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**Note**

# Gas chromatographic study of the histochemical reaction for isopropanol dehydrogenase

I. PLOC and L. STÁRKA

*Research Institute of Endocrinology, 116 94 Prague 1 (Czechoslovakia)*

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The histochemical reaction typical for some steroidogenic tissues and based on the oxidation of isopropanol was introduced by Hardonk in 1965[1]. He called the reaction, now generally known as "isopropanol dehydrogenase", a "secondary" alcohol dehydrogenase", because the system also oxidized other secondary alcohols, though not so extensively as isopropanol. The reaction was not identical with those concerning secondary alcohol groups on the steroid nucleus, *e.g.* 3$\beta$-hydroxysteroid dehydrogenase (EC 1.1.1.51), which often showed an inversed localization in the steroidogenic tissues. Hardonk suggested that the enzyme could be connected with the oxidation of the secondary alcohol groups in the cholesterol side-chain leading to the cleavage of the side-chain.

Though the reaction is widely used for the characterization of adrenal and gonadal tissues, its histochemical back-ground and subcellular localization have remained obscure until now.

## MATERIALS AND METHODS

### *Tissue*

Male Wistar rats weighing 200 $\pm$ 30 g were used in groups of 20–30 animals for each experiment. The animals were killed in ether anaesthesia and the adrenals were removed, cleaned and kept in ice-cold Krebs–Ringer buffer, pH 7.2. The adrenals, of average weight 25–30 mg, were pooled from the whole experimental group, homogenized 5 min at 0° in 0.25 *M* sucrose (in tissue–medium ratio 1:5, w/v) in an all-glass homogenizer.

The subcellular fractions were prepared by centrifugation in 0.25 *M* sucrose at 0° in a Beckman-Spinco Model L2-50B ultracentrifuge according to the method of Breuer and Knuppen[2]. The mitochondrial fraction was separated at 20,000 *g* for 30 min and the microsomal fraction and cytosol at 105,000 *g* for 60 min, repeated twice. The particulate fractions were washed and recentrifuged.

### *Incubations*

In each experiment 10 $\mu$mol of substrate (aliphatic alcohol or ketone) were incubated in 3 ml of Krebs–Ringer phosphate buffer, pH 7.0 or 7.6. Alternatively 0.2 *M* Tris–maleate buffer, pH 6.0–8.6, was used for the examination of pH d-

pendence. The incubation mixture was supplemented with 1 μmol of $NAD^+$, NADH or $NADP^+$, and the reaction was started by the addition of the adrenal subcellular fraction in 1 ml of 0.25 *M* sucrose. The protein concentration varied in individual experiments and was determined by the method of Lowry *et al.*[3]. The incubation was carried out at 37.0° ± 0.2° under air in glass-stoppered flasks under constant shaking for 60 min. It was stopped by extraction into 4 ml of ethyl acetate, except for the experiments with ethanol as substrate when extraction was into methyl ethyl ketone.

*Gas chromatography*

Alcohols and ketones were determined by gas chromatography using a flame-ionization detector and a Packard Model 871 gas chromatograph. The columns were 100 × 0.25 cm I.D. The column temperature was 130°, the inlet and outlet temperatures 180°. For ethanol, acetaldehyde, isopropanol and acetone the column was packed with Porapak Q (80–100 mesh, batch No. 690), for 4-methyl-1-pentanol, 4-methyl-1-pentanal, 6-methyl-2-heptanol and 6-methyl-2-heptanone with Porapak P (80–100 mesh, batch No. 800). The carrier gas was nitrogen. The inlet pressure was 55 $N \cdot m^{-2}$ for Porapak Q, and 38 $N \cdot m^{-2}$ for Porapak P. The detector sensitivity ranged from 1 to $3 \cdot 10^{-11}$ A. The amount of the extract of the incubation medium injected was 10 μl.

The yields of alcohols and ketones were corrected for extraction losses according to distribution coefficients determined by gas chromatography.

## RESULTS

*Gas chromatographic method*

The retention times for the alcohols and corresponding carbonyl compounds are listed in Table I. The reliability data are summarized in Table II. The method allowed the metabolic fate of the alcohols and ketones during incubation to be monitored with sufficient sensitivity, accuracy and precision.

TABLE I

RETENTION TIME DATA FOR ALCOHOLS AND CORRESPONDING CARBONYL COMPOUNDS

| $C_n$ | *Compound* | *Retention time (min)* | *Column* |
|---|---|---|---|
| $C_2$ | Acetaldehyde | 3.6 | Porapak Q |
| | Ethanol | 5.6 | Porapak Q |
| $C_3$ | Acetone | 9.2 | Porapak Q |
| | Isopropanol | 12.0 | Porapak Q |
| $C_6$ | 4-Methyl-1-pentanal | 7.6 | Porapak P |
| | 4-Methyl-1-pentanol | 11.6 | Porapak P |
| $C_8$ | 6-Methyl-2-heptanone | 18.0 | Porapak P |
| | 6-Methyl-2-heptanol | 32.0 | Porapak P |

*Biochemical characterization of the "secondary alcohol dehydrogenase" from rat adrenals*

*Subcellular localization and cofactor requirements.* To localize the enzyme activity, $C_2$, $C_3$, $C_6$ and $C_8$ alcohols were incubated with cytoplasma, microsomes and

TABLE II

CRITERIA OF RELIABILITY OF THE GAS CHROMATOGRAPHIC DETERMINATION OF ALCOHOLS AND ACETONE FROM THE INCUBATION SAMPLES

| *Criterion* | *Compound* | | | | |
|---|---|---|---|---|---|
| | *Ethanol* | *Acetone* | *Isopropanol* | *4-Methyl-1-pentanol* | *6-Methyl-2-heptanol* |
| Losses at incubation and extraction [mean ± S.D. (%)] | 11.6 ± 4.2 | 7.2 ± 3.5 | 9.5 ± 3.8 | 3.9 ± 2.8 | 3.0 ± 2.5 |
| Coefficient of variation (%)* | 2.4 | 2.1 | 2.0 | 2.6 | 2.5 |
| Sensitivity (ng) | 4.0 | 4.0 | 4.0 | 2.0 | 2.0 |

* From 10 determinations at the concentration 0.1 $g \cdot l^{-1}$.

mitochondria of the rat adrenals using $NAD^+$ as cosubstrate. As shown in Table III the primary alcohols, ethanol and 4-methyl-1-pentanol, were preferentialy oxidized in cytoplasma, and the secondary alcohols, isopropanol and 6-methyl-2-heptanol, in mitochondria. The microsomal alcohol dehydrogenase activity was negligible in both types of alcohol, and this subsellular fraction was not further investigated.

TABLE III

SUBCELLULAR LOCALIZATION OF THE OXIDATION OF PRIMARY AND SECONDARY ALCOHOLS IN RAT ADRENALS

Each alcohol (10 $\mu$mol) was incubated for 20 min at 37° in 3 ml of Krebs–Ringer phosphate buffer at pH 7.6 with 1 $\mu$mol $NAD^+$ and subcellular adrenal fraction equivalent to 120 ng of rat adrenals in 1 ml of 0.25 *M* sucrose.

| *Compound* | *Rate of alcohol oxidation (nmol/min)* | | |
|---|---|---|---|
| | *Mitochondria** | *Cytoplasma*** | *Microsomes**** |
| Ethanol | 44.8 | 94.3 | 0.0 |
| Isopropanol | 58.0 | 21.7 | 4.3 |
| 4-Methyl-1-pentanol | 41.0 | 79.5 | 0.0 |
| 6-Methyl-2-heptanol | 76.9 | 3.8 | 3.8 |

* 6.50 mg of protein per incubation flask.
** 5.88 mg of protein per incunation flask.
*** 0.78 mg of protein per incubation flask.

The cofactor requirements are shown in Table IV.

*Time dependence of the enzyme activity.* The time course of the reaction was followed for 60 min at 5-min intervals. Kinetic analysis of the data revealed that under the conditions used, the reaction is first order with respect to the substrate. The apparent velocity constants for the enzyme reaction in cytosol and mitochondria are summarized in Table V.

*Effect of pH on the enzyme activity.* Optimal pH values for the oxidation of the alcohols in subcellular fractions of rat adrenals were determined in Tris–maleate buffer in the range 6.0–8.6. The pH optimum was a relatively broad one for the oxidation of all alcohols investigated: in mitochondria it was in the range 6.4–7.0, in cytoplasma at pH 8.2.

TABLE IV

COFACTOR REQUIREMENTS OF THE ENZYME ACTIVITY

Alcohol (10 μmol) was incubated under conditions given in Table III.

| *Compound* | *Rate of oxidation (nmol/min)* | | | |
|---|---|---|---|---|
| | *Mitochondria** | | *Cytoplasma*** | |
| | *Cofactor: NAD⁺* | *Cofactor: NADP⁺* | *Cofactor: NAD⁺* | *Cofactor: NADP⁺* |
| Ethanol | 47.1 | 49.4 | 101.1 | 71.3 |
| Isopropanol | 55.1 | 84.1 | 21.7 | 20.3 |
| 4-Methyl-1-pentanol | 41.0 | 53.8 | 84.6 | 59.0 |
| 6-Methyl-2-heptanol | 76.9 | 80.8 | 11.5 | 3.8 |

* 6.55 mg of protein per incubation flask.
** 5.80 mg of protein per incubation flask.

TABLE V

APPARENT VELOCITY CONSTANTS FOR OXIDATION OF ALCOHOL IN RAT ADRENAL SUBCELLULAR FRACTION

| *Substrate** | *Subcellular fraction, $k_1$ ($sec^{-1}$)* | |
|---|---|---|
| | *Mitochondria* | *Cytoplasma* |
| Ethanol | $0.065 \cdot 10^{-3}$ | $0.233 \cdot 10^{-3}$ |
| 4-Methyl-1-pentanol | $0.070 \cdot 10^{-3}$ | $0.138 \cdot 10^{-3}$ |
| Isopropanol | $0.247 \cdot 10^{-3}$ | $0.075 \cdot 10^{-3}$ |
| 6-Methyl-2-heptanol | $0.107 \cdot 10^{-3}$ | $0.005 \cdot 10^{-3}$ |

* $NAD^+$ was used as cosubstrate at pH 7.6.

*Menten–Michaelis constants.* The Menten–Michaelis constants were determined for the systems that optimally oxidized the alcohols: for primary alcohols it was cytoplasma with $NAD^+$ as cosubstrate at pH 8.2 and for secondary alcohols mitochondria supplemented with $NADP^+$ at pH 6.4. The results are shown in Table VI. The constants for primary alcohols are approximately one order lower than those for secondary alcohols.

TABLE VI

MENTEN–MICHAELIS CONSTANTS RELATIVE TO SUBSTRATE AND COFACTOR

| *Alcohol* | *Cofactor* | *Menten–Michaelis constant, $K_m$* | |
|---|---|---|---|
| | | *Relative to substrate* | *Relative to cofactor* |
| Ethanol | $NAD^+$ | $0.24 \cdot 10^{-3}$ | $0.12 \cdot 10^{-3}$ |
| Isopropanol | $NADP^+$ | $1.78 \cdot 10^{-3}$ | $1.70 \cdot 10^{-3}$ |
| 4-Methyl-1-pentanol | $NAD^+$ | $0.71 \cdot 10^{-3}$ | $0.54 \cdot 10^{-3}$ |
| 6-Methyl-2-heptanol | $NADP^+$ | $2.59 \cdot 10^{-3}$ | $2.48 \cdot 10^{-3}$ |

## DISCUSSION

From the data obtained it can be seen that in the rat adrenal several (at least two) alcohol dehydrogenases exist. The cytoplasmatic alcohol dehydrogenase prefers primary alcohols and $NAD^+$ as cosubstrate and its pH optimum is in the alkaline

range. In this respect it is similar to the alcohol dehydrogenase EC 1.1.1.1, such as found in horse liver[4]. Our results neither prove nor disprove their identity; however, the differences in cosubstrate requirements favour the latter possibility.

Mitochondrial alcohol dehydrogenase(s) is probably the enzyme that is responsible for the histochemical "isopropanol dehydrogenase" reaction. This enzyme differs from the classical alcohol dehydrogenase EC 1.1.1.1 not only in its subcellular localization but also in the preference of secondary alcohols to the primary ones. In some ways it resembles bacterial alcohol dehydrogenase EC 1.1.1.2, in which $NADP^+$ is also preferred and the pH optimum is in the range 6.4–7.0.

It may be of interest that it is mitochondria in which the cleavage of the cholesterol side-chain occurs during formation of 3$\beta$-hydroxy-5-pregnen-20-one as the main precursor of adrenal steroid hormones. In this reaction isocaproylaldehyde (4-methyl-1-pentanal) is formed from the side-chain. Other reaction pathways lead to $C_{19}$ steroids[5]. In the cholesterol side-chain cleavage, according to Jungman[6], a $C_8$ unit, 6-methyl-2-heptanone, is split off. Tait[7–9] suggested that in the so-called sesquiterpene pathway, 23,24-dinor-5-cholen-3$\beta$-ol may be utilised as a steroid hormone precursor. Splitting off a $C_3$ unit, acetone, should lead to dehydroepiandrosterone. It is possible that the secondary alcohol dehydrogenase takes part in the further metabolic reactions of the fragments formed from the side-chains of the steroid hormone precursors in the adrenals or gonads.

## CONCLUSIONS

Gas chromatography of primary and secondary alcohols and of their oxidation products may be used for the characterization of their metabolism in the subcellular fractions of rat adrenals. It can be demonstrated that besides the cytoplasmic alcohol dehydrogenase which prefers primary alcohols and $NAD^+$, there exists alcohol dehydrogenase(s) in mitochondria which differs in cosubstrate requirements, pH optimum, velocity constants and substrate affinity. This enzyme system possibly takes part in the metabolism of the fragments formed from the side-chain of the precursors of steroid hormones. This secondary alcohol dehydrogenase apparently represents the enzyme activity responsible for the histochemical "isopropanol dehydrogenase" reaction.

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