508. *Isotope Dilution Analysis of Amino-acids. Part I. A Check on the Absence of Racemisation during Benzyloxycarbonylation.*

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Reverse isotope dilution analysis is shown to provide a convenient method for determining the optical purity of L-amino-acids. Use of this technique has confirmed the absence of racemisation during acylation of nine monobasic amino-acids with benzyloxycarbonyl chlorides. The preparation of several 4-bromobenzyloxycarbonyl derivatives is described.

Reverse isotope dilution analysis is an established method [1] for the determination of small amounts of radioactive compounds in the presence of larger quantities of other radioactive material, but its application to the study of optical enantiomorphs has received less attention, even though its potential value in this field was demonstrated [2] more than twenty years ago. The success of the method depends on the observation that recrystallisation of mixtures of enantiomorphs present in unequal amounts may give rise to crystals having a different composition from the original mixture and, in particular, that when a large excess of one enantiomorph is present repeated recrystallisation tends to give the

[1] Catch, "Carbon-14 Compounds," Butterworths Scientific Publns., London, 1961, Chapter 6.
[2] Rittenberg and Foster, *J. Biol. Chem.*, 1940, **133**, 737.

optically pure form. Thus it is possible to " resolve " isotopically labelled DL-amino-acids [3,4] or their derivatives [5] by adding an excess of radiochemically inactive optically pure carrier and recrystallising this mixture to constant specific radioactivity (which should be half that resulting from a similar dilution with DL-carrier).

The dangers inherent in the use of isotopically labelled L-amino-acids prepared by such procedures have been noted by Arnstein and his co-workers,[6] who point out that the radiochemical purity is always less than the optical purity by an amount given by the initial dilution factor, which is defined as the weight of L-amino-acid (carrier and labelled) divided by the weight of D-amino-acid (labelled). These workers diluted DL-[^{14}C]lysine with five times its weight of carrier L-lysine (dilution factor = 11). After this carrier had been recrystallised to constant specific radioactivity it was found by independent methods that about 10% of this activity was due to the D-enantiomorph. However, a control dilution analysis with DL-lysine as carrier would have detected this contamination, and it is probable that the low dilution factor made the separation difficult. It appears that dilution factors of at least 100 are necessary to provide a reliable method of separation; under these conditions tracer-level L-amino-acids are readily prepared with >99% radiochemical purity.[7]

The principle illustrated above also provides the most sensitive method available for determining small amounts of D-amino-acid in its L-form, or the reverse. The amino-acid may itself be isotopically labelled or it may be converted into an isotopically labelled derivative. For example, Bayly [4] used the former method to study the racemisation of amino-acids in alkali or strong acids, and Keston, Udenfriend, and Cannan [8] showed by the use of *p*-[^{131}I]iodobenzenesulphonyl derivatives that β-lactoglobulin contains less than 0·01% of D-alanine.

The present paper describes some initial experiments undertaken to ascertain the scope and reliability of these techniques.

Nine ^{14}C-labelled nominally L-amino-acids were diluted with various quantities of their respective D-carriers and recrystallised to constant specific radioactivity. With certain amino-acids, notably aspartic and glutamic acid, the last traces of labile radioactivity were difficult to remove by direct crystallisation, although in all cases less than 1% of the total removable activity remained after four recrystallisations. The remaining 1% could be effectively removed by adding inactive " scavenger " L-amino-acids in such amount as to reconstitute the original dilution factor (D/L), followed by four similar recrystallisations. In all cases satisfactory separation was obtained without seeding. With the present method of counting it was possible to take 1 mg. (50 μC) of L-amino-acid and to determine its optical purity to greater than 99·9% purity. A reproducibility of ±0·2% was achieved when detectable amounts of radioactivity remained as D-amino-acid (see Tables). These values do not represent the limit of the sensitivity of this method, for by using liquid scintillation-counting techniques and purified amino-acids substantial improvements can be obtained.[9] In addition to the limits set by the specific radioactivity of the labelled compound, and the counting efficiency and background radiation, which are encountered in all tracer studies,[10] the sensitivity is also inversely proportional to the initial dilution factor. Increased sensitivity could have been attained during the present work by using lower dilution factors, but the possibility of co-crystallisation of D- and L-forms would have been greatly increased (see above). The above results appear to be

[3] Weinhouse and Millington, *J. Biol. Chem.*, 1948, **175**, 995; Kögl, Halberstadt, and Barendregt, *Rec. Trav. chim.*, 1949, **68**, 387.
[4] Bayly, Conference on the Use of Radioisotopes in the Physical Sciences and Industry, Copenhagen, 1960.
[5] Wood and Gutmann, *J. Biol. Chem.*, 1949, **179**, 535.
[6] Arnstein, Hunter, Muir, and Neuberger, *J.*, 1952, 1329.
[7] Unpublished work at the Radiochemical Centre.
[8] Keston, Udenfriend, and Cannan, *J. Amer. Chem. Soc.*, 1946, **68**, 1390; 1949, **71**, 249.
[9] Waterfield, unpublished results.
[10] Cf. Whitehead, *Biochem. J.*, 1958, **68**, 662.

reliable, although a discrepancy was found on checking the value of 2·4% of D-[^{14}C]alanine obtained for one sample of L-[^{14}C]alanine. Treatment with hog-kidney D-amino-acid oxidase gave a chemical content of 1% of D-alanine, and subsequent isotope dilution analysis indicated that the sample so treated then contained less than 0·1% of D-[^{14}C]-alanine. This [^{14}C]alanine had not been diluted with inactive L-alanine carrier during its isolation from uniformly labelled *Chlorella* [^{14}C]protein.

In any study of the purity of optical enantiomorphs by use of labelled derivatives it is essential to ensure that no racemisation occurs during their formation. Details of the micropreparation of labelled *p*-iodophenylsulphonyl,[8] acetyl,[10] and 2,4-dinitrophenyl [11] derivatives of amino-acids are available, but these compounds were not ideal for the present study. It was preferable to use benzyloxycarbonyl derivatives which are known to be formed without detectable racemisation.[12] We have confirmed this observation by using labelled L-amino-acids as a preliminary to the preparation of optically pure benzyloxycarbonyl derivatives from labelled benzyloxycarbonyl chloride. The amino-acids were acylated in high radiochemical yield (see Table 2) when treated with benzyloxycarbonyl chloride or substituted benzyloxycarbonyl chlorides in aqueous sodium hydrogen carbonate by the method of Greenstein and Winitz.[13] The protecting groups were then removed by catalytic hydrogenation under normal conditions, and the recovered amino-acids were analysed for D-content by reverse isotope dilution analysis with D-carrier. The radiochemical yield at each stage was determined by dilution analysis with the appropriate DL-carrier. No racemisation was detected within the limits of experimental error (±0·2%).

Classical methods for the study of racemisation normally involve purification by crystallisation before determination of optical rotation. When the ratio of enantiomorphs in a mixture is large this can clearly lead to error. Isotope dilution analysis is free from this danger and can thus provide a reliable general method for the quantitative investigation of racemisation in all types of compound. In the particular case of the amino-acids, established microbiological procedures are available for the determination of optical purity. However, isotope dilution analysis of labelled derivatives can be used when no suitable enzyme is available, and it requires much less substrate for the same degree of accuracy. Some progress has been made in this direction and the results obtained by labelling inactive L-amino-acids with [^{14}C]benzyloxycarbonyl chloride are comparable with those described above.[9]

For the successful application of isotope dilution techniques to amino-acid derivatives these must be capable of repeated recrystallisation on a semimicro-scale, and this necessitates relatively high melting points. In the present work it was found that the melting points of some benzyloxycarbonylamino-acids were too low to allow repeated recrystallisation with ease, and 4-nitrobenzyloxycarbonyl and 4-bromobenzyloxycarbonyl derivatives were preferable. Carpenter and Gish [14,15] have shown that 4-nitrobenzyloxycarbonyl derivatives of all the common amino-acids have substantially higher melting points than the corresponding benzyloxycarbonyl compounds; however, the use of such derivatives labelled with carbon-14 or tritium could lead to measurement problems, since the nitro-group has a quenching effect in liquid scintillants.[16] Channing, Turner, and Young [17] reported that the 4-bromo-derivatives crystallised with greater ease than the unsubstituted materials, and further work [18] has shown that the melting points of several 4-bromobenzyloxycarbonyl-L-amino-acids are higher than those of the corresponding

[11] Whitehead, *Biochem. J.*, 1961, **80**, 35P; Zahn, Weigmann, and Nischwitz, *Colloid Z.*, 1961, **179**, 49.
[12] Bergmann and Zervas, *Ber.*, 1932, **65**, 1192.
[13] Greenstein and Winitz, "Chemistry of the Amino-Acids," John Wiley and Sons, Inc., New York, 1961, Vol. II, 891.
[14] Carpenter and Gish, *J. Amer. Chem. Soc.*, 1952, **74**, 3818.
[15] Gish and Carpenter, *J. Amer. Chem. Soc.*, 1953, **75**, 950.
[16] Helf and White, *Analyt. Chem.*, 1957, **29**, 13.
[17] Channing, Turner, and Young, *Nature*, 1951, **167**, 487.
[18] G. T. Young, personal communication.

benzyloxycarbonyl derivatives. The present work has confirmed these observations. 4-Bromobenzyloxycarbonyl chloride has been obtained pure and used to prepare highly crystalline derivatives of DL-leucine, DL-methionine, DL-phenylalanine, and DL-valine. These were obtained in lower yield than the corresponding 4-nitro-derivatives [16] but are well suited for use as carriers in isotope dilution analysis.

Experimental

M. p.s were observed on a Kofler block. Analytical-reagent grade solvents were used throughout. Carbon-14 samples were measured with an end-window Geiger counter as layers of barium [^{14}C]carbonate of infinite thickness, formed by burning the samples in oxygen and absorbing the [^{14}C]carbon dioxide in saturated aqueous barium hydroxide. Tritium samples were burnt in oxygen and measured as water by scintillation counting; the scintillant was a mixture of equal volumes of xylene, dioxan, and ethanol, containing naphthalene 8%, 2,5-diphenyloxazole 0·5%, and 2,2′-*p*-phenylenedi-(5-phenyloxazole) 0·05%. ^{14}C-Labelled amino-acids were obtained from *Chlorella* [^{14}C]protein by hydrolysis with 6N-hydrochloric acid at 120° (bath) for 48 hr., and separated by ion-exchange chromatography. They were purified by paper chromatography and crystallisation, and had been stored as freeze-dried solids in sealed, evacuated, glass ampoules for from 1 to 3 years. [^{3}H]Methionine was prepared by platinum-catalysed exchange [7] with tritiated water, and purified by repeated crystallisation. All optically active carrier amino-acids had satisfactory specific rotations.

4-Nitrobenzyloxycarbonyl Chloride.—Freshly recrystallised 4-nitrobenzyl alcohol (4 g.) was treated with carbonyl chloride (12 g.) in toluene (100 ml.) and kept at 60° for 7 hr. The solvent was distilled off *in vacuo* at 60° and the residue treated with toluene (10 ml.) and kept at 0° for 30 min. The unused 4-nitrobenzyl alcohol (1 g.) was filtered off and the filtrate was treated with light petroleum (b. p. 40—60°; 30 ml.) and kept at 0° overnight. The solution was decanted from the gummy precipitate, and light petroleum (100 ml.) was added. Crystallisation at −50° gave 4-nitrobenzyloxycarbonyl chloride (2·7 g., 65%), m. p. 32—33° (lit.,[14] 33·5—34°).

4-Bromobenzyloxycarbonyl Chloride.—A solution of 4-bromobenzyl alcohol (3·7 g.) in dioxan (5 ml.) was added to one of carbonyl chloride (2·5 g., excess) in toluene (20 ml.) and kept at room temperature for 24 hr. The solvent was distilled off *in vacuo* at 60°, leaving *4-bromobenzyl-carbonyl chloride* as a pale straw-coloured oil. Three crystallisations from hexane at −50° gave the product (3·9 g., 78%) as colourless needles, m. p. 12°, n_D^{20} 1·5588 (Found: C, 39·2: H, 2·7. $C_8H_6BrClO_2$ requires C, 38·5; H, 2·4%).

*Benzyloxycarbonyl- and 4-Nitrobenzyloxycarbonyl-*DL*-amino-acids.*—Standard procedures were used to prepare benzyloxycarbonyl-DL-glutamic acid,[12] m. p. 123°, -DL-aspartic acid,[13] m. p. 115—116°, and -DL-serine,[13] m. p. 125—125·5°, and 4-nitrobenzyloxycarbonyl-DL-alanine,[15] m. p. 132°, and -DL-threonine,[15] m. p. 124° (lit.,[15] 141·5—143°) (Found: C, 48·2; H, 4·7; N, 9·5. Calc. for $C_{12}H_{14}N_2O_7$: C, 48·3; H, 4·7; N, 9·4%).

*4-Bromobenzyloxycarbonyl-*DL*-valine.*—To a solution of DL-valine (0·285 g.) and sodium hydrogen carbonate (0·8 g.) in water (10 ml.) was added a solution of 4-bromobenzyloxycarbonyl chloride (0·7 g.) in dioxan (3 ml.). The mixture was vigorously shaken at room temperature for 3 hr., water (10 ml.) was added, and the solution washed with chloroform (10 ml.). Acidification with 6N-hydrochloric acid liberated an oil which was extracted with ethyl acetate (2 × 15 ml.). The combined extracts were dried (Na_2SO_4) and evaporated, giving *4-bromobenzyloxycarbonyl-*DL*-valine* (0·6 g., 74%) which solidified on trituration with hexane. The product crystallised from ethyl acetate–hexane as colourless prisms, m. p. 116·5—117·5° (Found: C, 47·5; H, 4·8; N, 4·6. $C_{13}H_{16}BrNO_4$ requires C, 47·3; H, 4·9; N, 4·2%).

*4-Bromobenzyloxycarbonyl-*DL*-methionine.*—DL-Methionine similarly gave *4-bromobenzyloxy-carbonyl-*DL*-methionine* (81%), prisms (from ethyl acetate–hexane), m. p. 129—130° (Found: C, 43·2; H, 4·8; N, 3·8. $C_{13}H_{15}BrNO_4S$ requires C, 43·1; H, 4·45; N, 3·9%).

*4-Bromobenzyloxycarbonyl-*DL*-leucine.*—Similar acylation of DL-leucine for 6 hr. gave *4-bromobenzyloxycarbonyl-*DL*-leucine* (54%), prisms (from benzene–hexane), m. p. 76—78° (Found: C, 49·1; H, 5·5; N, 4·55. $C_{14}H_{18}BrNO_4$ requires C, 48·8; H, 5·3; N, 4·1%).

*4-Bromobenzyloxycarbonyl-*DL*-phenylalanine.*—DL-Phenylalanine similarly gave *4-bromobenzyloxycarbonyl-*DL*-phenylalanine* (55%), needles (from ethyl acetate–hexane), m. p. 147° (Found: C, 54·3; H, 4·4; N, 3·8. $C_{17}H_6BrNO_4$ requires C, 54·0; H, 4·3; N, 3·7%).

Determination of the D-*Content of Isotopically Labelled Amino-acids.*—To the L-amino-acid (0·3—1·5 mg., *ca.* 50 μc) contained in a filter-beaker was added a weighed amount of the D-amino-acid carrier (150—250 mg.). The carrier was dissolved in the minimum amount of water, and hot ethanol was added to incipient crystallisation. The mixture was kept for 1 hr. at 0° and filtered. This crystallisation process was repeated until the carrier acquired constant specific radioactivity, as determined by analysis of a sample of the carrier at suitable intervals. Small amounts of " scavenger " L-amino-acid were added when necessary (see p. 2732). Some typical results are given in Table 1.

TABLE 1.

Behaviour of carrier D-amino-acids on recrystallisation.

L-Amino-acid	Wt. (mg.) of L-amino-acid	Initial dilution factor	Radioactivity (%) remaining after recrystn. no. 3	4	6	8	12	18
[^{14}C]Alanine	0·6	380	0·9		0·25 *	<0·1		
[^{14}C]Aspartic †	1·6	95	3·7		3·1		2·6	2·6
[^{14}C]Aspartic †	1·8	115		2·5 *		2·2	2·2	2·25
[^{14}C]Glutamic	2·1	120	2·3		1·1 *	1·0		
[^{14}C]Leucine	0·9	225		1·6		1·3	1·25	
[^{3}H]Methionine	0·15	1250		0·7 *		0·7		
[^{14}C]Phenylalanine ...	1·0	120		1·6		0·5 *	0·5	
[^{14}C]Serine	0·3	460		2·4		1·6	1·6	
[^{14}C]Threonine	1·4	140		0·3		<0·1		
[^{14}C]Valine	0·5	290		0·2		<0·2		

* " Scavenger " added at this stage. † Same batch.

Acylation of Isotopically Labelled L-*Amino-acids.*—*Benzyloxycarbonylation of* L-*serine.* Uniformly labelled [^{14}C]serine (0·55 mg., 91 μc), containing 1·6% of D-[^{14}C]serine as determined by dilution analysis with D-carrier, in water (1 ml.), was placed in a hard-glass test-tube (6″ × $\frac{5}{8}$″) together with sodium hydrogen carbonate (10 mg.) and benzyloxycarbonyl chloride (5 mg.). The mixture was shaken for 2 hr. at room temperature, diluted with water (0·5 ml.) and washed with hexane (2 × 1 ml.). 2N-Hydrochloric acid was added to pH 1, and the solution was extracted with ethyl acetate (5 × 1 ml.). A negligible amount of activity was contained in the fifth extract. The extracts were combined, dried (Na_2SO_4), and quantitatively filtered, giving 4·55 g. of solution. Carrier benzyloxycarbonyl-DL-serine (62 mg.) was added to a portion (168 mg.) of this solution. After two recrystallisations from ethyl acetate–hexane the carrier had a specific activity of 10·4 μc/mmole, giving the radiochemical yield of benzyloxycarbonylserine as 72 μc (79%). The remaining ethyl acetate solution was evaporated in a stream of nitrogen, and the residue treated with methanol (3 ml.) and 5% palladised charcoal (5 mg.). Hydrogen was slowly bubbled through the mixture for 90 min., water (3 ml.) was added, the solution was filtered, and the hydrogenation vessel and catalyst washed with water. Carrier DL-serine (65 mg.) was added to a portion (306 mg.) of the combined filtrate and washings (6·71 g.). After two recrystallisations from aqueous ethanol the carrier had a specific activity of 5·0 μc/mmole, indicating that the serine had been regenerated from its benzyloxycarbonyl derivative in 98% radiochemical yield. Carrier D-serine (184 mg.) was added to the remaining aqueous solution (containing 65 μc of serine) and recrystallised to a constant specific activity of 0·52 μc/mmole (1·4% of that of the original homogenised sample) as described previously. The above method was used to study the reaction of benzyloxycarbonyl chloride with [^{14}C]-aspartic and -glutamic acid, and the reaction of 4-bromobenzyloxycarbonyl chloride with [^{14}C]-leucine, -phenylalanine, and -valine, and with tritium-labelled methionine. The results are summarised in Table 2.

4-Nitrobenzyloxycarbonylation of L-*Alanine.*—To a solution of uniformly labelled L-[^{14}C]-alanine (0·55 mg., 94 μc) containing less than 0·1% of D-[^{14}C]alanine, and sodium hydrogen carbonate (10 mg.) in water (1·5 ml.) was added a solution of 4-nitrobenzyloxycarbonyl chloride (5 mg.) in dioxan (0·3 ml.). The mixture was shaken for 2 hr. at room temperature, diluted with water (1 ml.), and washed with ethyl acetate (2 × 1 ml.). After acidification to pH 1 with 2N-hydrochloric acid the procedure was identical with that described for serine. The results of this analysis and of a similar analysis of L-[^{14}C]threonine are given in Table 2.

TABLE 2.

Reaction of isotopically labelled amino-acids with benzyloxycarbonyl chlorides.

Amino-acid	Specific activity (mc/mmole)	Activity (μc) used for reaction	4-Subst.	Yield (%)	Yield (%) after hydrogn.	D- (%) before acylation	D- (%) after acylation and hydrogn.
[^{14}C]Alanine	15·2	99	NO_2	91	95	2·4	2·5
[^{14}C]Alanine *	15·2	94	NO_2	93	94	<0·1	<0·1
[^{14}C]Aspartic acid...	22·5	98	H	79	98	0·3	0·2
[^{14}C]Glutamic acid	5·5	103	H	78	102	1·0	0·9
[^{14}C]Leucine	7·2	86	Br	74	93	1·1	1·25
[^{3}H]Methionine ...	36·0	300	Br	67	95	0·6	0·7
[^{14}C]Phenylalanine	10·6	70	Br	78	97	0·5	0·7
[^{14}C]Serine	17·0	91	H	79	98	1·6	1·4
[^{14}C]Threonine	3·9	102	NO_2	94	97	<0·1	<0·1
[^{14}C]Valine	6·5	63	Br	73	102	<0·2	<0·2

* Preceding sample after treatment with hog-kidney D-amino-acid oxidase and purification by ion-exchange chromatography.

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