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PREPARATION OF DL-VALINE-4,4'-C_2^{14}, DL-NORVALINE-3-C^{14}, DL-LEUCINE-3-C^{14} DL-NORLEUCINE-3-C^{14}

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PREPARATION OF DL-VALINE-4,4'-C_2^{14}, DL-NORVALINE-3-C^{14}, DL-LEUCINE-3-C^{14}, DL-NORLEUCINE-3-C^{14} *

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ABSTRACT

1) DL-valine-4,4'-C_2^{14}, DL-norvaline-3-C^{14}, DL-leucine-3-C^{14} and DL-norleucine-3-C^{14} have been prepared with high specific activity.

2) The appearance of lower molecular weight amino acids and other by-products was observed in the course of these preparations. Some possibilities as to their origins and identitites are discussed.

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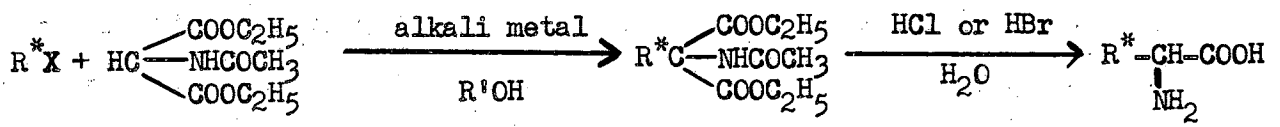
Introduction:

In a continuation of previous work (1,2) we have undertaken the preparation of valine-4,4'-C_2^{14}, norvaline-3-C^{14}, leucine-3-C^{14} and norleucine-3-C^{14}. The method of synthesis as represented by the following scheme was chosen because of its wide application, the generally high yields of amino acids reported by this method (3,4,5,6,7) and the availability of the required labeled halides in this laboratory (8,9):



$$R^*X + HC\begin{cases}COOC_2H_5\\NHCOCH_3\\COOC_2H_5\end{cases} \xrightarrow[R'OH]{\text{alkali metal}} R^*C\begin{cases}COOC_2H_5\\NHCOCH_3\\COOC_2H_5\end{cases} \xrightarrow[H_2O]{HCl \text{ or } HBr} R^*\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}COOH$$

$$+ CO_2 + 2\ C_2H_5OH + CH_3COOH$$

The following preparations of these isotopically labeled amino acids have been reported: DL-valine-1-C^{14} and DL-leucine-1-C^{14} by the Strecker method from $HC^{14}N$ (10,11); DL-leucine-2-C^{14} from isocaproic acid-2-C^{14} (12), DL-valine-4,4'-C_2^{13} from $C^{13}H_3I$ and diethyl-ethylidene-malonate (13) and uniformly labeled DL-valine and DL-leucine by microbiological methods (17). DL-leucine-3-C^{14} and DL-leucine-4-C^{14} have been prepared (15) in 42% yield by the same method as is described in this paper. No reports on the preparation of C^{14}-labeled norvaline or norleucine have come to the author's attention.

Experimental

1) Norvaline-3-C^{14} hydrochloride:

The preparation of norvaline-3-C^{14} will be described in detail as an example of the procedures used:

n-Propyl-1-C^{14}-iodide was prepared according to the following scheme (8):

$$CH_3CH_2C^*OOH \xrightarrow[10\%\ Ni(OH)_2]{90\%\ Cd(OH)_2} (CH_3CH_2C^*OO)\begin{cases}90\%\ Cd\\10\%\ Ni\end{cases} \xrightarrow[\substack{CuCrO\\ \Delta}]{H_2}$$

$$CH_3CH_2C^*H_2OH \xrightarrow[I_2]{P_4} CH_3CH_2C^*H_2I.$$

30 millimoles of diethylacetamidomalonate (Winthrop-Stearns, C.P. recrystallized twice from hot water, dried) was dissolved in a solution of 17 millimoles of potassium in 25 cc. of tertiary butylalcohol (dried by distillation from sodium). This mixture was placed in a long Carius bomb-tube fitted with a stopcock and a water condenser. After the tube was frozen and evacuated, 16.6 millimoles of n-propyl-1-C^{14}-iodide containing 34.9 mc. were added on a vacuum manifold and the mixture was then refluxed for four hours on a steambath. (Previous experiments had shown this time to give optimum yields of the condensation product.) The salt was removed by centrifugation and the solvent distilled off under vacuum, yielding 76% of the condensation product:

$$CH_3CH_2C^*H_2 - C\begin{cases}COOC_2H_5\\NHCOCH_3,\\COOC_2H_5\end{cases}$$ containing 25.4 mc. (73%)

The product was hydrolized and decarboxylated by refluxing it with saturated aqueous hydrogen bromide for 72 hours. (In subsequent preparations concentrated hydrochloric acid was used, giving similar yields and a somewhat purer product.) The solution was distilled to dryness <u>in vacuo</u> and traces of acid were removed by adding three small portions of water and distilling to dryness.

A sample of the product so obtained was subjected to paper chromatography using water-saturated phenol and n-butanol-propionic acid-water (2:1:1.4) solvents (16). The paper was radioautographed and the radioactive

spots counted with a Scott G. M. tube and then sprayed with 0.1% ninhydrin in 95% ethanol. The product proved to consist of 94% norvaline, 0.5% radioactive alanine, 1% of a radioactive compound whose chromatographic behavior indicates it to be α-amino-butyric acid (it will be referred to as "α-aminobutyric acid", since no other tests have been applied to establish its identity), 0.2% and 5% of two unknown, ninhydrin-negative, radioactive compounds (R_f's (Phenol) 0.49 and 0.90, R_f's (Butanol-Prop.) 0.70 and 0.80, respectively), and non-radioactive glycine. The origin of these contaminants will be discussed below.

The norvaline was purified by fractional elution from an ion-exchange column: industrial glass pipe (2.5 cm. in diameter, 50 cm. long) filled with 305 cc. of Dowex 50 (250-500 mesh; in the hydrogen form) to a height of 44 cm. was used. Ordinary pyrex tubing was not used as it is often cracked by the expanding resin. After the sample had been applied, the column was washed with 2 l. of water. This effluent contained 33 μc. (0.3% of the total activity). The amino acids were then eluted with 0.25 N hydrochloric acid at a flow rate of 80 cc./hr. or 0.28 cm./min.; every fifth fraction (about 20 cc. per fraction) was counted and chromatographed on paper with butanol-propionic acid-water solvent. (In the other amino acid preparations the column dimensions, acid strength and flow rates were changed as described later.) For details on this ion-exchange purification (and for those of the following amino acid preparations) see Table I.

The yield of norvaline·HCl was 1.83 gr. (74% of theor.) with a spec. activity of 14.4 μc/mg. (theor. 14.0 μc/mg.), a total of 29.5 mc. (75% of theor.). Paper chromatography showed that this fraction contained about 2% of radioactive impurities: 1.2% of an unknown [R_f (Phenol) 0.72, R_f (Butanol-Propionic) 0.62] and 0.4% each of alanine and "α-aminobutyric acid". A chlorine analysis of the norvaline hydrochloride showed 24.6% Cl (theor. 23.0%).

2) Valine-4,4'-C_2^{14} hydrochloride

(My thanks are due Mrs. P. Adams for the preparation of this compound.)

Isopropyl-2,2'-C_2^{14}-iodide was prepared according to the following scheme (18):

$$C^*O_2 \longrightarrow (C^*H_3COO)_2\text{-}Ba \xrightarrow{\Delta} C^*H_3 - \overset{O}{\overset{\|}{C}} - C^*H_3 \xrightarrow[3)\ P_4,\ I_2]{1)\ H_2;\ 2)\ \text{Copper chromite}}$$

C^*H_3-CHI-C^*H_3 in 75% overall yield.

13.2 millimoles of isopropyl-2,2'-C_2^{14}-iodide containing 11.1 mc. were condensed with 26 millimoles of diethylacetamidomalonate in 20 cc. tertiary butanol, containing 13.2 millimoles potassium tertiary butoxide. The condensation was carried out in a three-neck flask fitted with a stirrer, reflux condenser and dropping funnel through which the halide was slowly added to the reaction mixture. After the addition the mixture was refluxed for 24 hours. After hydrolysis and decarboxylation with concentrated hydrochloric acid, the crude amino acid mixture (8.2 mc., 73% of the initial activity) was found to contain valine, non-radioactive glycine, small amounts of radioactive alanine, "α-aminobutyric acid" and less than 3% of a ninhydrin negative, radioactive unknown compound.

The product was purified on a Dowex 50 column (2.5 cm. in diameter, 160 cm. long) filled with 700 cc. resin (100-200 mesh) with a capacity of 2100 meq. After the amino acid had been applied and washed with 1500 cc. of water (this effluent contained 2.1 μc.) they were eluted with 1 N hydrochloric acid at a flow rate of 20 cc./hour (0.07 cm./m.).

The hydrochloric acid solution of the fractions containing pure valine was distilled to dryness, dissolved in water, evaporated to dryness

on a steam bath and dried *in vacuo*. The dry valine-4,4'-C_2^{14} hydrochloride weighed 1.07 g. (54% of theoretical) and had a specific activity of 6.0 μc/mg. (theory: 5.5 μc/mg.). The discrepancy in specific activity is within the limits of accuracy of the assay of the isopropyl iodide. The yield of pure valine was 6.41 mc. (58%). Two-dimensional paper chromatographic analysis showed the valine to be completely free from radioactive and amino acid contamination.

An additional 1.73 mc. of valine (15%) contaminated by a trace (less than 1%) of "amino butyric acid" was isolated from the earlier elution fractions.

3) Norleucine-3-C^{14}

n-Butyl-1-C^{14}-bromide was prepared in a manner analogous to that used for n-propyl-iodide (8). Two batches of n-butyl-bromide-1-C^{14}, 6.6 milimoles containing 13.8 mc. and 8.5 millimoles containing 20.7 mc., respectively, were separately condensed with diethylacetaminomalonate and hydrolized to the amino acids as described for norvaline. These crude hydrolysis products representing 62% and 71% of the initial activity were pooled for purification. This pool contained non-radioactive glycine, traces of radioactive alanine, "α-aminobutyric acid" and an amino acid whose chromatographic behavior indicates it to be valine (in absence of more rigorous tests for identity it will be referred to as "valine") and about 2% of an unknown radioactive, ninhydrin negative compound (R_f (Phenol) 0.83, R_f (Butanol-propionic) 0.82).

The ion-exchange column for the separation was 570 cc. of Dowex 50 (250-500 mesh, 2.3 x 116 cm.) in the hydrogen form. The capacity was 1710 meq., loaded with 2% of this capacity. After the sample had been applied, 6 l. water

were passed through the column. The effluent contained 0.01% of the activity. The amino acids were eluted with 1 N hydrochloric acid at a flow rate of 14 ml./hour or 0.05 cm./min. Every fifth fraction (about 15 cc. per fraction) was tested for radioactivity and ninhydrin-sensitive materials. The active fractions were identified by one-dimensional paper chromatography and pooled accordingly. The norleucine hydrochloride so obtained, a gummy, brown material, was converted to the free amino acid by extraction with absolute alcohol and precipitation with gaseous ethylene oxide.

The overall yield of norleucine from the bromide was 62% (2.129 g.) containing 69% of the starting activity (23.80 mc.). The specific activity was 21 μc/mg. The recovery of the activity from the column was quantitative. On a two-dimensional chromatogram the material proved to contain no other α-amino acid and about 2% of the same radioactive, ninhydrin negative compound as mentioned above.

4) Leucine-3-C^{14} hydrochloride

Isobutyl-1-C^{14}-iodide was prepared in a manner analogous to that used for n-propyl iodide (8).

Three batches of halide, 8.8 millimoles containing 11.2 mc., 9.3 millimoles containing 12.1 mc. and 8.5 millimoles containing 5.0 mc., were condensed separately with diethylacetamidomalonate and hydrolized to the amino acid as described for norvaline. The crude hydrolysis products contained 6.7, 6.6 and 2.1 mc., respectively, representing 60%, 54% and 42% of the starting activity. They were pooled for purification. The pool proved to contain non-radioactive glycine, radioactive alanine and "α-aminobutyric acid" (0.2% each), radioactive leucine and a radioactive, ninhydrin negative compound appearing near leucine (R_f values slightly greater than those for leucine). The ion-exchange column for the separation of these compounds was

the same as described for norleucine. The washwater contained 2.2 μc. The fractions containing only leucine and 3% of an unknown were purified by recrystallization from water, after boiling with charcoal. This treatment removed the unknown compound. The total yield of DL-leucine hydrochloride was 2.958 g. (66% of theoretical) with a specific activity of 5.6 μc/mg. The yield on a radioactivity basis is 16.5 mc. (59% of theoretical). The recovery of radioactivity from the ion-exchange column was quantitative.

Discussion

The appearance of several by-products in the course of the condensation of diethylacetamidomalonate with various aliphatic halides in an interesting and previously unreported observation. These products are partly α-amino-acid in nature and partly non-amino acid, as judged by their behavior towards the ninhydrin reagent. Their observation was made possible only by the application of paper chromatography and radioautography. The usual methods for the establishment of purity and identity (Micro-Kjeldahl, C, H analysis, melting points) do not show the small percentages of these contaminants, especially when homologous amino acids are involved (see for instance (15)).

With the exception of glycine, which arises from the excess of diethyl-acetamidomalonate used, the origin of these compounds is obscure. There are several alternatives to account for the presence of these compounds: a) The halides which were used were contaminated with lower molecular weight halides, b) there was some decomposition or rearrangement in the course of the condensation or hydrolysis, or c) there was some decomposition or rearrangement in the course of the chromatographic procedures.

However, the data so far accumulated do not give an unequivocal answer as to the origin of the various by-products. The purity of the

halides is difficult to establish. The absence of contaminants can only be determined within ± 2% when tested by density, refractive index and elementary analysis. Mass spectrography does not tell more because of the lack of standards for comparisons. When carefully purified (fractionally distilled) samples of commercial or radioactive isopropyliodide (pure by these standards) were used for the preparation of valine, very little or no alanine could be seen. Also when two norleucine samples were compared, one made from not fractionally distilled and therefore possibly contaminated radioactive n-butylbromide, the other from the same halide diluted 5 times with commercial C.P. n-butyl bromide, the amount of alanine was very much less in the second case. On the other hand, in the one case when alanine (containing 12% of the starting activity) was separated in weighable amounts from a radioactive leucine preparation, the specific activity of the starting isobutyliodide and of the resulting alanine were identical (1.54×10^7 dis./min./mmole); it is therefore indicated that the alanine is formed from the isobutylbromide and not from a chance contamination with labeled methyl halide.

The data in the case of the "α-aminobutyric acid" are similarly inconclusive. This amino acid was seen in all valine preparations even those where there was no alanine. It was invariably not radioactive with our methods of detection. It is difficult to imagine any pathway from isopropyl-2,2'-C^{14}_2 halide to non-radioactive ethyl halide.

The same "α-aminobutyric acid" was also seen in all preparations of norvaline, leucine and norleucine. It was sometimes radioactive, in other cases it was not. It was usually present in smaller amounts than alanine. In the one case where this amino acid was separated in weighable amounts from a radioactive leucine preparation its specific activity (1.78×10^5 dis./min./mmole) was very much smaller than the specific activity of the isobutyliodide

used (1.53×10^7 dis./min./mmole). It contained about 1.5% of the starting activity. If rearrangements of the halide were to account for these data it would have to be extensive and the splitting would have to occur in several different places to result in radioactive and non-radioactive ethylhalide in order to produce α-aminobutyric acid of low specific activity. On the other hand, to account for the high spec. act. of the alanine one has to assume that the isobutyl iodide is giving only radioactive methylhalide. If chance contamination is the origin of these compounds one would have to assume that ethylhalide is harder to remove than methylhalide, since the valine preparations from highly purified isopropylhalide showed very little or no alanine, but did contain "α-aminobutyric acid".

A radioactive amino acid whose chromatographic behavior strongly indicates that it is valine was seen in the leucine and norleucine preparations. It is noteworthy that the same contaminant is present in commercial DL-leucine and L-leucine. The concentration varied from traces to about 10% of the activity of the main product. Similar by-products were seen in preparations of alanine-3-C^{14} by this method (2) and in preparations of α-amino-butyric acid-3-C^{14} (19).

The origin of the ninhydrin-negative, radioactive compounds with higher R_f in butanol-propionic acid than the main product seems to be connected with the chromatographic procedures. When, for instance, a norleucine spot and the accompanying unknown were cut out from a paper, eluted with water and re-chromatographed separately, the norleucine spot was again accompanied by 5-8% of the unknown while the unknown re-appeared unchanged. Heating of this unknown compound with conc. hydrochloric acid in a sealed tube did not change its chromatographic properties.

Other observations lead one to suspect that the ninhydrin-negative, radioactive spots result from an alteration of the amino acids during chromatography or ion-exchange. For instance, occasionally an amino acid mixture showed a different composition, qualitatively and/or quantitatively, before and after the fractional elution from a Dowex 50 column.

The fact that there appear unexpected by-products in the course of the synthesis of amino acids and that these observations were made possible only by the use of paper chromatography and radioautography seem important enough to present these data, although unfortunately they do not permit us to advance an unambiguous explanation of their origin.

Summary:

1) DL-valine-4,4'-C_2^{14}, DL-norvaline-3-C^{14}, DL-leucine-3-C^{14} and DL-norleucine-3-C^{14} have been prepared with high specific activity.

2) The appearance of lower molecular weight amino acids and other by-products was observed in the course of these preparations. Some possibilities as to their origins and identities are discussed.

Table I

Fractional Elutions of Amino Acid Mixtures from Dowex 50 Ion-exchange Resin Columns

Amino Acid Preparation	cc. 1.0 N HCl	C. C.[1]	μc.	% of Starting[2] Activity	Non-radio-active Components	Radioactive Components[3]
Norvaline[4]	0-5300	0-1.42	440	4	Glycine	Norvaline, alanine, "α-aminobutyric acid", two unknowns[7] (varying proportions).
	5300-8500	1.42-2.30	2.95×10^4	75	—	Norvaline (98%), alanine (0.4%), "α-aminobutyric acid" (0.4%), Unknown[7] (1.2%).
Valine	0-2275	0-1.08	2.1	<0.1	(5)	(5)
	2275-2785	1.08-1.33	28.4	0.3	Glycine	Alanine
	2785-2870	1.33-1.37	—	—	—	—
	2870-3190	1.37-1.52	1.72×10^3	15	—	Valine, "α-aminobutyric acid"[6]
	3190-4745	1.52-2.26	6.41×10^3	58	—	Valine
Norleucine	0-2560	0-1.5	60	0.2	(5)	(5)
	2560-3120	1.5-1.82	12	<0.1	Glycine	Alanine
	3120-3340	1.82-1.90	—	—	—	—
	3340-3750	1.90-2.20	8	<0.1	—	"α-aminobutyric acid"
	3750-5050	2.20-3.0	—	—	—	—
	5050-5600	3.0-3.5	2	<0.1	—	"Valine"
	5600-6540	3.5-3.83	8	<0.1	—	"Valine", norleucine
	6540-10,590	3.83-6.2	2.38×10^4	69	—	Norleucine (98%), unknown[7] (2%)
Leucine[8]	0-1600	0-0.93	0.65	<0.1	Glycine	Traces of various amino acids.
	1600-2930	0.93-1.70	4	<0.1	Glycine	Alanine (75%), leucine (17%).
	2930-3355	1.70-1.95	0.6	<0.1	—	"α-aminobutyric acid" (70%), leucine (25%), unknown[7] (5%).
	3355-4865	1.95-2.85	1.3×10^3	8.4	—	Leucine (92%), unknown[7] (8%), traces "valine".
	4865-8575	2.85-5.89	1.53×10^4	54.0	—	Leucine (97%), unknown[7] (3%).

Footnotes to Table:

(1) $C_o C_o = \frac{\text{meq. of acid passed through the column}}{\text{capacity of column in meq. acid}}$; this expression makes possible a comparison of elutions on columns of different capacities and with different eluants.

(2) Total radioactivity of the halide used in the preparation. This is not identical with the activity applied to the column.

(3) The percentages are the percentages of the radioactivity in the given fraction.

(4) This preparation was eluted with 0.25 N hydrochloric acid.

(5) Was not chromatographed.

(6) Very faintly radioactive.

(7) Gives no color reaction with the ninhydrin reagent.

(8) These pools include the fractions on either side which proved to contain neither radioactivity nor ninhydrin sensitive compounds.

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