PREPARATION OF TRITIATED POTASSIUM DIHYDROGEN THREO-D_s (+)-ISOCITRATE.

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A number of real or potential procedures have been described for the chemical and enzymatic synthesis of isotopically-labelled isocitric acid (1-5). However, none are readily adaptable to preparing large quantities of material with high specific activity in the isomeric form of interest in biochemistry, namely, threo-D_s(+)-isocitrate (6). Although a demand for highly labelled threo-D_s(+)-isocitrate has become apparent with the recent characterization of several enzymes involved in its metabolism (7-9), there are no commercial isotope suppliers that market this single isomer free of its racemic enantiomorph.

In connection with our investigations of the ligand-binding properties of yeast NAD^+-specific isocitrate dehydrogenase (ICDH) (10), it became essential that this labelled isomer be available for unambiguous study of the interactions among the various ligands which modulate or participate in the ICDH-catalyzed reaction. A limited supply of the highly purified enzyme also dictated that the substrate have a high specific activity. This led us to develop a general means for preparing tritiated isocitrate with these properties. The procedure takes advantage of the relatively low water-solubility of the monopotassium salt of threo-D_s(+)-isocitrate at pH 3.5 (5). This property facilitated its differential crystallization from an apparent mixture of isomers which one would expect to be generated on treatment of a small sample of the pure threo-D_s(+)-isomer by commerical tritium exchange.

Methods

The monopotassium salt of threo-D_s(+)-isocitrate was purchased from Sigma Chemical Co. (Lot No. 97B-7374, 99% pure). Twenty-three milligrams was sent to New England Nuclear Corporation for tritiation by radiation catalyzed-exchange using tritium gas (11). The exchange was effected in 0.2 ml water at pH 8 (adjusted with triethylamine) under 25 Ci of tritium gas and in the presence of 300 mg of 5% rhodium on alumina. The reaction mixture was stirred for two days at room temperature, after which labile tritium was washed out by twice adding 10 ml of water and removing the solvent by vacuum distillation. The labelled compound was received from NEN as a turbid, 10-ml solution containing the insoluble rhodium-alumina catalyst and 125 mCi of non-labile tritium.

This mixture was lyophilized overnight, then taken up in 0.8 ml of water. To hydrolyze any of the γ-lactone of isocitrate which slowly forms in warm aqueous solution, 0.25 ml of saturated KOH was added and the solution was heated for 15 min on a steam bath (4). After cooling to ice temperature the pH was lowered to 2 by adding 1 gm of wet-packed Dowex 50W X 4 (H^+ form). The resin was swirled in the solution for one minute, then filtered and rinsed with 0.8 ml of water using a suction assembly. This filtration step also removed the rhodium-alumina catalyst added for the earlier tritiation exchange reaction. To

the filtrate was added 0.542 gm of unlabelled potassium dihydrogen threo-$D_s(+)$-isocitrate which dissolved completely in approximately 2 minutes when held over a steam bath. The warm solution was immediately chilled on ice and the pH was adjusted to 3.5 with 0.1 M KOH. Crystals of the monopotassium salt formed within minutes and were incubated at refrigerator temperature in the mother liquor for 4 hours. They were subsequently collected in the cold by vacuum filtration and were sequentially washed with 1-ml portions of cold 95% ethanol, absolute ethanol, and finally absolute ethyl ether. These crystals were then transferred to a small vial and dried for 12 hours at 40°C under continuous vacuum (oil pump). The final dry weight of the product from this first crystallization was 0.484 gm which had a specific activity of 1.26 mCi/mmole as summarized in Table 1. Specific activities were determined by counting appropriate dilutions of weighed samples in a Packard Model 2002 liquid scintillation spectrometer. The scintillation solvent used was prepared by mixing 1 part of Biosolv III solubilizer (Beckman Instruments, Palo Alto, California), 5 parts of scintillation-grade toluene, and Omnifluor (New England Nuclear), 4 gm per liter.

Following the above initial crystallization, the salt was recrystallized 5 times to constant specific activity. Each time the compound recovered from the previous recrystallization was dissolved in 1.2 ml of water, heated in strong KOH solution, chilled, and then adjusted to pH 3.5 for crystal formation. Table 1 summarizes these 5 recrystallization steps.

The chemical purity of each recrystallized product was determined by enzymatic assay using the assay mixture containing 1 mM 5'-adenosine monophosphate (AMP) and homogeneous yeast NAD^+-specific isocitrate dehydrogenase previously described (7). This assay is accurate to approximately ±2 percent. A sample of the fifth recrystallization-product contained 16.8 percent potassium, versus theory for $KH_2C_6H_5O_7$, 16.99 percent. The specific rotation was $[\alpha]^{25}_D = +20.5°$ (0.1 M solution in water). When stored at pH 6.0 in frozen state, a 0.1 M solution lost negligible amounts of radioactivity into water within 6 months.

Radiochemical purity was also established enzymatically by coupling the reactions catalyzed by isocitrate dihydrogenase and bovine liver glutamic dehydrogenase (Sigma Chemical Co.). The L-glutamate thus formed was separated and counted for tritium content. A typical 3-ml assay mixture contained: 100 mM N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid (HEPES) adjusted to pH 7.6 with KOH, 4 mM $MgSO_4$, 1.5 mM NAD^+, 1 mM AMP, 3 mM dithiothreitol, 1 mM tritiated threo-$D_s(+)$-isocitrate, 1.5 mM NADPH, 10 mM NH_4Cl, 0.10 mg yeast isocitrate dehydrogenase, and 0.10 mg bovine liver glutamic dehydrogenase. This reaction mixture was incubated at 30° for 1 hour, then stopped by immersion in a boiling water bath for 1 minute. Unlabelled carrier L-glutamate was added to 10 mM final concentration and 20 µl aliquots of the reaction mixture and standard glutamate were subjected in quadruplet to paper chromatography with butanol-propionic acid-water (150:72:98 by volume). The location of labelled L-glutamate on the paper was determined by exact coincidence of the spot on a radioautogram with the ninhydrin-positive spot due to standard glutamate. These areas were cut out, eluted with 3 ml of 0.1 N HCl, and the eluant counted in 10 ml of Biosolv scintillation solvent. The radiochemical purities of the various crystallization-products calculated as percent of the original isocitrate label recovered as L-glutamate are given in Table 1.

The final product after five recrystallizations was of sufficiently high specific activity (1.14 mCi/mmole) for most types of enzyme-substrate binding experiments (10). It appears doubtful, however, that preparations of tritiated threo-$D_s(+)$-isocitrate with specific activities significantly greater than that reported here can be achieved by this method. The limiting factor in attaining

higher specific activities being the expected facility with which the other isomers of isocitrate, namely, erythro-D_s(-)-, erthyro-L_s(+)-, and threo-L_s(-)-isomers, are generated during catalytic tritium exchange (6).

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TABLE 1. Recrystallization of tritiated potassium dihydrogen threo-D_s(+)-isocitrate to constant specific activity.

Fraction	Specific Activity mCi/mmole	Recovery gm	% Chemical Purity[a]	% Radiochemical Purity
First Crystallization	1.26	0.484	98	89.9
1st Recrystallization	1.15	0.440	95	97.0
2nd Recrystallization	1.14	0.372	92	98.7
3rd Recrystallization	1.14	0.332	97	99.3
4th Recrystallization	1.14	0.290	99	99.1
5th Recrystallization	1.14	0.250	101	99.4

[a] Definition and assay method described in text.

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