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Aldehyde dehydrogenase isoenzymes in tumours — assay with possible prognostic value for oxazaphosphorine chemotherapy*☆○

Piotr Wroczyński[1✉], Agnieszka Laskowska[1], Jacek Wierzchowski[1], Artur Szubert[2], Jerzy Polański[2] and Marian Słowiaczek[3]

[1]*Department of Physical Chemistry, Faculty of Pharmacy, S. Banacha 1, 02-097 Warsaw, Poland,*
[2]*3rd Department of Surgery, 2nd Faculty of Medicine, Medical School, Stępinska 19, 02-739 Warsaw, Poland,*
[3]*3rd Department of General Surgery, Collegium Medicum, Jagiellonian University, Prądnicka 37, 31-202 Cracow, Poland*

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A novel fluorimetric assay, allowing independent measurement of the activities of two principal cytosolic forms of human aldehyde dehydrogenase, ALDH-1 and ALDH-3 (known as a tumour-associated ALDH) was applied to estimate the activities of these isoenzymes in human liver and thyroid tumours. The assay is based on two artificial substrates, 6-methoxy-2-naphthaldehyde (MONAL-62) and 7-methoxy-1-naphthaldehyde (MONAL-71), exhibiting excellent substrate properties toward various forms of human ALDH (see Wierzchowski *et al.*, 1997, *Anal. Biochem.* 245, 69–78).

We have found significant differences in ALDH activities between malignant and non-malignant tissue fragments, particularly in cancerous livers. Out of 16 tumours examined, only 4 exhibited ALDH-1 activities comparable to that found in the tumour-free tissue (0.5–2.5 U/g), while in the remaining 12 this activity was at least 10-fold

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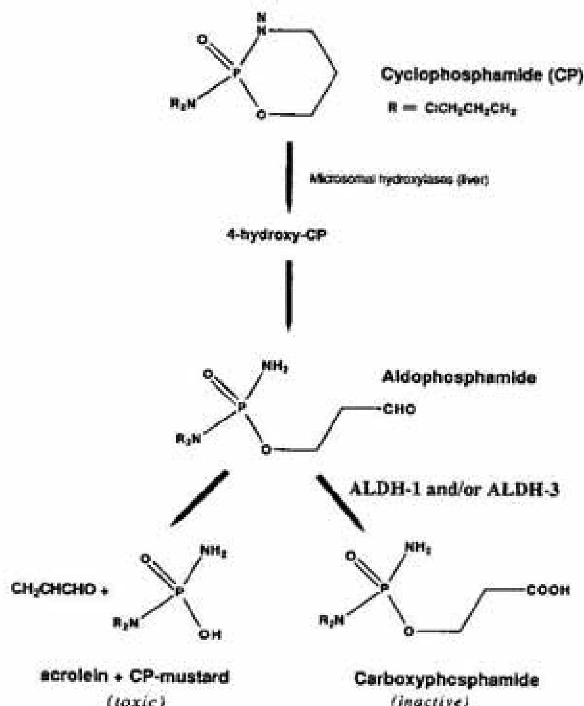
✉Dr Piotr Wroczyński, Department of Physical Chemistry, Faculty of Pharmacy, S. Banacha 1, 02-097 Warsaw, Poland; phone: (48-22) 823 6411 ext. 2310; fax: (48-22) 823 1487; e-mail: wropio@farm.amwaw.edu.pl

Abbreviations: ALDH, aldehyde dehydrogenase; MONAL-71, 7-methoxy-1-naphthaldehyde, MONAL-62, 6-methoxy-2-naphthaldehyde; DANAL-62, 6-dimethylamino-2-naphthaldehyde; DTT, dithiothreitol; C.V., coefficient of variation.

lower. The ALDH-3 activity was detectable in about 40% of both tumour and tumour-free liver samples (maximum value 1.5 U/g). Comparison of 13 pathological thyroid fragments revealed ALDH activities in the range of 0.02 to 0.35 U/g, with two malignant samples showing activities of 0.27 and 0.18 U/g. Both substrate specificity and kinetic behaviour of the thyroid ALDH (K_m values for the fluorogenic naphthaldehydes as well as propanal inhibition profile) were similar to those of the purified ALDH-1. In 5 thyroid samples traces of ALDH-3 activity was detected, using MONAL-62 and $NADP^+$ as substrates (maximum value 0.04 U/g). Possible prognostic value of the foregoing measurements for cyclophosphamide chemotherapy is discussed.

Aldehyde dehydrogenase (ALDH, EC 1.2.1.3) is a polymorphic enzyme responsible for detoxication of many drugs [1], including an alkylating agent cyclophosphamide (CP) widely utilized in cancer chemotherapy [1, 2], usually in combinations with other drugs [3]. The low ALDH activity in tumours is supposed to be crucial for the effectiveness of the CP chemotherapy [1, 2], since this enzyme inactivates aldophosphamide, a key intermediate of CP biotransformation (Scheme 1). The main ALDH isoenzyme responsible for CP inactivation and, consequently, for the cellular resistance to this drug, is the constitutive cytosolic ALDH-1 form [4], as recently confirmed by gene-transfer experiments [5], although some role of the inducible ALDH-3 isoenzyme



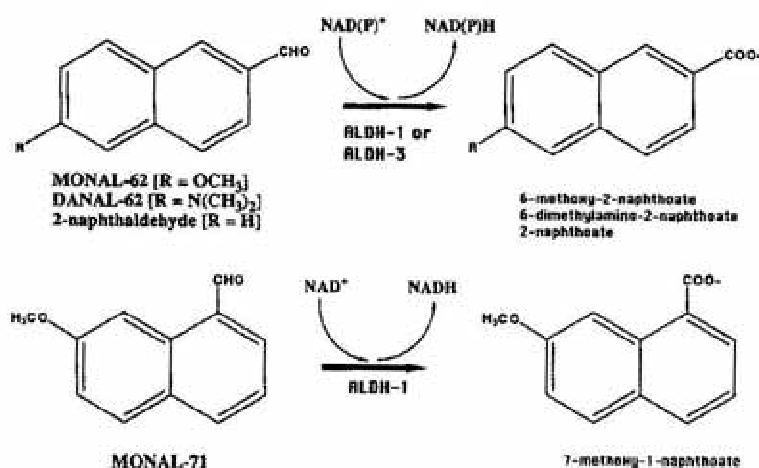
Scheme 1. Biotransformation of cyclophosphamide (adapted from ref. [2]).

(known as tumour-associated ALDH) has also been documented [6, 7]. The specific activities of the foregoing isoenzymes in tumours may therefore have a diagnostic, and possibly also a prognostic value for cancer chemotherapy.

We have recently developed selective, fluorimetric assays for both ALDH-1 and ALDH-3 activities in human tissue homogenates [8]. These assays utilize two artificial, and highly fluorogenic ALDH substrates, 7-methoxy-1-naphthaldehyde (MONAL-71) and 6-methoxy-2-naphthaldehyde (MONAL-62), acting with NAD^+ or $NADP^+$ as co-substrates, to measure selectively the activities of ALDH-1 and ALDH-3 isoenzymes, respectively. Alternatively, the less specific, but more fluorogenic 6-dimethylamino-2-naphthaldehyde (DANAL-62) can be used (Scheme 2). The foregoing substrates exhibit excellent kinetic properties toward the cytosolic ALDH forms ($K_m < 1\,\mu$M, and reaction rates comparable to those of benzaldehyde oxidation), and are much less sensitive to the ubiquitous mitochondrial ALDH-2 isoenzyme, which is responsible for most of the acetaldehyde oxidation activity in cells [8, 9].

In the present paper, we describe an application of the foregoing assays to the measurements of ALDH activity in tumor samples. We have investigated homogenates of several liver tumours, excised during the surgical operations, together with fragments of the surrounding liver tissue, as well as several samples of thyroid tumours differing in the degree of malignancy.

Liver tumours were chosen as an object of our investigations because human liver ALDH is rather well characterized in the literature [2], and its kinetic properties toward the



Scheme 2.

fluorogenic naphthaldehyde substrates are known [9]. By contrast, thyroid ALDH has not so far been characterized, but thyroid tumours, particularly lymphomas, are frequently subjected to CP chemotherapy, usually in combination with other drugs [3]. As shown below, the kinetic characteristics and substrate specificity of the ALDH activity in thyroid samples is typical for the class I enzyme, or ALDH-1, and can be easily quantified by the fluorimetric method.

MATERIALS AND METHODS

Chemicals. Syntheses of the naphthaldehyde substrates and the corresponding naphthoates have been described elsewhere [9, 10], and 2-naphthaldehyde was purchased from Merck (Darmstadt, Germany).

NAD^+, $NADP^+$, DTT, propanal and the protein micro assay kit were from Sigma (St. Louis, MO, U.S.A.), and all other chemicals were of analytical grade. Deionized and filtered water (Milli-Q system, Millipore Corp.) was used throughout.

Clinical material. Tumour and other pathological samples were obtained from the surgical operations, performed at the Clinical Hospital (Stępińska 19/25, Warsaw) (livers) and at the Collegium Medicum, Jagiellonian University (Cracow) (thyroid samples), with permission of the respective Ethical Committees. Post mortem samples (24 h) were obtained from the Department of Pathology, Central Clinical Hospital (S. Banacha 1, Warsaw). Liver fragments were stored at −80°C, and thyroid at −20°C. After thawing, samples were homogenized in sucrose solution (0.25 M), buffered with 5 mM phosphate, pH 7.5, containing EDTA (1 mM) and DTT (2 mM). The homogenates were spun at 9000 ***g*** to remove the mitochondrial fraction(s). The remaining supernatant (fraction S-9) contained 3–15 mg protein/ml, as determined by the Bradford method [11].

A purified sample of the recombinant human ALDH-1 was a gift from Dr. R.L. Blakley (St. Jude Childrens Research Hospital, Memphis, TN, U.S.A.). Its specific activity was about 260 U/g at pH 8.1 and 25°C, determined with MONAL-71 as a substrate.

Fluorimetric procedure. This procedure has been described in details elsewhere [8, 9]. Typical fluorimetric assays were run in 50 mM pyrophosphate/HCl buffer, pH 8.1, at 25°C, in the presence of 0.5 mM DTT and 0.5 mM EDTA. The pyrophosphate buffer was also used for the pH-dependence studies. Crude homogenates added corresponded to 0.03–0.2 mg protein. In the case of liver sample analysis, 0.4 mM 4-methylpyrazole was occasionally used to inhibit the alcohol dehydrogenase reaction, but it had only a minor effect on the obtained ALDH activities [8]. Typical naphthaldehyde concentrations were 3–5 μM. Purified reaction product(s) at concentrations of 2–3 μM were used as internal standards, to

obtain absolute reaction rates. Coenzymes NAD^+ and $NADP^+$ (Sigma) were used at concentrations of 100 μM and 300 μM, respectively [8], and in each case the oxidase activity was recorded prior to coenzyme addition, and subtracted from the final reaction rate. Assays were run on SPEX Fluoro-Max instruments, equipped with 150 W xenon lamp, with excitations at 330 nm for MONAL-71 and 310 nm for MONAL-62 [8], with fluorescence emission monitored at 390 nm and 360 nm, respectively.

RESULTS AND DISCUSSION

The objective of the present work was to examine the possibility of a selective assay of the two principal cytosolic forms of human ALDH, namely, ALDH-1 (constitutive) and ALDH-3 (inducible) in tumour samples, using the isoenzyme-specific fluorimetric method [8], and compare the obtained activities to that of the non-malignant tissue. Since ALDH plays a critical role in detoxication of cyclophosphamide [1, 4, 6], information on its activity in the excised tumour may be important for choosing therapeutic strategies in those cases in which chemotherapy follows surgical treatment.

In the previous paper [8] we have shown that two fluorogenic naphthaldehyde substrates, MONAL-71 and MONAL-62, acting with NAD^+ and $NADP^+$ as co-substrates, respectively, can be used to measure specific activities of ALDH-1 and ALDH-3, respectively, in a postmortem liver tissue. We now present preliminary data on the analogous ALDH activity determinations in liver and thyroid tumours, as well as on kinetic examination of the thyroid ALDH, which so far has not been characterized.

Liver tumours

We have examined a total of 19 samples of various pathological livers, mostly tumours, together with small samples of an apparently tumour-free liver tissue, both excised during the surgical treatment. Routine histopathological examinations of the tumours showed in 8 cases metastases from colon carcinoma, another 8 were diagnosed as *haemoangioma*, there were also cystes (1 case) and primary liver tumours (2 cases). In 3 cases no tumorous changes in liver were detected, but the excised liver fragments were nevertheless included for statistical purposes. Three postmortem liver samples were also examined for comparison.

It is known that the principal cytosolic ALDH form in human liver is the constitutive ALDH-1 isoenzyme, exhibiting broad substrate specificity toward both aliphatic and

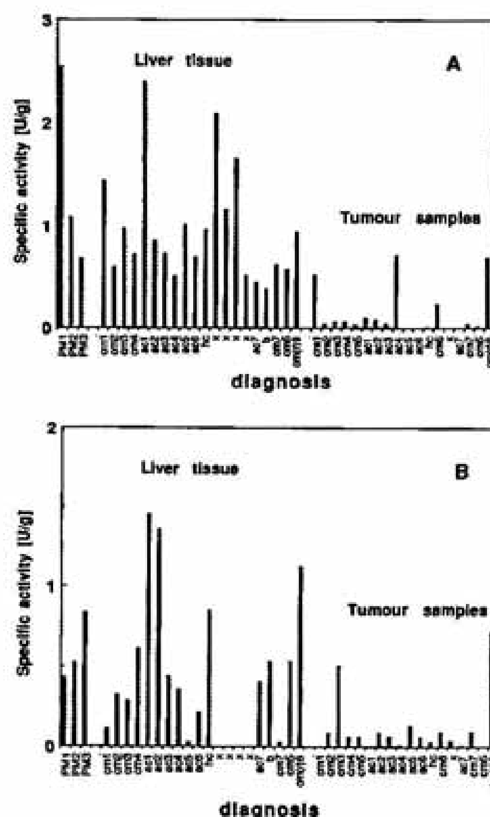


Figure 1. Specific activities of ALDH-1 (A) and ALDH-3 (B), in liver tumours and in the surrounding liver tissue, as determined by the fluorimetric method.

Three post mortem samples of non-cancerous livers (denoted PM) are given for comparison. Tumour diagnoses are: cm(r), colon carcinoma metastasis (recurrent); ac, *haemoangioma*; hc, primary liver carcinoma; b, biliary tract carcinoma; x, diagnosis unknown.

aromatic aldehydes [2, 8]. We have measured the activity of this isoenzyme, using a selective substrate, MONAL-71 [8]. A summary of the results is given in Fig. 1A, B. The activity of the ALDH-1 in tumour-free liver tissue exhibits moderate variability, ranging from 0.5 to 3 U/g, with C.V. of 53% [12]. Similar values were obtained for the postmortem samples (Fig. 1A, left). By contrast, the ALDH-1 activity in tumours was lower by approximately 1 order of magnitude (Fig. 1A, right part), with 3–4 exceptions in which this activity was close to the normal level.

In about 30% of cases, high activity of ALDH-3 in the tumour-free part of the liver was recorded (up to 1.5 U/g, see Fig. 1B, left), but in 6 cancerous livers this isoenzyme was hardly detectable. In tumours the activity of ALDH-3 was usually lower (< 0.1 U/g; with 3 exceptions), apparently in contrast to the literature data (obtained with the use of immunochemical methods [13]). The cases of high ALDH-3 activity were not necessarily coincident with those of high ALDH-1 (Fig. 1A, B).

In the tumour-free liver tissue of cancerous patients a highly variable activity of aldehyde oxidase was also detected, manifested as fluorescence background drift, observed prior to the coenzyme addition. This activity, occasionally amounting up to 30–35% of the recorded ALDH-1 activity, was the principal factor limiting the sensitivity of ALDH determinations. In tumours, as well as in the postmortem samples, this activity was significantly lower. We think therefore that the former enzyme, occasionally observed also in blood plasma (unpublished data) might have some additional diagnostic value. No ALDH activity was detected in blood plasma of the cancerous patients.

In conclusion, our data demonstrate that selective determination of both ALDH-1 and ALDH-3 activities in tumours is possible, and the obtained values differ significantly from those of the surrounding liver tissue. These values are, both in the case of ALDH-1 and ALDH-3, highly variable, and may affect the outcome of chemotherapy.

Thyroid tumours

We have examined also 13 thyroid samples excised in the course of strumectomy. Of these, 5 cases were histopathologically diagnosed as *struma nodosa*, or *struma nodosa toxica*, in 3 cases Graves-Basedov disease was

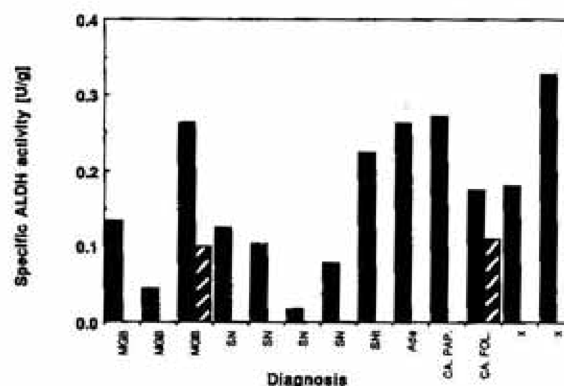


Figure 2. Specific activities of ALDH-1 in the surgically excised pathological thyroid fragments.

Activities were determined by the fluorimetric method with MONAL-71, 3 μM, as a substrate. Diagnoses are: MGB, Graves-Basedov disease; SN(t), *struma nodosa* (*toxica*); Ade, *adenoma folliculare* (non-malignant); CA. PAP. and CA. FOL. represent malignant cases of *carcinoma papillare* and *carcinoma folliculare*, respectively. Double bars represent activities of different fragments of the same samples.

detected, in 1 case *adenoma folliculare* (non-malignant), and there were 2 cases of malignant tumours: *carcinoma papillare* and *carcinoma folliculare*. Two of the examined samples were clearly morphologically heterogeneous (one case was diagnosed as Graves-Basedov disease and another as *carcinoma folliculare*), and, since it was possible to separate the two parts, differing in colour and density, they were examined separately. All the thyroid samples exhibited measurable ALDH ac-

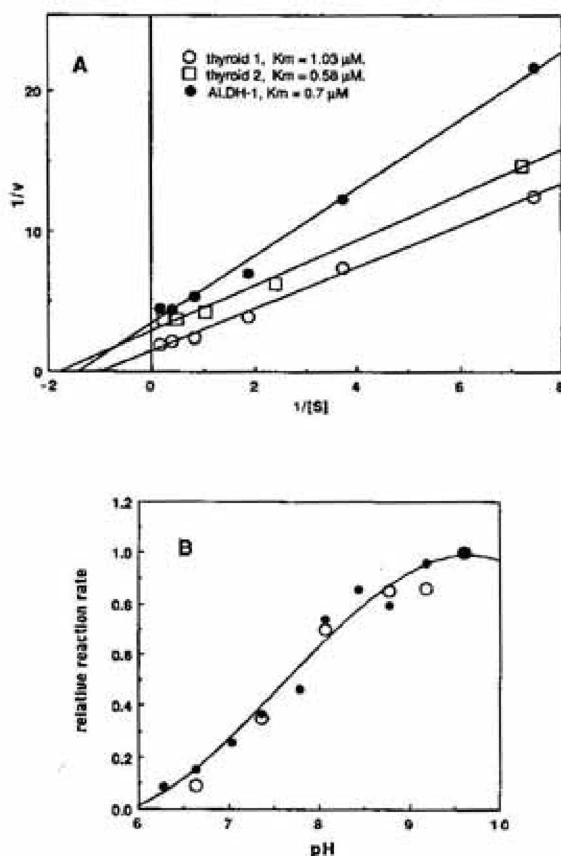


Figure 3. Kinetic characteristics of the thyroid ALDH (open symbols), as compared to that of the purified recombinant ALDH-1 (closed symbols).

Rates were measured with MONAL-71 and NAD^+ as substrates. (A) Determination of the (apparent) K_m constants; (B) Comparison of pH-dependence of the reaction rate(s) in the nearly saturating conditions (MONAL-71, 3.7 μM, NAD^+, 100 μM).

tivities, when assayed with MONAL-71 and NAD^+ as substrates, with apparent lack of both aldehyde oxidase and alcohol dehydrogenase activity (i.e., the fluorescence background was stable in the absence of NAD^+ and in the presence of NADH instead of NAD^+). The ALDH activities were approximately lower by 1 order of magnitude than those detected in the liver fragments (Fig. 2).

To better characterize the ALDH activity in thyroid, we have examined its substrate specificity and kinetic characteristics, and compared it to those of a purified sample of the recombinant ALDH-1. Comparison of the apparent Michaelis constants (K_m) for ALDH activity (measured with MONAL-71 as a substrate) in thyroid fragments with those of the purified ALDH-1 sample (Fig. 3A) demonstrated close similarity of the thyroid ALDH and ALDH-1. This was also confirmed by the comparison of the pH-dependence of the oxidation of MONAL-71 by thyroid homogenate and purified ALDH-1 (Fig. 3B), as well as by the inhibition profile, measured for the propanal inhibition of the latter reaction (Fig. 4). In the inhibition experiment, the oxidation of MONAL-71 was followed separately from the propanal oxidation thanks to the high fluorescence of 7-methoxy-1-naphthoate, not coinciding with NADH emission [9]. We therefore conclude that in thyroid fragments MONAL-71 is oxidized primarily by the ALDH-1 isoenzyme, very similar to that found in liver. This result is not unexpected, since ALDH-1 is known to be an ubiquitous enzyme, constitutively expressed in nearly all human tissues examined to date [2, 8].

Several other naphthaldehyde substrates were also examined as possible indicators of the ALDH activity in thyroid, all of them showing comparable catalytic constants (Table 1), as expected for ALDH-1 isoenzyme [9]. There was, however, an unexpectedly high activity of propanal oxidation (Table 1), suggesting the presence of some other ALDH forms, apparently inactive toward the aromatic substrates. Further investigation is needed to identify these isoenzyme(s).

Table 1. Substrate specificity of human thyroid ALDH, compared to that of the purified ALDH-1.

Rates were determined spectrophotometrically and/or fluorimetrically. Assay conditions: 50 mM pyrophosphate buffer, pH 8.1, containing 0.5 mM EDTA and 0.5 mM DTT, 100 mM NAD^+ and 4–5 μM substrate (except for propanal), at 25°C. Mean error about 10%.

Substrate	Relative activity	
	Thyroid ALDH	ALDH-1
MONAL - 71	100	100
MONAL - 62	40–210[a]	113
DANAL - 62	84	105
2-Naphthaldehyde	115	98
Propanal (100 μM)	414	about 140

[a]Two different thyroid preparations examined.

Average ALDH-1 activity in thyroid samples was found to be 0.16 U/g, with C.V. of 57%. In the most frequent cases of the (non-neoplastic) *struma nodosa*, we have observed relatively low ALDH-1 activity (0.02–0.13 U/g), with the exception of the *struma nodosa toxica*, in which this activity was 0.23 U/g. Malignant samples (*carcinoma papillare* and *folliculare*) as well as nonmalignant *adenoma folliculare* had somewhat higher values of the ALDH-1 activity (0.27, 0.17 and 0.26 U/g, respectively). Samples diagnosed as Graves-Basedov disease exhibited variable activity of ALDH-1 (0.05–0.26 U/g). It seems therefore that there is no simple relation between ALDH activity and malignancy of the sample.

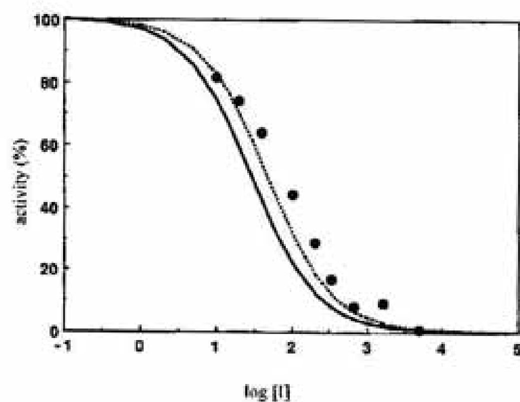


Figure 4. Inhibition of the enzymatic oxidation of MONAL-71 by thyroid homogenate in the presence of propanal.

Oxidation of MONAL-71 was followed selectively using the fluorimetric procedure (see Methods). Theoretical curves were drawn assuming K_m values for MONAL-71 of 0.7 μM (solid line) or 0.4 μM (broken line) and a fully competitive mode of inhibition, with K_m for propanal of 6 μM [14].

When MONAL-62 and $NADP^+$ were applied as substrates, (not shown) measurable activity of ALDH was detected in 6 samples originating from 5 operated thyroids (maximal value 0.043 U/g) while in 3 other samples the activity was on the verge of sensitivity of the method (0.004 U/g). The highest activities were observed in *carcinoma papillare* and Graves-Basedov disease samples, while no such activity was recorded in pre-neoplastic *struma nodosa* samples. By analogy with the results for the liver samples, we interpret these data as traces of the inducible tumour-specific ALDH-3 activity, although low reaction rates did not permit detailed examination of its kinetics.

While all the presented data must be regarded as preliminary, and errors resulting from freezing and/or handling samples cannot be excluded, they nevertheless demonstrate the possibility of detecting and quantifying the two main cytosolic ALDH isoenzyme activities (ALDH-1 and ALDH-3) in tumour samples, and probably in tissue biopsies. Due to high sensitivity of the assay, these examinations require only a minimal amount of clinical material (a few milligrams should theoreti-

cally suffice). We think that such examinations might be helpful in choosing therapeutic strategies in those cases, in which surgical treatment of tumours is to be followed by chemotherapy. In particular, the use of cyclophosphamide therapy in the treatment of thyroid tumours [3], could be preceded by ALDH activity examination in the tumour.

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