

## A Colorimetric and Isotopic Assay of Leucine Aminotransferase

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A rapid and sensitive leucine aminotransferase (EC 2.6.1.6) assay is presented. In this assay the absorbance of 2,4-dinitrophenylhydrazones of branched chain keto acids produced by transamination is measured after a single extraction with toluene. The method is suitable for aminotransferase assays where leucine, isoleucine, or valine serves as amino group donor to 2-oxoglutarate. When uniformly labeled amino acids were used as markers, the sensitivity of the method was of about the same order as that of the spectrophotometric assay of the keto acid hydrazones.

It is likely that a single aminotransferase, leucine aminotransferase, EC 2.6.1.6, catalyzes the reversible transamination reaction where leucine, isoleucine, or valine serves as amino group donor to 2-oxoglutarate.<sup>1-4</sup> The methods presented previously for the assay of this enzyme are based on the estimation of glutamate after a fractionation step or on the estimation of a formed monocarboxylic keto acid as its 2,4-dinitrophenylhydrazone.<sup>5</sup> The separate determination of the monocarboxylic keto acid and 2-oxoglutaric acid is based mainly on the distribution of the 2,4-dinitrophenylhydrazones between an aqueous phase and an aromatic hydrocarbon.<sup>6,7</sup> The hydrazones prepared from a branched chain amino acid-2-oxoglutarate transamination reaction mixture have been extracted selectively with toluene<sup>2,6</sup> or cyclohexane.<sup>8</sup> Cyclohexane selectively extracts the hydrazone of the keto acid derived from leucine but does not extract greater amounts of the hydrazones of keto acids derived from isoleucine and valine.

In this paper a simple colorimetric method for measuring leucine aminotransferase activity is described in which toluene is employed as a selective extracting solvent. This method was briefly described earlier.<sup>9</sup> The method is suitable also for the radioactive products of enzymatic branched chain amino acid-2-oxoglutarate transamination reactions.

## MATERIALS AND METHODS

*Reaction mixture.* A solution was prepared that contained 25  $\mu$ moles of an amino acid, leucine, isoleucine, or valine, 25  $\mu$ moles of 2-oxoglutaric acid, 0.01  $\mu$ moles of pyridoxal-5'-phosphate and 50  $\mu$ moles of Tris-HCl buffer, pH 8.0 in 0.5 ml. The 2-oxoglutaric acid was neutralized to pH 8.0 with a few drops of 5 N sodium hydroxide. The reactions were initiated with 0.5 ml of an enzyme extract containing 0.05–0.150 mg protein. The total volume of the reaction mixture in a 13  $\times$  120 mm test tube was thus 1.0 ml. The reaction mixture was incubated at 30° and the reaction stopped with 0.2 ml of 10 N sulfuric acid.

*Estimation of monocarboxylic keto acid.* 1.0 ml of a solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid containing approximately 15  $\mu$ moles of the hydrazine was pipetted into the acidified reaction mixture and the tube was allowed to stand at room temperature (18–23°) for 5 min. After the formation of the hydrazones, 5 ml of toluene was added with an automatic pipet. The tube was closed with a rubber stopper and shaken vigorously by hand for 1 min. The intensity of the yellow color of the toluene layer was then measured with a Beckman DU spectrophotometer at 348 nm (leucine as amino donor in the reaction) or at 362 nm (isoleucine or valine as amino donor). A tube in which the enzyme extract was replaced by distilled water served as a blank.

*Enzyme extract.* *Escherichia coli* cells harvested in the late exponential phase from a glucose-mineral salt medium<sup>10</sup> were broken in a 0.05 M Tris-HCl buffer, pH 8.0, in a MSE 60W ultrasonic disintegrator during 8 min. The suspension was centrifuged in a preparative MSE L-50 ultracentrifuge at 100 000 *g* for 1 h. The supernatant was used as enzyme source.

*Protein estimation.* Protein was determined by the method described by Lowry *et al.*<sup>11</sup> using bovine serum albumin as standard.

*Isotopic method.* In the isotopic method the procedure was the same as in the colorimetric assay except that the reaction mixture contained, in addition to the compounds mentioned above 0.5  $\mu$ C of uniformly labeled <sup>14</sup>C-L-amino acid. After the extraction of the hydrazones with toluene, a 1.0 ml aliquot of the toluene layer was pipetted into a scintillation liquid containing 2,5-diphenyloxazole (POP) and 1,4-di[2-(5-phenyloxalyl)]-benzene (POPOP).<sup>12</sup> A Packard 3115 liquid scintillation counter was used for counting.

*Materials.* The following commercially available compounds were purchased from Fluka AG., Buchs, Switzerland: pyridoxal-5'-phosphate (*purum*, purity higher than 98 %), 2-oxoisocaproic acid (*puriss.*), 2-oxoisovaleric acid (*puriss.*), L-leucine, L-isoleucine, L-valine, and 2-oxoglutaric acid (*puriss.*). Toluene (G.R.) and 2,4-dinitrophenylhydrazine (G.R.) were purchased from E. Merck AG, Darmstadt, Germany. 2-Oxo-3-methylvaleric acid, prepared by organic synthesis, was a generous gift from Mr. J. Soimajärvi of this laboratory. L-Leucine-<sup>14</sup>C(U), 10 mC/mmole, and L-isoleucine-<sup>14</sup>C(U), 8.7 mC/mmole, were purchased from The Radiochemical Centre, Amersham, England.

## RESULTS

*Extraction time of hydrazones.* It is known that 2,4-dinitrophenylhydrazones of monocarboxylic keto acids are formed faster (in 5 min.) than the corresponding dicarboxylic keto acid hydrazones.<sup>7</sup> Therefore a 5-min incubation time was employed for the formation of the hydrazone derivatives of the branched chain keto acids. To test the time needed for extraction of hydrazones into toluene, the absorbance of the organic layer was measured at intervals during the extraction. The results are presented in Fig. 1. The results show that a one-minute extraction time is needed to extract the hydrazones of branched chain keto acids with manual mixing and therefore this time was chosen for the assay.

*Stability of hydrazones.* A very important aspect of the method is the stability of the hydrazones in the toluene layer and therefore the absorbance of the

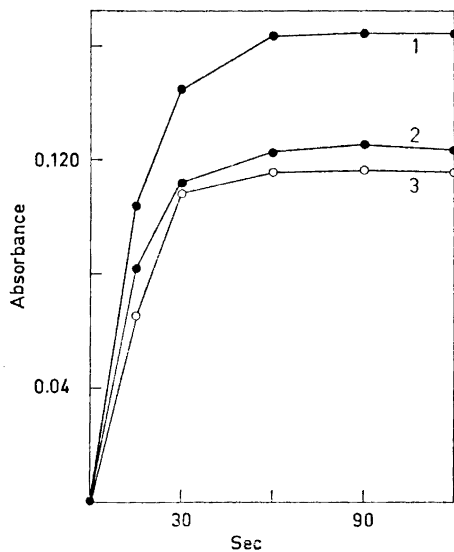


Fig. 1. Extraction of 2,4-dinitrophenylhydrazones of branched chain keto acids into the toluene layer. Three incubated reaction mixtures were treated as presented in "Materials and Methods" and the absorbance of the organic layer was measured during the extraction. A reaction mixture without enzyme extract but extracted after the same periods of time served as a blank. 1=2-oxoisocaproate 2,4-dinitrophenylhydrazone, 2=2-oxo-3-methylvalerate 2,4-dinitrophenylhydrazone and 3=2-oxoisovalerate 2,4-dinitrophenylhydrazone.

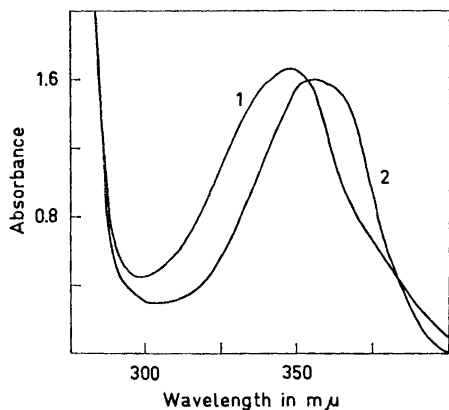


Fig. 2. Spectra of 2,4-dinitrophenylhydrazones of 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid. 0.5 ml of substrate solution and 0.5 ml of branched chain keto acid solution in 0.05 M Tris-HCl buffer, pH 8.0, was treated as presented in "Materials and Methods". The substrate solution contained the L-amino acid corresponding to the keto acid. 1=spectrum of 2-oxoisocaproate 2,4-dinitrophenylhydrazone, and 2=spectrum of 2-oxo-3-methylvalerate 2,4-dinitrophenylhydrazone. The spectrum of 2-oxoisovalerate 2,4-dinitrophenylhydrazone was similar to 2.

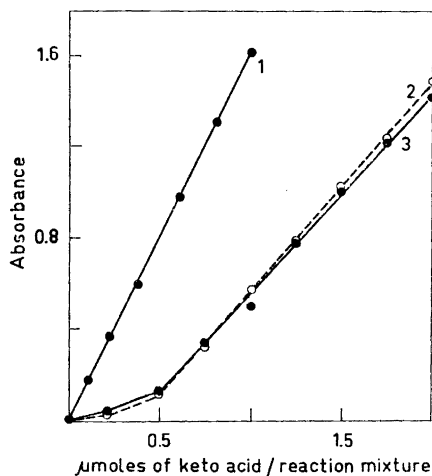


Fig. 3. Standard curves for hydrazones of three branched chain keto acids. The procedure was that presented in Fig. 2. 1=2-oxoisocaproate 2,4-dinitrophenylhydrazone, 2=2-oxoisovalerate 2,4-dinitrophenylhydrazone, and 3=2-oxo-3-methylvalerate 2,4-dinitrophenylhydrazone.

layer was measured at intervals. The results showed that the hydrazones are stable for at least 2 h at room temperature.

*Spectra of the hydrazones.* The spectra of two branched chain keto acid 2,4-dinitrophenylhydrazones are given in Fig. 2. The maximum at 348 nm for 2-oxoisocaproate hydrazone and that at 362 nm for the hydrazones of the keto acid analogs of isoleucine and valine were chosen for the assay.

*Standard curves for keto acid hydrazones.* Standard curves for hydrazones of branched chain keto acids are presented in Fig. 3.

It is seen that the standard curve for the 2-oxoisocaproate hydrazone is linear, but the standard curves for the hydrazones of 2-oxo-3-methylvalerate (ketoisoleucine) and 2-oxoisovalerate (ketovaline) are not linear at low keto acid concentrations. The reason for this non-linearity is unknown. When a solution of 2-oxoisovalerate hydrazone in toluene was diluted with toluene, no irregularity was found, and therefore the lack of linearity is not due to deviation from the Lambert-Beer law. The standard curves for 2-oxo-3-methylvaleric acid and 2-oxoisovaleric acid were, however, linear after the initial rise in the curve. The absorbance of the toluene layer was 0.258 against toluene when the reaction mixture without enzyme contained 2-oxoglutaric acid and L-leucine.

*Correlation of the rate of reaction and enzyme content.* The correlation between the rate of the reaction and the enzyme content of the reaction mixture is shown in Fig. 4. The correlation is linear.

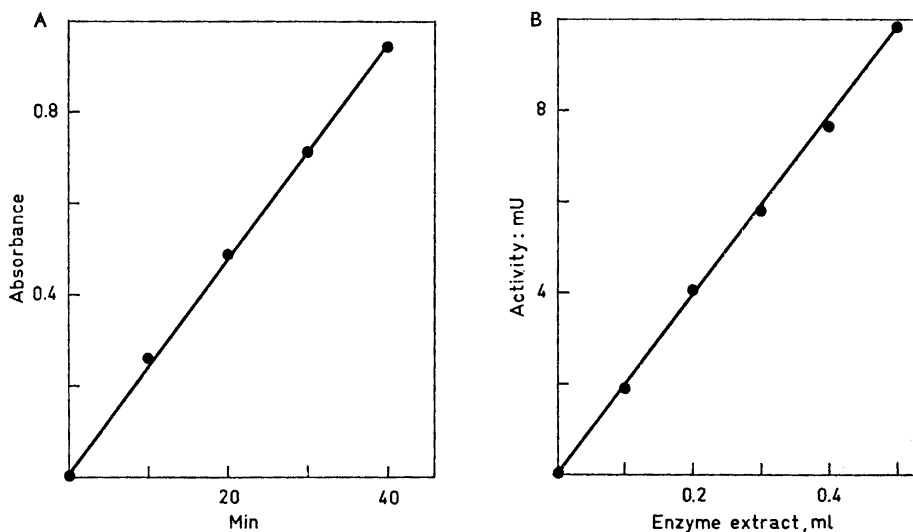


Fig. 4. Dependence of the extent of the L-leucine-2-oxoglutarate reaction on time (A) and enzyme content (B). A=absorbance of the 2-oxoisocaproate 2,4-dinitrophenylhydrazone at 348 nm. The weight of protein in the reaction mixture was 68  $\mu$ g. B=formation of ketoleucine hydrazone with different amounts of 100 000 g supernatant of *E. coli* containing 136  $\mu$ g of protein per ml. Incubation time at 30° was 20 min.

*Accuracy of the method.* The accuracy of the method was determined using L-leucine as amino group donor to 2-oxoglutarate. The standard deviation was  $\pm 0.31$  milliunits and the mean value 5.25 milliunits ( $n=11$ ). The relative standard deviation is thus  $\pm 6.0\%$ . The protein concentration in the reaction mixture was 0.034 mg and therefore the mean specific activity was 155 milliunits/mg protein.

*Isotopes as markers in the assay.* The results of experiments where 0.5 microcuries of uniformly labeled L-leucine or L-isoleucine were added to a reaction mixture containing 25  $\mu$ moles of unlabeled L-amino acid as amino group donor to 2-oxoglutarate are presented in Fig. 5. The data show that

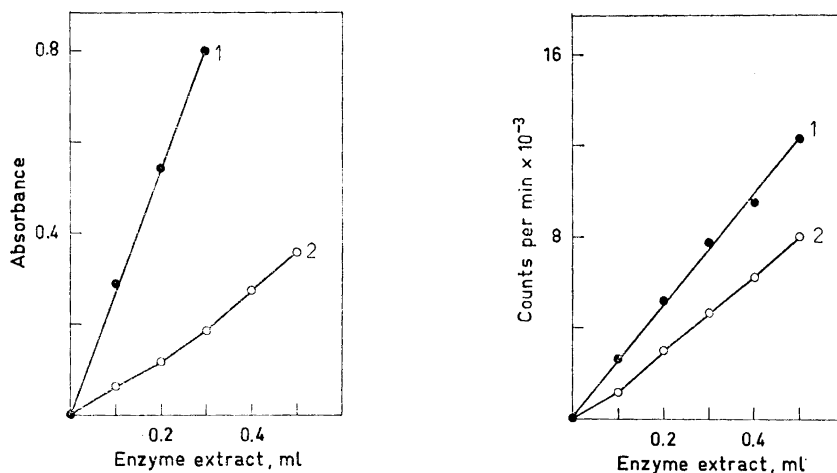


Fig. 5. Correlation between rate of reaction and the formation of branched chain keto acid 2,4-dinitrophenylhydrazone determined spectrophotometrically (A) and by  $^{14}\text{C}$ -counting (B). 0.5  $\mu\text{C}$  of L-leucine- $^{14}\text{C}$  or L-isoleucine- $^{14}\text{C}$  was added to the reaction mixture containing 232  $\mu\text{g}$  of protein per ml. After 20 min incubation the hydrazones were extracted from the water layer with toluene and the absorbance of the toluene layer measured at 348 nm (for ketoleucine) or at 362 nm (for ketoisoleucine). 1 ml of the organic layer was added to 10 ml of POPOP-toluene scintillation liquid and counted with a Packard counter.

there is good linear correlation between the enzyme content and the rate of reaction when leucine is the amino group donor and fairly good correlation when isoleucine is the amino group donor. The results were the same whether the rate was measured using the spectrophotometric or isotopic assay. It will be noted, however, that the ratio of ketoleucine to ketoisoleucine was different in the spectrophotometric assay than in the isotopic assay.

## DISCUSSION

The leucine aminotransferase assay presented here includes a simple one-step extraction. Sodium carbonate solution is used in other methods as a second extracting agent. Its effect is based on its selective extraction of unchanged 2,4-dinitrophenylhydrazine, neutral hydrazones, and acidic hydrazones.<sup>6</sup> It is reported, however, that sodium carbonate solution selectively extracts also monocarboxylic keto acid 2,4-dinitrophenylhydrazones.<sup>13</sup>

Toluene is a highly selective extracting agent for monocarboxylic keto acid hydrazones. The hydrazone of 2-oxoglutaric acid is retained in the aqueous layer, but its concentration has an effect on the sensitivity of the assay of other keto acids. The blank reading in this procedure was, however, low (0.258). It is likely that the method is suitable for the study of all monocarboxylic keto acid-2-oxoglutaric acid transamination reactions; only possible irregularities in the standard curves may limit the use of the method. It has been reported earlier<sup>13</sup> that linear standard curves are obtained for 2-oxoisocaproic acid, phenylpyruvic acid, hydroxyphenylpyruvic acid, and pyruvic acid, but non-linear curves for dimethylpyruvic acid and 2-oxo-3-methylvaleric acid.

Isotopes make possible the use of the method for the assay of the activity of a branched chain amino acid-oxoglutaric acid transaminase, *e.g.*, in kinetic experiments, because each keto acid can be measured separately by choosing the appropriate labeled amino acid. The sensitivity of the isotopic assay is of about the same order as that of the spectrophotometric assay. Higher sensitivities are obtained, of course, by increasing the ratio of labeled to unlabeled amino acid. One can calculate from Fig. 3 that the lowest amounts of keto acid measurable in a reaction mixture are in the range of 0.05–0.1  $\mu$ moles. Therefore initial rates can be measured when 0.2–0.4 % of a substrate amino acid (25 micromoles) is converted to the corresponding keto acid. Linear initial rate curves were usually obtained up to at least 10 % of the over-all reaction. The standard curves for ketoisoleucine and ketovaline are non-linear at low keto acid concentrations, but are linear at high keto acid levels, and thus an absorbance of 0.200 or more has to be obtained to reach the linear standard curve (Fig. 3).

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