanced above.

N-Acetyl-DL-methionine was chosen as a test substance for the investigation of labelling procedures. The du Vigneaud procedure [8] gave, as expected, products of low specific activity due to the unavoidable dilution of label by the aqueous medium and is not reported in detail. There was a rough correlation between the efficiency of labelling and the proportion of acetic anhydride used.

Methods involving formation of the oxazolone in hot tritiated solvent gave materials of high specific activity and showed high efficiency of isotope incorporation. The percentage efficiency of incorporation of label was in excess of the theoretical amount assuming incorporation only at the α-carbon atom. However, when amino acid was prepared from the *N*-acetyl derivative it was found to be labelled to a much smaller extent. Accordingly, a sample of *N*-acetyl-DL-methionine was hydrolysed with acid and the specific activities of the generated DL-methionine and acetic acid (isolated as the *S*-benzylthiouronium salt) were determined by inverse isotope dilution analysis. All the activity present in the original *N*-acetyl-DL-methionine was accounted for within the limits of experimental error. The presence of tritium in the acetyl

group of the *N*-acetyl derivatives, which has not been reported previously, suggests the involvement of pseudo-oxazolone species in the reaction mechanism. This observation provides a novel procedure for obtaining [^{3}H]-acetic acid and presumably other [α-^{3}H] labelled fatty acids.

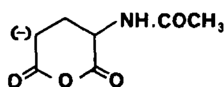
The Scheme is thus shown to be a much simplified description of the labelling reaction and calculations of radiochemical yield and percentage efficiency of labelling are therefore not strictly valid. They have been retained, however, as they provide a valuable assessment of procedural efficiency. The average equilibration efficiency (Table 2), about 70%, indicates that the considerations on which this study was based are tenable. Nevertheless, it should be emphasised that, because of the assumptions involved, the figures do not provide a basis for argument. The presence of tritium at the α-carbon atom and in the methyl group of the *N*-acetyl substituent did not produce an isotope effect outside the limits of experimental error in the rate of enzymic hydrolysis of the compound with renal acylase I.

The labelling experiments on N^{α},N^{ε}-diacetyllysine provide two points of information. Firstly, the excess of water added to terminate the reaction is irrelevant (Table 2, expts. 8 and 9). This agrees with the conclusion of Crawhall and Smyth [2] that the oxazolone ring is opened rapidly under aqueous conditions stabilising the label introduced by racemisation. Secondly, the use of extended reaction times, which previous authors have used without justification, gave material of a slightly lower degree of labelling than could be obtained by milder conditions, probably due to traces of water present on the inner surfaces of glassware becoming incoıporated into the reaction mixture during the reflux procedure (Table 2, expts. 9 and 10). In general, full equilibration of label was seen to have occurred between most *N*-acetyl-amino acids and the solvent after three minutes reaction at 100°. In certain cases, use of more vigorous reaction conditions either gave products of lower specific activity for the reason explained above (Table 2, expts. 4, 5; 13, 14; 24, 25; 31, 32) and/or gave rise to products of lower radiochemical purity due to decomposition (Table 2, expts. 4, 5; 14, 15; 18, 19; 24, 25, 26; 27, 28). *N*-Acetylproline needed, as expected, more vigorous treatment due to the fact that the cyclic structure of the amino acid places a constraint on the molecule as regards oxazolone formation. Maximum equilibration could be achieved in this case by heating the reaction mixture to boiling point (Table 2, expts. 20 and 21).

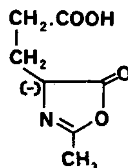
N-Acetyl-*O*-benzylserine (*N*-acetylserine is destroyed by the labelling procedure) was seen to decompose appreciably under the reaction conditions for labelling. The results in Table 2 show that maximum equilibration is achieved after 3 min at 100° at which time less than 4% destruction (which was regarded as acceptable) has occurred.

Where *N*-acetylamino acids bore additional unprotected functional groups, dilution of the labelling medium occurred resulting in decreased specific activity of the recovered amino acid (Table 2, expts. 1, 2, 6). Solvated

crystalline starting materials are obviously highly unsuitable for labelling (e.g. those of arginine and histidine) as would be expected, but even when allowance has been made for this, equilibration is still inexplicably inefficient. The low value obtained for *N*-acetylglutamic acid may be rationalised on the basis of competition between oxazolone and anhydride formation as has been discussed by Wenzel *et al.* [19] and Hochreiter and Schellenberg [9]. This need not mean that label should also be found at the γ-carbon atom of glutamic acid as the anion (I) derived from the anhydride is not stabilised to the same extent as is (II) derived from the oxazolone.



(I)



(II)

When anhydride formation is eliminated by protection of the γ-carboxyl function as the benzyl ester the degree of labelling at the α-carbon atom is comparable with that observed for acetylamino acids with inert side-chains (Table 2, expt. 3). Similarly, N^{im}-benzyl protected histidine and N^{ω}-benzyloxycarbonyl protected ornithine and lysine derivatives are obtained at higher specific activities at the α-carbon atom than the unsubstituted analogues (Table 2, expts. 7, 11, 12, 16 and 17). The N^{ω}-benzyloxycarbonyl-ornithine and -lysine derivatives show specific activities of greater than 100% of that expected. This may mean that the acidity of the remaining hydrogen atom on the ω-nitrogen atom is decreased by the substituting function. This consideration has been put forward to explain the resistance towards racemisation conferred on amino acids by the benzyloxycarbonyl group when present as an N^{α}-substituent [20]. In this way, the hydrogen atom would be prevented in some measure from ionising and participating in the dilution of the labelling medium.

As no suitable fully protected derivative of arginine is available for labelling, a synthetic approach *via* ornithine is suggested. A synthetic procedure has been developed using unlabelled compounds and is not reported here. N^{α}-Boc-N^{G}-nitro-L-arginine was obtained in 36% overall yield from *N*-benzyloxycarbonyl-L-ornithine by a 3-stage reaction sequence. *O*-Methyl-*N*-nitroisourea was preferred to the thio analogue for the final nitroguanylation as the latter reagent gave, for unknown reasons, very little of the desired reaction product and a large amount of an unidentified side-product.

N-Acetyl-DL-glutamic acid γ-benzyl ester was resolved without difficulty using mould acylase. It was essential when preparing the solution of the

material that adjustment to pH 7.5 (with NaOH) was effected under vigorous stirring on a pH-stat. In this way the pH could be kept below 10.5 and hydrolysis of the ester avoided.

A satisfactory method for obtaining α-^{3}H labelled histidine has not been found. N^{α}-Acetyl-N^{im}-dinitrophenyl-DL-histidine was prepared [21], labelled and digested with mould acylase. Separation of the N^{im}-dinitrophenyl-L-histidine under mild conditions was not possible by the usual conditions of ethanol precipitation as the amino acid is soluble in ethanol. Attempted separation by ion-exchange chromatography was also unsuccessful. Labelling of N^{α}-acetyl-N^{im}-benzylhistidine under mild conditions gave a yellow oil from which no N^{im}-benzyl-L-histidine could be obtained after mould acylase digestion. The impurities in the reaction mixture both inhibited the action of the enzyme and prevented the crystallisation of the amino acid. A small yield (6% overall) of material of high specific activity was obtained by the resolution of material obtained from the impure oil by crystallisation (Table 3, expt. 3). N^{im}-Benzyl-L-histidine is not the ideal protected derivative of histidine for use in peptide synthesis due to the harsh conditions needed for deprotection.

N^{α}-Acetyl-lysine and -ornithine were labelled at the α-position as their N^{ω}-benzyloxycarbonyl derivatives with high efficiency as has already been discussed. Resolution of these products with mould acylase directly afforded the crystalline ω-substituted amino acids in good yield. Mould acylase had previously been used in this way to resolve N^{α}-acetyl-N^{ε}-benzoyl-DL-lysine [22].

Proline is usually resolved by the action of renal amidase on the amide [23]. This enzyme is not available commercially and we have not attempted a preparation. Nevertheless, N-acetyl-DL-proline was converted to radiochemically pure proline amide by a 5-stage procedure without crystallisation of intermediates in overall yield of 61% by a standard method [24].

Resolution of N-acetyl-O-benzyl-DL-serine by mould acylase [25] was inhibited by the impurities introduced by the labelling procedure. O-Benzyl-L-serine failed to crystallise from the digestion mixture either directly or after deproteinisation and concentration, presumably due to these impurities and/or to impurities from the enzyme preparation. Renal acylase I was found to resolve the mixture and use of this much cleaner enzyme preparation allowed isolation of the amino acid after a solvent extraction step with ethyl acetate to remove the bulk of the unhydrolysed acetyl derivative. After a further treatment of the extracted acetyl derivative with acylase to ensure complete digestion (Table 3, expt. 10) and removal of generated amino acid by passage through Dowex 50 (H^{+} form) resin, pure N-acetyl-O-benzyl-D-serine was obtained from which D-serine was recovered in 37% yield by strong acidic hydrolysis. The yield could certainly be improved if fewer mechanical losses were involved. It would probably be advisable to resolve either with a small amount of enzyme to recover O-benzyl-L-serine or with a larger amount of enzyme to recover N-acetyl-O-benzyl-D-serine directly by column chromatography and not to attempt to isolate both products from one single experiment.

This situation may not hold, however, when scaling up the reaction eases the mechanical problems involved. It is interesting to note that Dowex 50 (H^+ form) resin retains a large amount of the acetylbenzylserine applied if eluted with water. Elution with 6% acetic acid was necessary to effect complete elution. The same effect was observed with mixtures of phenylalanine and *N*-acetylphenylalanine and is presumably due to adsorption effects between the aromatic residues and the aromatic matrix of the resin. The effect was not reported by Baker and Sober [(26)] who recommended the use of such resins for small-scale separations of enzymic digests. Recovery of D-amino acid in this way would be possible for all other amino acids examined.

Labelled *N*-acetyltryptophan was resolved by use of mould acylase [(27)]. Due to the impurity of the enzyme preparation, which hampered isolation of the amino acid, a small amount of enzyme and a prolonged digestion time had to be used (Table 3, expt. 11).

Tyrosine was recovered in excellent yield *via* the *N*-acetyl compound which was generated by saponification of diacetyltyrosine but not isolated. Mould acylase has been used previously to resolve *N*-chloroacetyltyrosine [(28)] but not the *N*-acetyl derivative.

The remaining *N*-acetyl derivatives, namely of methionine, phenylalanine and valine, were labelled and resolved with renal acylase I without difficulty under standard conditions [(14)]. Acetylglycine was labelled similarly and the amino acid recovered by acidic hydrolysis.

It cannot be assumed that scaling up the amounts of materials used in the foregoing experiments will give results comparable to those reported here. The problem of raising larger volumes of solution quickly in temperature to ensure maximum equilibration of label will increase the reaction time necessary as the volume handled is increased. As already mentioned, it is to be anticipated that working on a larger scale should show the advantage of higher yields of end-product as mechanical losses should become less.

The amino-acids recovered by enzymic resolution were examined for stereochemical purity by digestion with L-amino acid oxidase (or D-amino acid oxidase in the case of D-serine) and were seen to be totally susceptible to oxidation. Moreover, significant amounts of activity did not appear on the TLC strips at positions different to the amino acids after digestion. This indicates that, within the limits of detection (0.01%), isotope is localised wholly at the α-position of the amino acid in every case. Isotope at the α-position is released by the enzyme into the solvent and is lost from the thin-layer by evaporation during the experimental procedure.

L-Glutamic acid γ-benzyl ester, N^{im}-benzyl-L-histidine, N^{δ}-benzyloxycarbonyl-L-ornithine and *O*-benzyl-L-serine were all seen to be totally digested by the enzyme although it is known that glutamic acid, ornithine and serine are totally resistant and histidine only slowly attacked. These results demonstrate the low specificity of the enzyme given the one condition that the side-chain of the L-amino acid contains no ionisable group. L-Methionine gave

methionine sulphoxide to the extent of about 10% of the starting material when treated with the oxidase in the presence of oxygen, thus invalidating the analysis. Parikh *et al.* [15] did not mention this side-reaction in their study. Accordingly, methionine was examined by the method of Manning and Moore [16] which involves formation of the L-leucyl dipeptide by treatment of the amino acid with L-leucine *N*-carboxyanhydride and examination of the product on the amino acid analyser. L-Leucyl-D-methionine was not detectable in the sample. It was thought desirable to analyse D-serine by the same procedure as the preparation of this amino acid in the optically pure form is, unlike all the other preparations described, dependent on the absolute completion of an enzymic digestion and the total removal of L-amino acid. The D-serine was not contamined with the L-acid.

The L-amino acids as recovered sometimes contained small amounts (~1%) of radiochemical impurity which in all cases was *N*-acetyl-D-amino acid (Table 3). This was not considered a serious matter because, as all amino acids are intended for synthetic use, subsequent derivatisation will allow purification at a later stage. The inevitable loss of material that would accompany purification by recrystallisation of the amino acids would also not justify the gain in purity.

The nature of the derivatives of the polyfunctional amino acids made was largely determined during the course of the study by the fact that commercially available mould acylase is very impure. This requires that the deacetylation product of the acetylamino acid should be insoluble in water, thereby avoiding the difficulty of separating the amino acid from the low molecular weight material in the enzyme preparation. This was possible for all amino acids for which the enzyme was used with the exception of tryptophan. In this case, the difficulties of isolation were overcome by using a smaller amount of enzyme and an extended digestion time.

Although acid-stable protecting groups, e.g. benzyloxycarbonyl for side chain amino protection, have been used in this study, the mildness of the labelling procedure would be expected to permit the use of the acid-labile [29] t-butyloxycarbonyl group.

The tritium-labelling method for determining the C-terminal amino acids in peptides proposed by Matsuo *et al.* [1] was briefly investigated on account of its obvious connection with the present study. Matsuo and his co-workers have reported various methods of labelling *via* the oxazolone depending on the individual amino acid which is C-terminal. This approach rather limits the usefulness of the technique except as a confirmatory method. Holcomb, James and Ward [30] investigated some aspects of Matsuo's work and levelled certain criticisms of which the most significant was that under specific conditions non-C-terminal glutamic and aspartic acid residues are labelled whereas glutamine and asparagine are not. We were able to extend this result by demonstrating this effect under different conditions using the two synthetic peptides Hypertensin and Synacthen. Our main interest in the Matsuo tech-

nique was to investigate the "acid catalysed" procedure which is obligatory for a peptide bearing C-terminal proline (i.e. Synacthen) in the light of the results obtained in the present study. Accordingly, Synacthen and Hypertensin were labelled by the mildest conditions known to produce complete equilibration of label between *N*-acetylproline and solvent. In both peptides, histidine was heavily labelled, a finding not reported by Holcomb and co-workers and only recently acknowledged by Matsuo [31]. In our hands, racemisation labelling of N^{α}-acetyl-N^{im}-benzylhistidine introduced label exclusively at the α-position. Plentl and Kelly [6] showed that 92 % of total label entered the α-position on racemisation treatment of N^{α}-acetylhistidine while Matsuo's group has claimed total exchange of all protons under such conditions [31].

Synacthen was labelled heavily at proline, glutamic acid, glycine, methionine, tyrosine, and histidine. With Hypertensin, in contrast, tyrosine was not labelled. Holcomb *et al.* [30] reported a low non-specific incorporation of isotope into all the basic amino acids irrespective of the labelling method. We were unable to detect any labelling of lysine or arginine by our method of analysis which is several orders of magnitude more sensitive than those of the Matsuo or Holcomb groups, while small amounts of labelling were detectable in other amino acids (Tables 4 and 5). The mechanism by which glycine, methionine, tyrosine and histidine in Synacthen incorporate isotope is not evident from consideration of previous reports or by inspection of the structure of Synacthen.

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