Kinetics of Alcohol Dehydrogenases, Studied with the Aid of a Fluorescence Recorder

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The kinetics of reactions (1), (2) and (3)

$$\text{DPNH} + \text{ADH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{DPNH}\cdot\text{ADH} \quad (1)$$

$$\text{DPNH}\cdot\text{ADH} + \text{CH}_3\text{CHO} + \text{H}^+ \underset{k_4'}{\overset{k_4}{\rightleftharpoons}} \text{DPN}\cdot\text{ADH} + \text{C}_2\text{H}_5\text{OH} \quad (2)$$

$$\text{DPN}\cdot\text{ADH} \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \text{DPN} + \text{ADH} \quad (3)$$

have previously been studied spectrophotometrically [1,2].

From the formula derived for the initial reaction velocity at $t = 0$

$$\frac{1}{e} \times \frac{da}{dt} = \frac{1}{\frac{1}{k_1 x} + \frac{1}{k_4 a}\left(1 + \frac{k_2}{k_1 x}\right) + \frac{1}{k_3}} \quad (4)$$

it is evident that all the velocity constants can be determined from initial reaction velocities if x and a can be varied within wide enough limits, and e is kept low. Since the whole system is perfectly reversible, a corresponding formula can be obtained for the reverse reaction: ADH + DPN + alcohol.

We have now found that fluorescence measurements are much more sensitive than spectrophotometric measurements, not only with flavin systems [3,4] but with DPNH systems as well. DPN does not fluoresce, but DPNH does, and this fluorescence is not quenched by the coupling of DPNH to ADH. With [DPNH] = 10^{-6} M deflections of 5 inches are observed in our apparatus with S = 0.1 and the narrowest light entrance slit (1 mm). Larger slits give proportionally higher deflections. The light absorption of this solution in 1 cm layer would be as low as $\log I_0/I = 0.006$. All the velocity constants were determined for liver ADH at pH 7.15 and 10.2; at 23.5° and 36°; and in phosphate buffers with or without 0.15 M NaCl. For yeast ADH, which is unstable at pH 10, the investigations have so far been restricted to pH 7.15. Some interesting effects have been observed.

Liver ADH: Increasing the pH from 7.15 to 10.2 at 23.5° in 0.1 M phosphate decreases k_1 ("on velocity" for DPNH) from 6.6×10^6 to 0.148×10^6; increases k_2 ("off velocity" for DPNH) from 2.7 to 5.8; scarcely changes k_5 ("on velocity" for DPN): 0.19×10^6 to 0.16×10^6; and *decreases* k_3 ("off velocity" for DPN) from 66 to 0.74. k_4 decreases from 390×10^3 to 5.6×10^6, k_4' from 9.5×10^3 to 1.6×10^3 *.

0.15 M NaCl decreases the "on velocities" (k_1 and k_5) to about half, both at pH 7.15 and pH 10.2: increases k_2 by 50% at pH 7.15 but *decreases* k_3 from 66 to 18 at pH 7.15. These effects are much less pronounced at pH 10.2. Chloride has no effect on k_4 at 23.5°, but reduced k_4' from 9.5 to 3.7×10^3. The temperature coefficient is in some cases positive, in others negative.

The *yeast ADH* differs very much from the liver ADH. Chloride decreases both k_1 and k_5, but does not interfere with the other velocity constants.

Discussion. As a result of these complicated changes it happens that chloride may stimulate or inhibit the reaction velocity in the liver ADH system, depending on the concentration of the different components. For example, if in a mixture of ADH + DPN + ethanol at 23.5° and pH 7.15 both [DPN] and [ethanol] are high, chloride increases the reaction velocity, which is then dependent mainly on k_2 that is increased by chloride. But chloride inhibits if either [DPN] or [ethanol] are low,

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* The equilibrium constant with small amounts of ADH is expressed as

$$K = \frac{[\text{CH}_3\text{CHO}]\,[\text{DPNH}]\,[\text{H}^+]}{[\text{C}_2\text{H}_5\text{OH}]\,[\text{DPN}]} = 10^{-11}$$

In the presence of ADH in excess, the equilibrium constant will be

$$K_E = \frac{k_4'}{k_4} \times [\text{H}^+]$$

because the decreasing effects of chloride on k_5, respectively k_4' then predominate. Such effects would have been hard to understand before the theory of the system was developed.

Finally, we can now calculate the influence of any of the variables on the redox potential of a solution in equilibrium. For instance, if DPN, DPNH and ADH, all in the concentration 10^{-4} *M*, were mixed with ethanol, 2×10^{-3} *M*, the system would have a potential 34 mV higher than for the same system at low [ADH]. If now 0.15 *M* NaCl were added, the potential would drop not less than 9 mV. We think such anion effects may be of physiological importance in regulating the potentials in the living tissues, where chloride-free cells are surrounded by chloride containing intercellular fluid.

The effects of chloride seem to differ considerably in various enzyme-coenzyme systems. For instance, the redox potential of the yeast ADH system is not influenced by chloride. For the oxidized form of the old yellow ferment the "off" velocity constant is increased by chloride, whereas it is decreased for the DPN · ADH complex.

The complete data will be given in a forthcoming paper in this journal.

1. Theorell, H. and Bonnichsen, R. *Acta Chem. Scand.* **5** (1951) 1105.
2. Theorell, H. and Chance, B. *Acta Chem. Scand.* **5** (1951) 1127.
3. Theorell, H. and Nygaard, A. P. *Acta Chem. Scand.* **8** (1954) 877.
4. Theorell, H. and Nygaard, A. P. *Acta Chem. Scand.* (*In press*).

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Studies on Antimetabolites

IV. Synthesis of the α,α-Dimethyl Analogue of (+)-Panthothenic Acid *

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Following up our investigations on the possibility of designing antimetabolites by the introduction of a suitably situated *gem*dimethyl group into the molecule of an essential metabolite (outlined in Part I[1] of this series), we have now prepared the α,α-dimethyl analogue (I) of (+)-panthothenic acid (II).

The synthetical method used was essentially adopted from Kuhn and Wieland's synthesis [2] of panthothenic acid, the purification of the crude material being, however, carried out in a different way. (−)-Pantholactone was condensed with the benzylester (III) of α,α-dimethyl-β-alanine, the ester group was removed by hydrogenolysis, and the crude acid, which could not be induced to crystallise, was converted into its calcium salt, which was obtained as a microcrystalline powder. The intermediate benzylester (III) was prepared from α,α-dimethyl-β-alanine *via* the hydrochloride of α,α-dimethyl-β-alanyl-chloride, obtained from the amino acid and phosphorus pentachloride in acetyl-chloride solution. No attempt was made to improve the rather low yield of panthothenic acid analogue.

Preliminary tests with *Lactobacillus arabinosus* 17-5, using an agar cup method, showed that the panthothenic acid analogue inhibited the growth of this organism, and that this inhibition was competitively reversed by addition of (+)-panthothenic acid. The inhibition index was about 10 000. A detailed report will be published by Dr. A. Bolinder, Department of Food Chemistry, Royal Institute of Technology, Stockholm.

Experimental *. *Benzyl α,α-dimethyl-β-alaninate hydrochloride.* Ethyl β-formamino-α,α-dimethylpropionate [3] (60 g) and 20 % hydrochloric acid (150 ml) were refluxed overnight. The mixture was evaporated to dryness *in vacuo*, the dry salt extracted twice with ether, powdered, and added in small portions to acetyl chloride (400 ml) with shaking. When all had been added, finely ground phosphorus pentachloride (80 g) was added and the stoppered flask shaken overnight. Anhydrous ether (750 ml) was added and the precipitated solid was centrifuged and repeatedly washed with dry ether, mixed with benzyl alcohol (100 ml) and heated on the water bath until the evolution of hydrogen chloride ceased. The solution was filtered and diluted with much dry ether, and the precipitated ester hydrochloride filtered off and washed with ether. For purification the product was dissolved in the minimum amount of dry acetone, filtered, and reprecipitated by the addition of much ether. Lustrous plates, m.p. 94.5—96°. Yield 41 g. (Found:

* Part III. *Acta Chem. Scand.* **8** (1954) 1389.

* All melting points uncorrected.