Studies of Enzyme-mediated Reactions. Part 11.[1] Experiments on the Dismutation of Aldehyde to Alcohol and Carboxylic Acid by the Complex of Liver Alcohol Dehydrogenase and NAD$^+$

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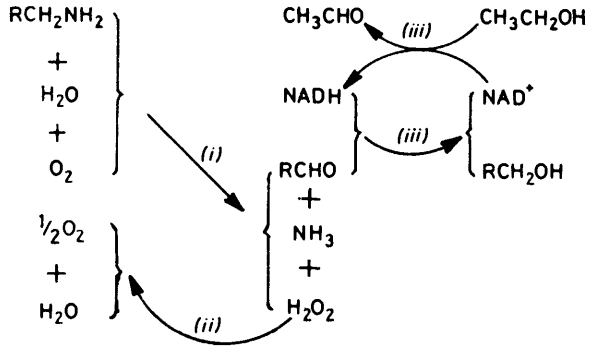
[1-$^3H_1$]Heptanal undergoes dismutation in the presence of the complex of liver alcohol dehydrogenase and NAD$^+$ to yield unlabelled heptanoic acid and (1*RS*)-[1-$^3H_1$]heptanol with double the specific radioactivity, thus demonstrating transfer of the formyl hydrogen from one half of the sample to the other. Conditions are found for reducing the incidence of this dismutation process.

For our studies of the stereochemical course of the dehydrogenation of primary amines by monoamine oxidase from liver mitochondria,[1] we added liver alcohol dehydrogenase and NADH to the reaction mixture to trap the initially formed heptanal by reduction to heptanol. Surprisingly, [1-$^3H_1$]heptanal afforded [1-$^3H_1$]heptan-1-ol of considerably higher specific activity than that of the starting material; the alcohol was *ca.* 1.6 times more radioactive than expected.[1]

RESULTS AND DISCUSSION

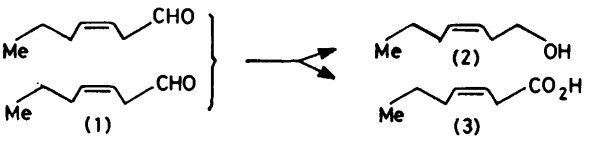
The first possible explanation considered was that [1-$^3H_1$]heptanal might be undergoing partial oxidation to heptanoic acid by oxygen or hydrogen peroxide, both of which were available in the reaction mixture.[1] Such an oxidation would be expected to enrich tritium in the *unchanged* aldehyde which could then be trapped by reduction. This expectation follows (*a*) from the fact that tritium was at tracer level and (*b*) from the reasonable assumption that oxidation of the aldehyde would involve a kinetic isotope effect in breaking the C–H rather than the C–T bond.

This possibility was tested by accurate measurement of the oxygen consumed during the incubations. Scheme 1 shows the interlocking set of reactions involved and it



Scheme 1 (*i*) amine oxidase; (*ii*) catalase; (*iii*) liver alcohol dehydrogenase

can be seen that the net requirement for oxygen is 0.5 mol, due to conversion of the initially formed hydrogen peroxide into oxygen and water by the added catalase. The results from two parallel incubations in Warburg manometers showed an oxygen consumption of 0.51 ± 0.02 mmol per mmol of 1-aminoheptane oxidised. Yet to account by oxidation for the increase of *ca.* 60% in the tritium content (found in experiments 1 and 2 of Table 1 in the preceding paper), would require a further 0.2 mmol of oxygen even assuming the maximum likely



Scheme 2

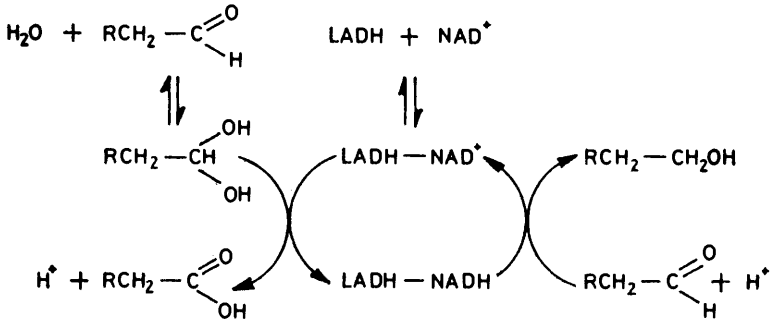
kinetic isotope effect K_H/K_T of 14. Still more additional oxygen would be needed if the isotope effect were lower. Thus, this first possible explanation of high tritium content was eliminated.

The clue to the true explanation came from studies which also gave direct evidence for the nature of the process involved. In these, the reduction of heptan-1-al was attempted using liver alcohol dehydrogenase and the coenzyme NAD$^+$ coupled to propan-2-ol as the reducing agent. This was tried, despite propan-2-ol being a poor substrate for the enzyme [2] because the highly effective secondary alcohols cyclohexanol [3] and cyclopentanol [4] would have been difficult to separate from the required product, heptan-1-ol. The run based on propan-2-ol slowly yielded heptan-1-ol, as shown by g.l.c. analysis, and this product was finally isolated in 32% yield. When [2-^{2}H]propan-2-ol was used in an attempted transfer of deuterium to (*Z*)-hept-3-enal (1) with ethanol-free liver alcohol dehydrogenase and NAD$^+$, the aldehyde was steadily used up and (*Z*)-hept-3-en-1-ol (2) was formed, at about the same rate as in the foregoing run with unlabelled propan-2-ol. The yield of the alcohol (2) was again low (28%) and, importantly, the alcohol carried no detectable amount of deuterium.

It was thus clear that the [2-^{2}H]propan-2-ol was not involved in the enzymic reaction and that we were observing the dismutation shown in Scheme 2, which has the same outcome as the well known Cannizzaro reaction. Work-up for acidic materials indeed afforded (*Z*)-hept-3-enoic acid (3) in 27% yield. Confirmation of this view of the process came from runs in which heptan-1-ol and heptanoic acid were enzymically produced from heptan-1-al in the absence of any added alcohol as reducing

agent. Conversely, heptan-1-al was recovered unchanged, even after prolonged incubations, when either liver alcohol dehydrogenase or the coenzyme NAD^+ was omitted from the reaction mixture.

A survey of the literature showed that there had been previous observations of dismutations of simple aldehydes brought about by the liver alcohol dehydrogenase–NAD^+ couple.[5-7] Studies with labelled materials had not been carried out but the stoicheiometry of the overall process has been established for formaldehyde, acetaldehyde, and butyraldehyde,[5-7] *i.e.* 1 mol each of acid and alcohol are formed from 2 mol of aldehyde. Scheme 3 shows a plausible interpretation of the earlier findings and of ours; it is based on the hydrate of the aldehyde being the substrate for the enzymic dehydrogenation.[8] Bell [9] has discussed the extent to which carbonyl compounds become hydrated in dilute aqueous solution and it appears that all simple aliphatic aldehydes of four or more carbon atoms are 15—30% in the hydrated form under physiological conditions. In addition, work reported on octanal [10] during our studies showed that the liver alcohol dehydrogenase–NAD^+ system again produced octanoic acid, but here NADH accumulated rather than being used to produce octanol. It would appear, surprisingly, that octanol is too poor a substrate for the reverse alcohol dehydrogenase reaction, but the authors did not comment on this aspect.



SCHEME 3 LADH = Liver alcohol dehydrogenase

To test further the mechanism in Scheme 3, [1-^{3}H]-heptanal was required. This was prepared by the dithian route [11] and it was treated with liver alcohol dehydrogenase and NAD^+ until the aldehyde was completely consumed. The results in the Table demonstrate complete transfer of the formyl hydrogen from one half of the sample to the other. The isolation of unlabelled heptanoic acid also shows that no scrambling of the label has occurred during the enzymic reaction. These findings and the earlier ones [5-7,9] strongly support Scheme 3. Since transfer of ' hydride ' from NADH to [1-^{3}H]heptanal produces (1*S*)-[1-$^3H_1$]heptanol whereas transfer of ' tritide ' from NAD^{3}H to unlabelled heptanal yields the (1*R*)-species, the alcohol produced in this way is the racemic (1*RS*)-[1-$^3H_1$]heptanol.

Incubation of tritiated heptanal with alcohol dehydrogenase [a] and NAD^+

[1-$^3H_1$]Heptanal [b] specific activity [e]	Expt. no.	Heptanoic acid [c] Isolated yield %	Heptanoic acid [c] Specific activity	Heptan-1-ol [d] Isolated yield %	Heptan-1-ol [d] Specific activity [e]
246	1	28	0	41	496
226	2	30	0	43	480

[a] From horse liver, ethanol-free (Sigma). [b] Radioassay as semicarbazone. [c] Characterised as the *S*-benzylthiuronium salt. [d] Radioassay as biphenyl-4-ylurethane. [e] μCi mmol^{-1}.

During various control experiments, it was found that a high buffer concentration reduced the incidence of the dismutase reaction and the omission of albumin and especially of catalase, was also helpful. These conditions were used for the later experiments in the preceding paper and they allowed the excess of tritium over the expected value to be reduced to *ca.* 12%.

This dismutation of aldehydes to acid and alcohol is not normally encountered *in vivo* [12] nor in laboratory experiments using the alcohol dehydrogenase–NADH system to prepare stereospecifically labelled alcohols from aldehydes.[1,3] In the latter case, high concentrations of an alcohol which is a good substrate for the enzyme are usually present and this normally brings about rapid reduction of the aldehyde. The occurrence of dismutation in the experiments where amine, amine oxidase, and alcohol dehydrogenase were mixed [1] is therefore interesting and merits further study.

EXPERIMENTAL

General directions are as given in ref. 1 with the following exception: gas–liquid chromatography (g.l.c.) was carried out on a Perkin-Elmer F11 analytical instrument using column I, a 7.6 m × 1-mm internal diameter glass column packed with 5% w/w Carbowax on Chromosorb W (70—80 mesh).

Initial Observations.—(*a*) Albumin (10 mg; Sigma) and NAD^+ (10 mg; free acid, Boehringer) were dissolved in a solution containing 200mM-phosphate (K^+) buffer, pH 7.0 (25 ml), distilled water (185 ml), and propan-2-ol (4 ml). This solution was added to heptanal (500 mg; 4.4 mmol) in dioxan (2 ml), the cloudy mixture was clarified by warming to 30 °C, and ethanol-free horse liver alcohol dehydrogenase (5 mg; *ca.* 1.4 units mg^{-1}, Sigma) was added. The reaction mixture was kept at 34 °C under an atmosphere of nitrogen in the dark; further alcohol dehydrogenase (5 mg) and NAD^+ (5 mg) were added at 16 h intervals. The course of the reaction was followed by g.l.c. as follows. The reaction mixture (*ca.* 0.5 ml) was shaken with a mixture of saturated

sodium hydrogen carbonate (0.5 ml), sodium chloride (200 mg), and ether (1 ml). The ether layer was dried (Na_2SO_4), concentrated to *ca.* 0.2 ml under a stream of nitrogen, and a 10-μl sample was injected onto the g.l.c. column. Using this procedure, carboxylic acids are not detected. After 48 h, no heptanal remained and only one product could be detected (column I, 110 °C, retention time 9.2 min), which corresponded to heptan-1-ol (retention time 9.2 min, one symmetrical peak on co-injection). The cloudy main solution was saturated with sodium chloride, water (30 ml) was added and the mixture was extracted continuously with ether for 18 h. The ether extract was concentrated to *ca.* 300 ml and was washed with 1% sodium hydrogen carbonate in saturated sodium chloride (3 × 10 ml), dried ($MgSO_4$), and concentrated (bath temperature < 30 °C), to give an oil (160 mg, 32%). Treatment with biphenyl-4-ylisocyanate as described previously [1] gave the biphenyl-4-ylurethane derivative of heptan-1-ol, m.p. and mixed m.p. with authentic material 104—105 °C.

(*b*) Acetone (9.0 ml, 4.55 mol equiv.) was reduced with lithium aluminium deuteride (1.0 g; >99.9% D_4) in ether (25 ml) and the reaction mixture was worked up as described previously for a related case [1] to give, finally, [2-^{2}H]propan-2-ol (4.15 g, 72%) in water (25 g). To this solution was added 70mM-phosphate (K^+) buffer, pH 7.0 (65 ml), albumin (50 mg; Sigma), NAD^+ (20 mg; free acid), and distilled water (420 ml). This solution was added to (*Z*)-hept-3-enal (600 mg, 5.35 mmol) in dioxan (3 ml), the cloudy solution was clarified by warming to 30 °C, and ethanol-free horse liver alcohol dehydrogenase (30 mg; Sigma) was then added. The reaction mixture was agitated gently at 30 °C under nitrogen in the dark for 16 h, when an aliquot was shown by g.l.c. as above to still contain the starting aldehyde (25%). More NAD^+ (10 mg) and the alcohol dehydrogenase (10 mg) were added and the reaction mixture was kept as before for a further 12 h, when analysis showed the absence of the starting aldehyde. The reaction mixture was worked up as for (*a*) above, except that the organic extracts were not washed with sodium hydrogen carbonate. The residual oil (500 mg) was deduced to be a *ca.* 1 : 1 mixture of unlabelled (*Z*)-hept-3-en-1-ol and the corresponding (*Z*)-hept-3-enoic acid; δ (CCl_4) 7.5 (2 H, br s, OH and CO_2H), 5.5 (4 H, m, *cis*-CH=CH), 3.35 (2 H, t, alcohol 1-H_2), 3.03 (2 H, d, *J* 5 Hz, acid 2-H_2), 2.3 (2 H, m, alcohol 2-H_2), 2.0 (4 H, m, 2 × 5-H_2), 1.4 (4 H, m, 2 × 6-H_2), and 0.91 (6 H, t, 2 × 7-H_3).

The above oil was partitioned between ether and saturated sodium hydrogen carbonate, the separated extracts were back-extracted with more of the appropriate phase, and the corresponding layers combined. The ether extract was dried (Na_2SO_4) and evaporated to give (*Z*)-hept-3-en-1-ol (140 mg, 28%) which gave after purification by p.l.c. on silica gel as above, a biphenyl-4-yluretane derivative, identical with authentic material.[1] The hydrogen carbonate extract was acidified with 3N-sulphuric acid (to pH 1) and was extracted with ether (3 × 30 ml). The ether extract was washed with saturated sodium chloride, dried (Na_2SO_4), and evaporated to give an oil which was bulb-to-bulb distilled (120 °C, 20 mmHg) to give (Z)-*hept-3-enoic acid* (135 mg, 27%) as a colourless oil (Found: M^+, 128.083 9. $C_7H_{12}O_2$ requires: *M*, 128.083 7; $\lambda_{max.}$ end absorption only; $\nu_{max.}$ (liquid film) 3 500—2 400, 1 705, and 1 220 cm^{-1}; δ (CCl_4) 11.2 (1 H, br s, CO_2H), 5.6 (2 H, m, *cis*-CH=CH), 3.1 (2 H, d, *J* 5 Hz, 2-H_2), 2.0 (2 H, m, 5-H_2), 1.4 (2 H, m, 6-H_2), and 0.90 (3 H, t, *J* 7 Hz, 7-H_3).

Enzymic Control Runs.—(*a*) A solution containing heptanal (250 mg, 2.19 mmol), dioxan (1 ml), 70mM-phosphate (K^+) buffer pH 7.0 (20 ml), and distilled water (180 ml) was kept in the dark under nitrogen at 30 °C for 36 h. Analysis of the reaction mixture by g.l.c. as usual showed that no heptan-1-ol was present, and that the heptanal remained. The reaction mixture was worked up as above to give heptanal (190 mg, 76%), which gave a semicarbazone, m.p. and mixed m.p. with authentic material 109—110 °C (lit.,[13] 109 °C). Acidification of the hydrogen carbonate washings from the work-up gave no organic material.

(*b*) A parallel experiment to (*a*) was carried out in which a solution comparable with that above was prepared and was then treated with albumin (14 mg), NAD^+ (8 mg) and ethanol-free horse liver alcohol dehydrogenase (10 mg; Sigma) before the solution was incubated and monitored by g.l.c. as before. The concentration of heptanal decreased rapidly over the first 12 h and then more slowly, while the concentration of heptan-1-ol increased slowly after 12 h, and the reaction was complete after a total of 48 h (absence of heptanal). The reaction mixture was worked up as above to give heptan-1-ol (112 mg, 45%), characterised as it biphenyl-4-ylurethane, m.p. 104—105 °C, together with heptanoic acid (75 mg, 30%), characterised as its *S-benzylthiuronium salt*, m.p. and mixed m.p. with the salt prepared from authentic heptanoic acid 150—151 °C (from ethanol) (Found: C, 60.9; H, 8.4; N, 9.4. $C_{15}H_{24}N_2O_2S$ requires C, 60.8; H, 8.2; N, 9.45%).

Quantitative Investigation using [1-^{3}H$_1$]*Heptanal.*—(*a*) 2-*Hexyl*-1,3-*dithian.* A stirred solution of heptanal (30 ml, 0.30 mol) and propane-1,3-dithiol (34.2 g, 0.30 mol) in chloroform (250 ml) at 0 °C was treated with a slow stream of dry hydrogen chloride for 15 min, and stirring was continued for a further 45 min, when two layers had separated. The mixture was washed with water, 1N-sodium hydroxide (3 × 50 ml), and saturated sodium chloride, dried (Na_2SO_4), and evaporated. The residual oil was distilled to give 2-*hexyl*-1,3-*dithian* (44.6 g, 73%), b.p. 82 °C at 0.1 mmHg (Found: C, 58.7; H, 9.7; S, 31.8%; M^+, 204. $C_{10}H_{20}S_2$ requires C, 58.8; H, 9.8; S, 31.4%; *M*, 204); δ 4.03 (1 H, t, 2-H), 2.8 (4 H, m, 4- and 6-H_2), 2.2—1.1 (12 H, m, CH_2), and 0.89 (3 M, distorted t, Me); *m/e* 204 (M^+, 100), 129 (45), 119 (95), and 106 (35).

(*b*) [2-^{3}H]-2-*Hexyl*-1,3-*dithian.* Tritiated water (30 mg, 1.67 mmol; 150 mCi) was transferred by vacuum line into trifluoroacetic anhydride (350 mg, 1.67 mmol); the tube was sealed and kept under vacuum for 16 h at 20 °C to yield [^{3}H]trifluoroacetic acid. A solution of n-butyl-lithium (15% w/w in hexane; 19.1 ml, 27.5 mmol) was added by syringe through a serum cap to a stirred solution of 2-hexyl-1,3-dithian (5.1 g, 25 mmol) in dry tetrahydrofuran (80 ml) at −78 °C under dry nitrogen. The coolant was changed to solid CO_2–CCl_4 and after the internal temperature had been maintained at −23 °C for a further 2 h an aliquot (20 ml, *ca.* 25%) was withdrawn and quenched with D_2O (1.5 ml). The remainder was treated with trifluoroacetic acid (150 mg; 1.3 mmol) followed after 5 min with the foregoing [^{3}H$_1$]trifluoroacetic acid (190 mg; 1.67 mmol; 60 mCi). Water (2 ml) was then added and the solution was warmed to 20 °C and evaporated. The residue, in pentane, was washed with cold water, saturated sodium hydrogen carbonate, and saturated sodium chloride, and then dried and evaporated to give an oil (3.2 g). Distillation gave [2-^{3}H]-2-hexyl-1,3-dithian (1.25 g, 44%; *ca.* 16 mCi), b.p. 82—83 °C at 0.1 mmHg.

The aliquot which was treated with D_2O was worked up similarly to give the deuteriated dithian (0.8 g, 16%) which had 0.8 ± 0.05 atom deuterium at C-2 (by n.m.r.).

(*c*) [1-^{3}H]*Heptanal.* Ammonium ceric nitrate (11.0 g; 20 mmol) was added in one portion to a vigorously stirred solution of [2-^{3}H]-2-hexyl-1,3-dithian (1.0 g; 5 mmol) in water–acetonitrile (1 : 3) (32 ml).[14] After 3 min, the solution was diluted with ice–water (100 g) and the mixture was extracted with pentane (1 × 50, 2 × 25 ml). The pentane extract was filtered, washed with water, saturated sodium hydrogen carbonate, and saturated sodium chloride, dried, and evaporated (at 0 °C) to an oil (0.5 g) which was diluted with redistilled heptanal (2.0 g). Bulb-to-bulb distillation (70—80 °C, 25 mmHg) gave [1-^{3}H]heptanal (1.9 g, 16.7 mmol; 4.1 mCi).

The semicarbazone crystallised from toluene as plates (246 μCi mmol^{-1}), m.p. 109—110 °C; δ 9.72 (0.85 H, br s, NH), 8.7 (0.15 H, br s, NH), 7.11 (0.85 H, t, *J* 6 Hz, 1-H), 6.45 (0.15 H, t, *J* 6 Hz, 1-H), 6.0—5.2 (2 H, br m, NH_2), 2.2 (1.7 H, m, 2-H_2), 1.92 (0.3 H, m, 2-H_2), 1.3 (8 H, m, $[CH_2]_4$), and 0.8 (3 H, distorted t, 7-H_3). Recrystallisation from ethanol gave unchanged material, m.p. 109—110 °C; the mixture of stereoisomers was identical with that for the unlabelled material prepared from authentic heptanal.

(*d*) *Incubation of* [1-^{3}H]*heptanal with the enzyme system.* Freshly prepared [1-^{3}H]heptanal (250 mg; 246 μCi mmol^{-1}) was incubated as under *Enzyme Control Runs* (*b*) and after all heptanal had been consumed (g.l.c.) the mixture was worked up as before to give radioinactive heptanoic acid (70 mg, 28%), characterised as its *S*-benzylthiuronium salt, m.p. 150—151 °C, and [1-$^3H_1$]heptan-1-ol (105 mg, 41%). The biphenyl-4-ylurethane derivative was prepared as before, m.p. and mixed m.p. 103.5—105 °C; repeated recrystallisation showed a constant specific activity of 496 μCi mmol^{-1}.

Repetition on a separate preparation of heptanal gave similar results: [1-^{3}H]heptanal (226 μCi mmol^{-1}) was converted as above into [1-$^3H_1$]heptan-1-ol (43%; 480 μCi mmol^{-1}) and radioinactive heptanoic acid (30%).

We thank Dr. P. J. Williams for valuable samples, St. Catharine's College, Cambridge, for a Research Fellowship (to D. G. B.), and the Nuffield Foundation, the S.R.C., and Roche Products Ltd. for financial support.

[8/2036 *Received*, 22*nd November*, 1978]

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