Biochemical Synthesis of Stereospecifically Hydrogen Labeled Compounds on a Preparative Scale, VI [1–3]

Synthesis of Further Substrates of NAD(P)-Linked Dehydrogenases of High Specific Tritium Content

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The preparation of (R) and (S) [2-^{3}H]lactate as well as (S) [2-^{3}H]glutamate *via* the coupled exchange reaction catalyzed by NAD linked dehydrogenases and NADH: lipoamide oxidoreductase (diaphorase) is described. The specific radioactivity of the hydrogen ions of the $^3HOH/H_2O$ can be obtained in the substrates (100% exchange) if equilibrium isotope effects are disregarded. By the exchange procedure substrates with higher specific radioactivity are obtained from positionally [^{3}H]labeled racemic mixtures prepared by chemical reductions with [^{3}H]labeled hydrides. The tritium content of one of the enantiomeres is "washed out" into water. As examples are presented the preparation of (R) [2-^{3}H] (S) [2-H]malate as well as the corresponding carnitine, glutamate and (R) and (S)lactate.

Abbreviations: (R)carnitine, (R)3-hydroxy-4-trimethylamino butyric acid; ADH, alcohol dehydrogenase (EC 1.1.1.1) from yeast; (S)LDH, (S)lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle; (R)LDH, (R)lactate dehydrogenase (EC 1.1.1.28) from *Lactobacillus leichmannii*; GDH, (S)glutamate dehydrogenase (EC 1.4.1.3) from beef liver; DP, α-lipoamide dehydrogenase (diaphorase) (EC 1.6.4.3) from pig heart; GR, glutathione reductase (EC 1.6.4.2) from yeast; CDH, (R)carnitine dehydrogenase (EC 1.1.1.108) from *Pseudomonas aeruginosa*.

Stereospecific hydrogen labeled chiral compounds are important for stereochemical and other mechanistic studies in organic chemistry and biochemistry [4–7]. Usually only enzymic procedures lead to complete stereospecificity of the labeling. A preparative scale is often limited by the high cost of the enzymes involved. One of the first examples of a preparative synthesis of a chiral compound by enzymes was reported by Levy *et al.* [8] who prepared (1S) [1-^{2}H]ethanol by reduction of [1-^{2}H]acetaldehyde with yeast alcohol dehydrogenase. A different approach for the biochemical preparation of stereospecifically hydrogen labeled substrates by microorganisms or enzymes was reported by us [1–3, 9–12]. This method makes use of the stereospecific hydrogen exchange which takes place between the substrate of a NAD(P)-linked dehydrogenase and water in the presence of a diaphorase. The latter can either be specific for NADH [9, 13–15] like lipoamide oxidoreductase [9, 15] or can exchange with both NADH and NADPH like glutathione reductase [16, 17].

In this reaction catalytic amounts of NAD or NADP are required as hydrogen carriers [2, 3, 9–11]. This exchange procedure was utilized for the preparation of (S) [2-^{3}H]malate [10] the (1S)- and (1R)-enantiomeres of propanol [2, 3], ethanol [3, 9] and butanol [3], as well as (R) [3-^{3}H]carnitine [11].

Some other different enzymatic procedures have been published. Konikova *et al.* [18] observed that transaminases are able to exchange the hydrogen atom at C-2 of an amino acid with the surrounding water. Based on that observation, Oshima and Tamiya [19] incubated (S)alanine with glutamic-pyruvic transaminase (EC 2.6.1.2) and pyridoxal phosphate in a medium containing ^2H_2O. However the (S)alanine isolated was not only labeled at the C-2 position but in addition hydrogen atoms of the methyl group had also been substituted by ^{2}H [19]. Wenzel and

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Brühmüller [20] used this reaction in their synthesis of (S) [2-^{3}H] glutamate. Hoberman and d'Adamo [21] prepared (R)- and (S) [2-^{2}H] lactate from (R)- and (S) [2-^{2}H] alanine which in turn were prepared by enzymatic resolution of (RS) N-acetyl-[2-^{2}H] alanines with the help of acylase (EC 3.5.1.14). (RS) N-Acetyl-[2-^{2}H] alanine was formed by exchange between (RS) N-acetyl alanine and [1-^{2}H] acetic acid [21]. Due to a non specific loss of ^{2}H during the acylase reaction, the maximal yield was about 75%. Both methods [20, 21] are restricted to the labeling of a particular substrate.

Brendel *et al.* [11] have recently reported that the release of ^{3}H into water from trace levels of (R) [3-^{3}H] carnitine was reversely proportional over a 500 fold concentration range to the non-labeled (R) carnitine present in the same medium. The exchange reaction takes place by the coupled enzymic action of (R) carnitine dehydrogenase and α-lipoamide dehydrogenase (diaphorase) utilizing catalytical amounts of NAD as hydrogen carrier. In this way a new specific and sensitive assay for (R) carnitine in biological fluids was developed [11]. Theoretically there is no reason why this procedure could not be extended to the assay of other substrates which are oxidized by an NAD(P)-linked dehydrogenase. This is of interest in the case of enzymes such as (R) carnitine dehydrogenase which exhibit a high sensitivity to keto-group trapping agents [22] such as the commonly used hydrazine, semi-carbazide, dimedone and aminooxyacetic acids. Since the exchange reaction takes place without displacing the equilibrium of the system, trapping agents are not necessary in this analytical approach.

Experiments with ^3H_2O are limited by the amount of radioactivity which can be safely handled. On the other hand, chemical reductions with [^{3}H] metal hydrides or $^3H_2$ gas are not governed by these limitations and, consequently, much higher specific radioactivities can be achieved [23]. Chemical reductions are usually carried out under achiral conditions and, consequently, lead to racemic mixtures. If such racemic mixtures are subjected to the procedure described here for synthesis, the ^{3}H content of one particular enantiomer can be specifically and quantitatively "washed out". In such cases in which it is imperative to utilize substrates with very high specific radioactivity, this variation might become of importance. Since the "natural" isomer would be the one which is lost in this procedure one would perform the experiment with the labeled racemate and perform a control experiment with the racemate which is only labeled in the "unphysiologic" enantiomer.

Results and Discussion

Tables I and II show results of the preparation of (R) [2-^{3}H] lactate, (S) [2-^{3}H] lactate and (S) [2-^{3}H] glutamate. The tritium incorporation into lactate occurs specifically and without racemization at the C-2 position, less than 0.5% of the tritium is incorporated into the methyl-group of lactate. While this work was in progress it was found [12] that the exchange of (R) lactate using (R) lactate dehydrogenase of *Lactobacillus leichmannii* is strongly increased at pH 8.5. The first three experiments of Table I show that the amounts of enzymes specified can handle total amounts of (S) lactate from 0.1 mmol to 1.0 mmol without significant differences in the exchange yield, and that the flavine enzyme component is vital for the success of the incorporation. The fourth experiment describes an exchange reaction with (R) lactate dehydrogenase which leads to results identical to those observed for the (S)-isomer. The maximum specific radioactivity in lactate is dependent on the specific radioactivity of the exchangeable hydrogen in the reaction medium. The first four experiments were performed in a medium containing approximately 20 mCi/ml ^3H_2O. Experiment five shows that (S) lactate of a high specific radioactivity can be synthesized when the incubation medium is first lyophilized and the freeze-dried material is then dissolved in ^3H_2O of high specific radioactivity as is commercially available. This confers a specific radioactivity to the medium 50 times higher than that used in the previous experiments. The last experiment in Table I shows that the exchange also takes place when glutathione-reductase is used instead of diaphorase. The reduced exchange yield obtained in this experiment is most likely due to the much lower diaphorase activity of glutathione-reductase. International units given are those for the primary activity of the enzymes α-lipoamide dehydrogenation and glutathione reduction which is not related to the enzymes diaphorase activity.

The first two experiments of Table II show that both α-lipoamide dehydrogenase and glutathione-reductase can facilitate incorporation of ^{3}H into the C-2 position of (S) glutamate when NAD and (S)

Table I. Synthesis of stereospecifically labeled (R) and (S) [2-^{3}H] lactates.

#	Lactate [mmol]	Time [hours]	Water [μatom hydrogen]	Specific radioactivity [dpm/μmol] Lactate Total	C-2	CH_3	Exchange Yield [%]	Enzyme units [IU]
1	0.1 (S) [a]	24	4.1×10^5	4.1×10^5	4.0×10^5	1.2×10^3	100	40 (S) LDH + 40 (DP)
2	1.0 (S) [a]	24	4.7×10^5	4.6×10^5	—	4.1×10^2	98	40 (S) LDH + 40 (DP)
3	0.1 (S) [a]	24	4.3×10^5	2.1×10^2	—	—	0.5	180 (S) LDH + 0 (DP)
4	0.3 (R) [b]	18	3.7×10^5	4.1×10^5	—	8.0×10^2	110	300 (R) LDH + 150 (DP)
5	0.3 (S) [a,c]	21	2.0×10^7	1.9×10^7	2.0×10^7	—	95	180 (S) LDH + 50 (DP)
6	0.2 (S) [a]	16	4.2×10^6	2.7×10^6	2.4×10^6	—	64	150 (S) LDH + 120 (GR)

(S) [a], Lactate containing 99.5% of the (S) isomer and 0.5% of the (R) isomer.
(R) [b], Lactate containing 95% of the (R) isomer and 5% of the (S) isomer.
[c] Incubation cocktail was lyophilized before addition of $^3HOH/H_2O$.
C-2, Specific radioactivity of the isolated lactates at the C-2 position as evident from transfer to NADH by the corresponding LDH.
CH_3, Specific radioactivity located at the methyl group of the isolated lactates by Kuhn-Roth oxidation.
Apart from the amounts of lactate and enzymes stated in the table, the following additions were made per ml of reaction mixture: 2.8 μmol NAD, 0.3 μmol NADH (omitted in # 6), 1.0 mg ovalbumin, 2.7 μmol EDTA, 100 μmol phosphate buffer at a pH of 6.5 and the reaction was carried out at 30 °C under N_2 in a total volume of 0.1 ml. After the reaction was completed, the incubation mixture was lyophilized and the residue taken up in 1 ml of boiling water. The lactates were isolated as described in the "Methods" section. Chemical yields were better than 97% in all experiments.

Table II. Synthesis of stereospecifically labeled (S) [2-^{3}H] glutamate.

#	(S) Glutamate [μmol]	Time [hours]	Water [μatom hydrogen]	Specific radioactivity [dpm/μmol] Total	(S) Glutamate C-2	Exchange Yield [%]	Enzyme units [IU]
1	19.2	24	2.0×10^7	1.9×10^7	1.8×10^7	95	25 (GDH) + 40 (DP)
2	8.6	20	1.5×10^6	1.3×10^6	1.3×10^6	86	26 (GDH) + 64 (GR)
3	5.2	19	1.1×10^6	9.0×10^5	8.9×10^5	82	25 (GDH) + 64 (GR)

C-2, Specific radioactivity of the isolated (S) glutamate at the C-2 position as evident from transfer to NADH by GDH.
Apart from the amounts of (S) glutamate and enzymes as stated in the Table, the following additions were made per ml incubation cocktail: 5 mg ovalbumine, 2.7 μmol EDTA, 100 μmol Tris-HCl buffer, 3.5 μmol NAD (Experiment # 1 & 2) and 4.2 μmol NADP (Experiment # 3) at pH 8 and the reaction was carried out under N_2 at 30 °C in a total volume of 0.1 ml.
After the reaction was completed, the incubation mixture was lyophilized and the residue taken up in 1 ml of boiling water. The (S) glutamate was isolated in approximately 98% yield as described in the "Methods" section.

glutamate dehydrogenase are present. Again, incorporation into other positions except C-2 is minimal. In the third experiment NADP was used with glutathione-reductase instead of NAD [16, 17]. (S) glutamate dehydrogenase can accept both NAD and NAD(P), but prefers the former [24].

The selective wash-out of the label from only one of the two enantiomers in the presence of the corresponding lactate dehydrogenase, NAD, and diaphorase is shown in Table III. In both experiments the product of the reaction consisted of a racemate which had its label almost entirely localized in one of the two enantiomers.

In Table IV an experiment is described for (RS) [2-^{3}H] glutamate with results similar to those described for the experiments in Table III. In this case, however, only one of the two glutamate enantiomers can be selectively "washed out". The differences in the three experiments described are the utilization of NADP instead of NAD and glutathione-reductase instead of diaphorase. Table V describes the preparation of (R) [2-^{3}H] (S) [2-H] malate from (RS) [2-^{3}H] malate with α-lipoamide dehydrogenase or glutathione-reductase. In Table VI label in (R) carnitine was "washed out" of a (RS) [3-^{3}H] carnitine with (R) carnitine dehydrogenase, NAD, and diaphorase yielding (R) [3-H] (S) [3-^{3}H] carnitine.

Table III. Preparation of (R) [2-H] (S) [2-^{3}H] lactate and ((R) [2-^{3}H] (S) [2-H] lactate by enzymic "wash out" of (RS) [2-^{3}H] lactate.

#	(RS) Lactate [μmol]	Time [hours]	Specific radioactivity [dpm/μmol] Original lactate	Isolated lactate			Exchange Yield [%]	Enzyme units [IU]
1	0.3	12	6.6×10^9	3.2×10^9	C-2 (R) / 4.8×10^6	C-2 (S) / 3.3×10^9	50	5 (R) LDH + 10 (DP)
2	0.4	14	6.6×10^9	3.3×10^9	3.1×10^9	5.7×10^6	47	10 (S) LDH + 10 (DP)

C-2 (R), Specific radioactivity of the product which can be transferred to NAD by (R) lactate dehydrogenase.
C-2 (S), Specific radioactivity of the product which can be transferred to NAD by (S) lactate dehydrogenase.
In addition per ml of reaction mixture were added: 0.2 μmol NAD, 2 mg ovalbumin, 1 mg EDTA, 50 μmol phosphate buffer at a pH of 7 and the reaction was carried out at 30 °C under N_2 in a total volume of 0.1 ml. The flow of N_2 was regulated such that most of the reaction water was evaporated at the end of the experiment. After 12 hours the mixture was taken up in 1 ml of boiling water and lyophilized. This procedure was repeated twice and the produced lactate analyzed. It is of paramount importance that during the determination of the ^{3}H content at the C-2 position of the (R) and (S) lactates formed, all the originally present diaphorase activity was completely inactivated.

Table IV. Preparation of (S) [2-H] (R) [2-^{3}H] glutamate by enzymic "wash out" of (RS) [2-^{3}H] glutamate.

#	(RS) Glutamate [μmol]	Time [hours]	Specific radioactivity [dpm/μmol] Original glutamate	Isolated glutamate		Exchange Yield [%]	Enzyme units [IU]
1	0.2	20	4.4×10^9	2.1×10^9	C-2 (S) / 1.7×10^7	48	12 (GDH) + 18 (DP)
2	0.2	22	4.4×10^9	2.2×10^9	1.4×10^7	50	12 (GDH) + 60 (GR)
3	0.2	14	4.4×10^7	2.3×10^9	2.3×10^7	52	12 (GDH) + 60 (GR)

In addition the reaction medium contained per ml: 2 mg ovalbumin, 1 mg EDTA, 50 μmol phosphate buffer 0.3 μmol NAD (experiments # 1 and 2) and 0.4 μmol NADP (in experiment # 3) at a pH of 7 and the reaction carried out at 30 °C under N_2 in a total volume of 0.1 ml. The nitrogen flow was regulated to evaporate most of the reaction water during the experiment. After the reaction was completed, the residue was taken up in 1 ml of boiling water and then lyophilized.

Table V. Preparation of (R) [2-^{3}H] (S) [2-H] malate by enzymic "wash out" of (RS) [2-^{3}H] malate.

#	(RS) Malate [μmol]	Time [hours]	Specific radioactivity [dpm/μmol] Original malate	Isolated malate		Exchange Yield [%]	Enzymes [IU]
				C-2 (S)			
1	0.3	16	3.9×10^9	1.9×10^9	1.5×10^7	49	40 (MDH) + 22 (DP)
2	0.2	22	3.9×10^9	2.0×10^9	1.1×10^7	50	33 (MDH) + 40 (GR)

In addition per ml of reaction mixture were added: 0.3 μmol NAD, 2 mg ovalbumine, 1 mg EDTA, 50 μmol phosphate buffer at a pH of 7 and the reaction carried out at 30 °C under N_2 in a total volume of 0.4 ml. Nitrogen flow was regulated as described in Tables III and IV. The reaction was performed under conditions as described in Table III and in the text. At the end of the incubation period the mixture was taken up in 1 ml of boiling water and lyophilized. This procedure was repeated twice. The same precautions in regard to diaphorase activity must be observed as stated in Tables III and IV.

Table VI. Preparation of (R) [3-H] (S) [3-^{3}H] carnitine by enzymic "wash out" of (RS) [3-^{3}H] carnitine.

#	(RS) Carnitine	Time [hours]	Specific radioactivity [dpm/μmol] Original carnitine	Isolated carnitine	Exchange Yield [%]	Enzymes [IU]
1	0.5	24	6.8×10^9	3.2×10^9	47	5 (CDH) + 10 (DP)

In addition per ml incubation mixture were added: 0.5 μmol NAD, 2 mg ovalbumin, 1 mg EDTA, 100 μmol Tris-HCl buffer at a pH of 8 and the reaction carried out at 30 °C under N_2 in a total volume of 0.2 ml under conditions described for Tables III and IV. After completion of the incubation the mixture was poured on a 1.5 × 50 cm cation exchange column and carnitine was eluted with 1 N HCl [10].

Material and Methods

Enzymes

(R) carnitine dehydrogenase was isolated from *Pseudomonas aeruginosa* ATCC 7700 grown in a minimal medium containing (RS) carnitine as sole carbon and nitrogen source as described by Aurich *et al.* [22]. The enzyme was precipitated with ammonium sulfate and partially purified by G-200 gel filtration. With the exception of (R) lactate dehydrogenase from *Lactobacillus leichmannii,* which was obtained from Boehringer, all other enzymes were purchased from Sigma Chemical Co. or Boehringer Mannheim Corporation.

Substrates

(R) carnitine was a gift of Otsuka Pharmaceutical Co. (RS) carnitine was purchased from Sigma. (RS) [2-^{3}H] lactate (3 mCi/μmol), (RS) [2-^{3}H] malate (2.3 mCi/μmol) were purchased from New England Nuclear and (RS) glutamate (1.8 mCi/μmol) from Amersham Searle. Acetyl-CoA was obtained from Calbiochem. (RS) [2-^{3}H] carnitine with a specific radioactivity of 3 mCi/μmol was synthesized as already described by a NaB^3H_4 reduction of dehydrocarnitine-ethylester bromide [11].

Methods

Radioactivity was determined by liquid scintillation counting using 10 – 15 ml of a solution containing 5 g Omnifluor (New England Nuclear) in 1000 ml of a mixture of 2:1 v/v toluene/triton X-100 or alternatively with Bray's solution [25]. The radioactivity of 6 to 10 mg Ag-acetate was determined by dissolution in 5 ml of ethanolamine and addition of 15 ml of a scintillation medium containing 5 g PPO and 0.3 g of dimethyl POPOP in 1 l of toluene. All liquid scintillation counting was performed in either a Beckman LS 330 or an Intertechnique SL-40 scintillation counter.

(R) carnitine was enzymatically determined after the method of Marquis and Fritz [26]. (S) lactate [27], (S) malate [28], and (S) glutamate [29] were determined according to the references given. (R) lactate was determined analogous to the method described for (S) lactate.

Isolation of NADH

The NADH produced during oxidation of a substrate by the corresponding substrate dehydrogenase was isolated on a DEAE-cellulose column (1 × 8 cm) in the bicarbonate form after the procedure of Silverstein [30]. The column was washed with 100 ml of 3.5 mM NH_4HCO_3 and the NADH was eluted with 10 – 15 ml 0.2 M NH_4HCO_3. The column eluates were concentrated at 40 °C in vacuo and the NADH determined enzymatically with Na-pyruvate and (S) lactate dehydrogenase [31].

(R) and (S) lactates and (S) malate were isolated on a Dowex-1 column (1.5 × 20 cm) in the formiate form with a formic acid gradient after the procedure of Bush *et al.* [32]. (S) glutamate was isolated on the same column with a linear gradient of 200 ml water – 200 ml 8 N formic acid. Fractions were collected, concentrated and re-evaporated in vacuo, and substrate concentrations were determined enzymatically.

(RS) carnitine was isolated on a high pressure, cation exchange column with citrate buffer as eluent, and analyzed with sodium triphenylcyanoborate [33].

Proof of position of the label was obtained for:

Lactate

The ^{3}H incorporation into the methyl group of the isolated lactate was determined by a Kuhn-Roth degradation [34, 35] in CrO_3/H_2SO_4. The produced acetic acid containing the radioactivity localized in the methyl group of the original lactate was neutralized with CO_2-free 0.1 N NaOH, concentrated in vacuo, taken up in 10 ml water, adjusted to a pH of 6 with diluted HNO_3 and precipitated as Ag-acetate by addition of $AgNO_3$ and acetone [34]. The Ag-acetate was recrystallized to constant specific radioactivity.

Lactate and other substrates

The ^{3}H incorporation into the C-2 position of (S) lactate, (R) lactate, (S) malate and (S) glutamate was checked by a transfer of the label to NAD *via* the reaction catalyzed by the respective NAD-linked dehydrogenase and the NADH produced was isolated [29] and radioactivity determined as described above.

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