Alanine Turnover in the Human: Determination of the Specific Activity (37692)

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Most current methods for the separation and analysis of amino acids, *e.g.*, silica gel chromatography of dinitrophenylated derivatives (1), ion exchange chromatography (Beckman 120 C Amino Acid Analyser), *etc.* (2, 3), are scaled to microquantities. Although the methods are quite precise, the quantity of substrate isolated is frequently insufficient when specific radioactivity analyses are desired.

Our approach to the specific activity assay of alanine employs an initial separation by ion exchange chromatography. Subsequent oxidation of the alanine with ninhydrin yields acetaldehyde, which when bubbled through cold buffered dimedon solution (5,5-dimethyl-1,3-cyclohexanedione), yields an acetaldehyde–dimedon complex (4, 5). This end product, a crystalline solid, is easily assayed by gravimetric means, after which it can be quantitatively solubilized in a liquid scintillation cocktail for radioactivity assay. Preliminary studies with radioactive alanine in the human indicate that the specific activity decreases by two orders of magnitude within 2 hr. The counting rates we obtain from the analysis of 10 ml blood at that time following the administration of 25 μCi of L-[^{14}C]alanine in a single injection are of the order of 80 cpm. The present communication describes this procedure in detail, including a summary description of the isolation for a specific activity assay of two of alanine's metabolic products, lactic acid and glucose.

Materials. Isotopic compounds L-[U-^{14}C]alanine, L-[U-^{14}C]lactic acid, and [U-^{14}C]glucose were obtained from New England Nuclear, Boston, MA. Nonradioactive L-alanine and ninhydrin were obtained from Sigma Chemical Co., St. Louis, MO. Perchloric acid, hydrochloric acid, potassium hydroxide, dibasic sodium phosphate, and sodium acetate were all Baker Chemical, Phillipsburg, NJ. Dimedon was obtained from Eastman Kodak Co., Rochester, NY. Ion exchange resins AG 50 $\times$ 8 H^+ and AG 1 $\times$ 8 Ac^-, both 200–400 mesh, were obtained from Bio Rad Laboratories, Richmond, CA.

Experimental procedure. Sample preparation. The proteins from 10-ml samples of whole blood are precipitated with 7% perchloric acid (80 ml). Following neutralization with 4 *N* KOH, the clear supernatant is brought to pH 2 with 2 *N* HCl, and water is added in a quantity sufficient to bring the final dilution to 100 ml.

Concentration analysis. Alanine was assayed by the enzymatic–fluorometric method described by Young and Lowry (6) with slight modifications. Lactic acid was assayed by a modification of the enzymatic–fluorometric method described by Loomis (7). Glucose was assayed by the enzymatic–spectrometric method employed with Glucostat (Worthington enzymes).

Ion-exchange column. AG 50 $\times$ 8 H is cycled with 2 *N* NaOH and HCl, washed to neutrality with deionized water, and poured to a column of 1.2 $\times$ 15 cm. AG 1 $\times$ 8 Ac is cycled with 2 *N* NaOH, 2 *N* HCl, and NaAc, washed with deionized water, and poured to a column of 1.2 $\times$ 15 cm.

The pH-2 filtrate with carrier alanine added and subsequent washings are applied to the above columns in sequence. Alanine, retained on the AG 50 $\times$ 8 H column, is eluted therefrom with 1 *N* HCl, appearing in the 50–80 ml fraction. The 30 ml 1 *N* HCl

containing alanine is added together with 10 g of $Na_2HPO_4 \cdot 7H_2O$ to a three-neck flask containing a pinch of alundum. N_2 is bubbled through the solution while it is brought to a boil. At this point, a receiving flask containing 15 ml of dimedon solution (4 mg dimedon/ml NaAc, pH 4.25) is connected, and 8 ml ninhydrin solution (ninhydrin 8% in 95% EtOH) is added dropwise to the reaction flask, oxidizing the alanine therein to acetaldehyde.

The reaction is allowed to proceed for 1 hr during which time the receiving flask is kept immersed in ice. At the end of this period, the receiving flask is removed from the apparatus, stoppered, and shaken. Shaking is repeated until a precipitate begins to form, whereupon the flask is left standing overnight at room temperature. The acetaldehyde–dimedon precipitate is filtered and washed five times with 95% ethanol–H_2O (30:70), dried, weighed, dissolved in toluene based liquid scintillation fluid, and counted for ^{14}C activity.

The initial sample volume, after passing the AG 50 × 8 H column, is passed over the AG 1 × 8 Ac column which retains lactic acid. The elution of lactate is achieved with 2 *N* NaAc. The 25–30-ml fraction containing lactate is transferred to the same apparatus employed for the oxidation of alanine and is oxidized with $KMnO_4$ to acetaldehyde, which is then processed in a manner identical to that just described for the acetaldehyde product of alanine oxidation.

The glucose specific activity assay involves volume reduction of the initial sample volume and washings passed through both ion exchange columns. Enzymatic (Glucostat) assay for glucose and liquid scintillation counting of an aliquot of the concentrate in Aquasol yield the necessary data.

TABLE I.

Solution	Alanine (mg)	[^{14}C]Alanine (μCi)	Mix I[a] (μM)	Mix II[b] (μM)
1	5	10^{-3}	—	—
2	5	10^{-3}	3.8	—
3	5	10^{-3}	—	28.2

[a] Mix I = Val, Leu, Ileu, Phe, Meth.

[b] Mix II = Glu-NH_2, Lys, Cys, Gly, Pro, Arg, His, Thr, Trp, Ser, Tyr, Glu, Asp.

Results. Table I lists the composition of three solutions that were processed by the method just described. These solutions prepared in water were not subject to deproteinization. Solution 1 containing no amino acids other than alanine was employed as a standard of reference. Solution 2 containing alanine in the same stable/radioactive ratio as solution 1 also contains those amino acids that yield aldehydes on oxidation with ninhydrin. Solution 3, again with the stable/radioactive alanine content of solution 1, contains all the other amino acids commonly found in blood. All amino acids other than alanine were added in concentrations that would approximate their concentration in a blood filtrate.

Table II lists the data from triplicate analysis of the solutions listed in Table I. It is apparent from a scan of columns 3 and 4 of this table that there are no significant differences in any of these analyses, which is the sought-for result. Generally, except for the final analysis of solution 3, the yields are reasonably good. While we have no explanation for the low yield from that analysis, we note that the low yield does not, apparently, jeopardize the validity of the specific activity determination.

Although no mass dilution was apparent in the analysis of solutions 2 and 3, we sought to obtain a more sensitive estimate of such an error by employing [^{14}C]valine, which, in the system we employ elutes from the resin very close to alanine and yields an aldehyde when subject to oxidation with ninhydrin. Starting with a sample of blood, we added [^{14}C]valine in trace quantity and alanine in the amount of 5 mg. Duplicate analysis of this solution yielded activity in the final alanine derivative amounting to 1.5 ± of the valine radioactivity added and, presumably, a like mass contribution to the sample from endogenous valine in the blood. We feel that this source of error can be considered to be negligible. Valine and alanine occur in plasma at approximately equal concentrations, 3 mg %. The addition of 5 mg carrier alanine to each 10 ml blood sample will, hence, render the small 1.5% error possible from valine to an approximate 0.085%

TABLE II.

Solution	Acetaldehyde–dimedon complex (mg)[a]	Net cpm	cpm/mg	Av specific activity	Yield (%)	Av
1	6.79	781	115		39.5	
	7.70	901	117		44.8	
	7.40	821	111	114	43.0	44.5
2	7.54	919	122		43.8	
	7.64	894	117		44.4	
	7.35	880	119	119	42.7	43.6
3	6.03	699	116		35.1	
	5.00	574	115		29.1	
	2.08	245	118	116	12.1	25.4

[a] Expected acetaldehyde–dimedon complex = 17.2 mg for all determinations.

error, which we feel can be considered inconsequential.

A more serious possible error to the determination of alanine specific activity could arise from the spill of label from circulating labeled metabolites that arise from the injected labeled alanine. Of principle importance are lactic acid and glucose. Both of these compounds should, theoretically, be excluded in the initial ion exchange steps of the method, but if not there, in the subsequent procedure of ninhydrin oxidation of alanine to yield acetaldehyde. To determine the validity of this reasoning, 1 μCi each of lactic acid and glucose were added to the alanine solutions which were then subjected to analysis for alanine specific activity. The activity from glucose found in the alanine fraction directly off the ion exchange column was negligible and was undetectable following oxidation of the alanine to acetaldehyde and isolation of that product as the acetaldehyde–dimedon complex. A small but significant quantity, 2.5%, of the activity from lactate or lactate contaminants was evident prior to oxidation of alanine. This was, however, reduced by a factor of 10 to a negligible amount in the final product, acetaldehyde–dimedon complex.

A further significant aspect of this isolation procedure is the question of isotopic contamination of the product metabolites of alanine which will be of interest in the study of alanine metabolism. Again, these are lactic acid and glucose. To examine this question, we turned our attention to the isolation and specific activity assay of the lactate and glucose fractions of deproteinized blood samples to which we had added alanine radioactivity. The lactate was eluted from the anion exchange columns and counted directly in the eluate, employing aquasol as the scintillation cocktail. The glucose as described above passes both cation and anion resins and is counted in the aqueous solution concentrated from the initial void volumes of the columns. The results of this assay indicate that a small but significant quantity of alanine or contaminant does spill into both lactate and glucose fractions, amounting to approximately 0.5% in the lactate fraction and 0.4% in the glucose fraction. This spill can easily be corrected for from the data of standard solutions assayed with each sample set.

Discussion. As noted before, the method of determination of alanine specific activity described here provides adequate sensitivity for the study of alanine turnover in the human given 25 μCi of ^{14}C-labeled alanine. The separation by ion exchange followed by specific chemical oxidation of alanine with ninhydrin yield a counting sample of reasonable mass (20 mg) which is demonstrably free of activity and/or mass contaminants that could logically arise from the metabolism of alanine in man.

While the development of this method was in progress, we noted publication of a paper employing enzymatic conversion of alanine

carbon to pyruvate with subsequent isolation of the pyruvate as the phenyl hydrazine for the specific activity determination of alanine (8). While it is not possible to assess the relative merit of these alternate methods at present, we have noted that these investigators employed larger injected doses and the technique of continuous infusion, which, on balance, should provide an average circulating alanine specific activity considerably greater than the minimum levels we are able to determine with good precision with the present method.

Summary. A method for specific radioactivity analysis of alanine in blood is described. This analysis involves the separation of alanine by an ion exchange column technique and its subsequent isolation as a gravimetrically assayable sample (acetaldehyde–dimedon). Mass contamination of the acetaldehyde–dimedon from valine, which elutes from the ion exchange column close to alanine, is demonstrated to be negligible. Activity contamination of the acetaldehyde–dimedon from lactate and glucose, two significant products of alanine metabolism, is likewise demonstrated to be negligible. Sample counting rates have been demonstrated to be significantly above background ($\times$ 3) 2 hr following a single injection of 25 μCi L-[^{14}C]alanine to a human subject.

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