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Rat Liver Alcohol Dehydrogenase

Purification and Properties

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Fairly exhaustive studies have been made on HLADH ** in this and other institutes but comparatively little work has been reported on other LADH's, for example on rat liver alcohol dehydrogenase, RLADH.

Previous to this investigation partial purification of RLADH had been described,[1] and many authors [2–5] used crude extracts or solutions after precipitation with ammonium sulfate between 35 and 75 % saturation for preliminary studies of RLADH.

Great difficulties in the purification were caused by the instability of RLADH, as it was found [1] that the enzyme was relatively stable only at pH's above 8. The procedures successfully used for purification of HLADH [6,7] could not be applied on RLADH.

In this paper a description is given of the complete purification of RLADH and some of its isoenzymes. Extraction and fractionated precipitation with ammonium sulfate was followed by removal of hemoglobin and acid protein contaminants with DEAE Sephadex A 50. Rechromatography on Sephadex G 100 gave a product which had 107 times higher specific activity than the crude extract and were electrophoretically homogeneous. The stabilizing effect of several reagents (DTE, ME, ethyl alcohol) was investigated. Changes in the relative amounts of the isoenzymes and in the enzymatic activity were noticed. In the presence of DTE some of previously lost activity was recovered.

MATERIALS AND METHODS

Purification of RLADH. Wistar rats of 200 – 250 g in weight were used. It was found most convenient to use about 15 rats (*ca.* 150 g of liver) for one preparation. Homogenization was performed with an Ultra-Turrax homogenizer, model TP 18/2, and 3 ml of

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** Abbreviations used in this work: rat liver alcohol dehydrogenase, RLADH; horse liver alcohol dehydrogenase, HLADH; nicotinamide adenine dinucleotide, NAD^+; reduced nicotine amide adenine dinucleotide, NADH; dithioerythritol, DTE; 2-mercaptoethanol, ME; nitroblue tetrazolium, NBT; phenazine methosulfate, PMS; 17β-hydroxy-5β-androstan-3-one, 5β-DHT; Tris(hydroxymethyl)aminomethane, Tris; *p*-chloro-mercuriphenylsulfonate, PCMS; 3β-hydroxy-5β-cholanoic acid, 3β-OHCA; 5β-androstan-3β-ol-17-one, 5β-A-3β-OH.

0.05 M Tris-HCl buffer (pH = 8.4) was used per gram of fresh rat liver. The homogenate was centrifuged for 45 min at 39 000 *g* in a Sorvall RC 2-B Automatic Superspeed Refrigerated Centrifuge.

The enzyme was precipitated with ammonium sulfate between 35 and 75 % saturation, and the pH maintained at 8 by addition of ammonia. The precipitate after centrifugation at 39 000 *g* was resuspended in a minimum amount of 0.05 M Tris-HCl buffer (pH = 8.4) and was dialyzed against 4 × 5 l of the same buffer. The deep red coloured solution was centrifuged for 30 min at 39 000 *g* and it (volume about 80 – 90 ml) was applied to a DEAE Sephadex A 50 column (equilibrated with 0.05 M Tris-HCl buffer, pH = 8.4; dimension of column 50 × 1000 mm, effluent rate: 1.4 ml/min/cm²). It was found useful to have a layer of about 1 cm of cellulose powder on the top of the column to avoid difficulties with applying the sample. The first portion of the effluent solution contained the beginning of the enzyme activity and was yellowish; the main fraction, which contained most of the enzyme, was colourless. The volume of the most active fraction was about 400 ml and this solution was concentrated by ultrafiltration in an Amicon Model 402 Ultrafiltration cell by using Diaflo Membrane PM30. The concentrated solution (12 – 15 ml), after centrifugation for 15 min at 13 000 *g*, was applied on a Sephadex G 100 column (equilibrated with 0.2 M Tris-HCl buffer, pH = 8.4; dimension of column 45 × 1350 mm; effluent rate 1 ml/min/cm²). The enzyme activity was found in the third peak (Fig. 1) and after rechromatography RLADH isoenzymes were obtained free of contaminating proteins.

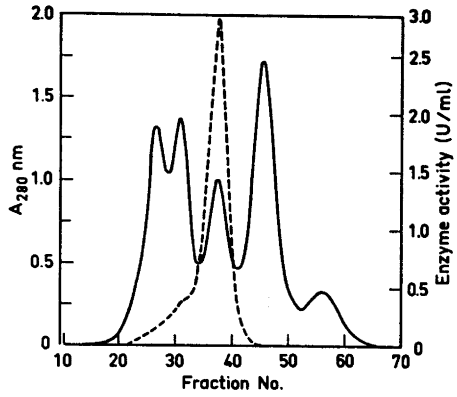

Fig. 1. Sephadex G 100 gel filtration of RLADH active fraction (after chromatography on DEAE Sephadex A 50). ——— conc. of protein; – – – enzyme activity (U/ml).

The same purification procedure was also performed in the presence of 5 % ethyl alcohol, 3.25×10^{-4} M DTE or 6.4×10^{-3} M ME.

All operations were performed at 4°C.

Electrophoresis. Discontinuous electrophoresis on polyacrylamide gel was tried at first for following the purification of RLADH. Good separation of proteins was obtained by polymerization at pH 5.0 and separation at pH 4.3 (β-alanine, acetic acid buffer), but the activity staining was unsatisfactory with such acid conditions in the gel. This technique was used only for checking the homogeneity of the end products.

The most suitable method seemed to be starch gel electrophoresis as used previously for separation of HLADH isoenzymes [5] and also for RLADH.[4] It was necessary to find new conditions for separation, however, because those used formerly did not give good resolution of the bands. The separation in phosphate buffer below pH 7 seemed best. Starch gels were prepared by adding 22.5 g Starch-Hydrolyzed (Connaught, Canada) per 200 ml 0.005 M phosphate buffer (pH = 6.85). Electrophoresis was performed on horizontal starch gels at 4°C for 16 – 18 h at 190 V constant voltage. The gel dimensions were 25.0 × 8.5 × 0.5 cm; 0.05 M phosphate buffer pH = 6.85 was used in the electrode vessels. After electrophoresis the starch gels were sliced horizontally into two sheets by means of a wire; one was stained for enzyme activity, the other for protein, as previously de-

scribed.[8] Staining for enzymatic activity was done in a solution of 20 mg NAD^+, 16 mg NBT, 1.5 mg PMS, and 0.15 ml 95 % ethyl alcohol in 30 ml 0.1 M Tris-HCl buffer, pH 8.8. For steroid staining 2 mg of 5β-A-3β-OH was used as substrate instead of ethanol. After 10 – 15 min incubation in the dark at room temperature the staining was interrupted by the addition of 5 % acetic acid, and the excess staining solution was washed away. For protein staining 0.02 % (w/v) solution of nigrosin in 40 % (v/v) ethyl alcohol in water containing 2.5 % trichloroacetic acid was used. The gels were kept in this solution in a closed box overnight. The excess staining solution was washed away with 5 % acetic acid.

Enzyme assays. RLADH ethanol activity was assayed at 340 nm at 23.5°C in 1 cm cuvettes containing 0.45 mM NAD^+, 8.6 mM ethanol and 0.1 M glycine buffer, pH 10.0, in a total volume of 3 ml with a Beckman DK 2 Spectrophotometer. One U of activity is the amount of enzyme which catalyzes the transformation of 1 μmole of substrate in 60 sec. For estimation of the specific activity with steroid substrate (RLADH steroid activity) 0.175 mM NADH, 0.11 mM 5β-DHT, and 0.1 M phosphate buffer, pH 7.0, in 3 ml total volume were used. The decrease of NADH was assayed and the same units were used as for the ethanol activity.

Kinetic studies were made with a Cary spectrophotometer, model 14, using the expanded scale, and fluorometric studies were made on the instrument previously described.[10]

PCMS titrations for sulfhydryl groups were made according to the method of Yonetani and Theorell.[11]

Comparison of molecular size of RLADH with HLADH. Two methods were used for the molecular size comparison; first, column gel chromatography on G 100 (0.15 M Tris-HCl buffer, pH 8.4; dimension of column: 30 × 140 mm: effluent rate 0.7 ml h^{-1} cm^{-2}). The effluent volumes of the examined samples of purified RLADH were compared with those of the standards (EE and SS types of isoenzymes of HLADH). The second method was thin-layer chromatography on Sephadex G 75 superfine (0.1 M Tris-HCl buffer, pH 8.4). The distance from the start was compared by using the same standards as for column chromatography.

Proteins were determined by the Lowry method.[12]

Reagents. NAD^+, NADH, NBT, PMS, DTE, Tris, 5β-A-3β-OH were all purchased from Sigma Chemical Company, St. Louis, Mo., USA; ME was a product of Eastman-Kodak Organic Chemicals, Rochester, N.Y., USA; 5β-DHT was a product from Ikapharm, Ramat-Gan, Israel; nigrosin was obtained from G. T. Gurr, London, England; pure samples of isopropanol, butanol and acetone were kindly supplied by NMR Laboratory of Karolinska Institutet, Stockholm, Sweden.

RESULTS

Fresh livers from rats which had not been perfused were used for purification.

It was not possible to estimate accurately the original enzyme content of the extract after homogenization, because the blank values without ethanol showed great variability. Therefore, the enzyme content of the solution after precipitation between 35 and 75 % ammonium sulfate saturation was used for calculating the yields in the purification steps. In the 35 – 75 % fraction twice as many enzyme units/kg of liver were found as previously reported[1] (1500 – 2100 U/kg, average of ten preparations). This is only three times lower than the enzyme content of horse liver (5500 – 5800 U/kg). It was found that the ratio of steroid activity units to ethanol activity units in rat liver was 1.2 – 1.8, which is about six times higher than that for horse liver (0.2 – 0.3). The steroid-active enzyme content in rat liver was found to be 3000 – 4000 U/kg.

During the purification procedure it was found that the precipitation between 35 and 75 % saturation of ammonium sulfate caused twofold puri-

Table 1. Purification of RLADH (mean values based on 5 preparations without stabilizing agents).

	Specific activity (mU/mg prot.)	Times purified	Yield %
First supernatant	16.0	1	
Precipitate (35 – 75 % Am_2SO_4)	31.7	1.98	87
Fraction after DEAE Sephadex A 50	163.5	10.2	53
Fraction after rechromatography on Sephadex G 100	853	53.3	5.5

fication. The DEAE Sephadex A 50 column step increases it to ten times. After the Sephadex G 100 the purest fraction had 40 – 60 times the original specific activity (Table 1). The activity of RLADH decreased with time at each stage of purification, even under optimal conditions (0°C, pH 8). The enzyme could be stored at −18°C after precipitation with 70 % saturation of ammonium sulfate. Under these conditions 80 % of the activity remained after six months.

RLADH in solution (pH > 8, 0°C) became more and more labile as purification progressed, as can be seen from Fig. 2. In the first extract and in the

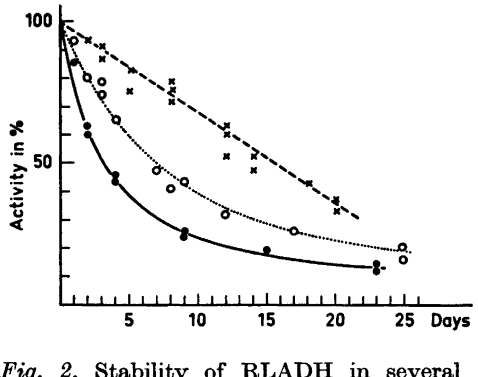

Fig. 2. Stability of RLADH in several stage of purification. Per cent of original value (mU/ml, enzyme activity with activity with ethanol as substrate) against time (in days). × first extract, or fraction after precipitation (35 – 75 %) Am_2SO_4; O fraction after DEAE Sephadex A 50 column; ● fraction after Sephadex G 100 column. All solutions were in Tris-HCl buffer (0.05 M, pH = 8.4), stored at 0°C.

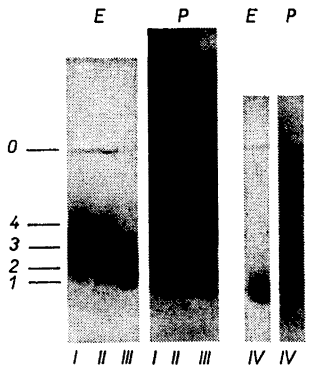

Fig. 3. Starch gel electrophoreses of RLADH (during purification). E: staining for enzyme activity; 0: origin; P: staining for protein; I. first extract; II. fraction after 35 – 75 % Am_2SO_4 precipitation; III. fraction after DEAE Sephadex A 50; IV. fraction after Sephadex G 100.

fraction after ammonium sulfate precipitation 80 % of the activity remained after five days, but in the purest fraction only 30 % remained after the same period.

Therefore, some known stabilizing reagents [13] were tried. DTE was found to be very convenient, since steroid activity could be estimated without dialysis even in relatively high concentrations of DTE (4 mg/ml). There was an apparent decrease in ethanol activity, however, in the presence of excess DTE (Table 3). Addition of 1 mg DTE/ml to the first extract, or to the fraction after ammonium sulfate precipitation, prevented loss of activity up to 10 to 14 days; the same effect was observed after addition of DTE to the pure product. When DTE was added in a concentration of 4 mg/ml to a pure product which had lost about 30 % of its activity, the activity after 5 days rose to a level higher than that originally measured (Table 3).

Table 2. Purification of RLADH in the presence of 5 % ethanol.

	Protein (mg)	Activity (mU)	Specific activity (mU/mg prot.)	Times purified	Yield %
First supernatant	19 600	314 000	16.1	1	
Precipitate (35 – 75 % Am_2SO_4)	7 650	281 000	36.7	2.3	90
Fraction after DEAE Sephadex A 50	900	193 400	214.8	13.3	61.5
Fraction after rechromatography on Sephadex G 100	26	45 000	1732.0	107.0	14.3

ME was not as effective a stabilizing agent as DTE and caused some inhibition of steroid and total inhibition of ethanol activity. ME in a concentration of 2 mg/ml was effective only for 4 – 5 days.

It was noticed during crystallization experiments that 5 % ethanol also has a stabilizing effect. The ethanol activity of a pure product was fully protected for 6 – 7 days, but there was a slight decrease in steroid activity.

Stabilizing reagents were therefore used in the purification procedure. The best results were obtained by using 5 % ethanol in all solutions during the purification; the specific activity of the end product was 1730 mU/mg protein and the product was 107 times purified (Table 2).

The purification in the presence of $3.25 \times 10^{4-}$ M DTE or 6.4×10^{-2} M ME gave similar results; it was possible to obtain products with a specific activity of about 1000 mU/mg protein and with a purification of about 90 times, using a different starting material.

Table 3. The effect of DTE on the enzyme activity of RLADH.

Days after addition of DTE	Without DTE		0.5 mg DTE/ml	1.0 mg DTE/ml	2 mg DTE/ml	4 mg DTE/ml
1	S	1860	2480	2400	2420 (2400) [a]	2520
	E	950	700	440	250 (1050) [a]	130
6	S	1200	3140	2800	3020	2950
	E	570	1530	1400	830	550
14	S	800	1520	2140	3100	3500
	E	440	750	1380	1630	1750
20	S	730	890	1400	1940	2400
	E	340	500	900	1340	1340
31	S	390	400	800	1060	1100
	E	280	260	520	650	700

S: enzyme activity with 5β-DHT as substrate (in mU/ml); E: enzyme activity with ethanol as substrate (in mU/ml). The samples were in 0.05 M Tris-HCl buffer, pH = 8.4, at 0°C.
[a] values after dialysis.

After rechromatography on Sephadex G 100, the pure fraction was concentrated by ultrafiltration until there was 4 – 5 % protein content. This solution was dialyzed against gradually increasing concentrations of ethanol (5, 12, and 18 %) and the temperature was gradually lowered to – 7°C; after 3 – 4 days needle-like crystals similar to those of HLADH appeared. These were fully active on ethanol and steroids, but the crystals were not stable at temperatures above 0°C.

The purification of RLADH was checked by starch gel electrophoresis. Four separate bands (isoenzymes) were detected by enzyme activity staining. All the enzyme bands were on the cathodic side of the origin. The relative intensities of the bands of RLADH varied during the purification and in the original homogenate the most intensive bands were Nos. 2 and 3. In preparations with no stabilizing agents or with 5 % ethyl alcohol 4 bands were observed. The staining with ethanol as substrate was generally more intense than the steroid staining, but the relative intensities of the bands were the same for both substrates (Fig. 4).

After addition of either DTE or ME there were extensive changes in the electrophoretic picture; two of the bands (Nos. 3 and 4) gradually disappeared and the final result was a very intensive band No. 1 and a rather weak band No. 2 (Figs. 4 and 5). Moreover, when DTE or ME was used throughout the preparation, only these two bands (protein and enzyme staining) appeared

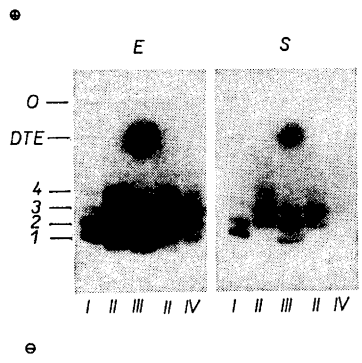

Fig. 4. Starch gel electrophoreses of RLADH (staining for enzyme activity). E: staining with ethanol as substrate; S: staining with 3β-A-5β-OH as substrate; 0: origin; I. fraction after Sephadex G 100, action of DTE during 10 h, dialyzed; II. the same sample without DTE; III. the same sample, action of DTE during 4 h, undialyzed; IV. fraction after Sephadex G 100, prepared in presence of DTE after crystallization in 18 % ethanol.

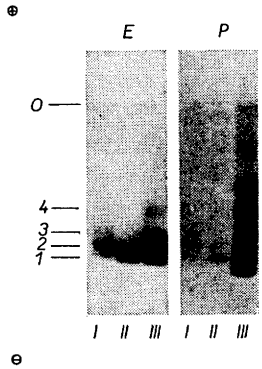

Fig. 5. Starch gel electrophoreses of RLADH (effects of DTE). E: staining for enzyme activity with ethanol as substrate; P: staining for protein; I. fraction after Sephadex G 100; II. the same sample, action of DTE during 24 h, dialyzed; III. fraction after DEAE Sephadex A 50 from the same purification.

in the final product. On re-electrophoresis of each of these two bands on starch gel the mobility was not changed. The same result was obtained with discontinuous polyacrylamide gel electrophoresis. Treatment of this product with ethanol during crystallization gave the original electrophoretic picture (Fig. 4, Sample IV), consisting of four bands.

Table 4. K_m values of RLADH for different substrates.

Substrate	K_m (mM)
Ethanol	2.13
Isopropanol	76.4
Butanol	8.75
Cyclohexanol	1.15
5β-A-3βOH	0.0185
Cyclohexanone	1.54
5β-DHT	0.117

A comparison of molecular size was made between RLADH and the EE and SS types of HLADH by use of a Sephadex G 100 column and thin-layer chromatography on Sephadex G. 75. No differences were found in effluent volume or distance from the origin, respectively, which indicates that the molecular sizes are the same or similar.

It was of interest to make some kinetic studies on RLADH at this stage of purification in order to determine the Michaelis constants for various substrates. Previously,[1,14] ethanol, retinol and a few steroids had been used as substrates for the kinetic studies. In the present case this list has been further extended (Table 4). Both the oxidation and reduction reactions were studied (Figs. 6 and 7). These kinetic experiments were of further interest because of the comparative values for RLADH prepared with and without stabilizing agents (Table 5).

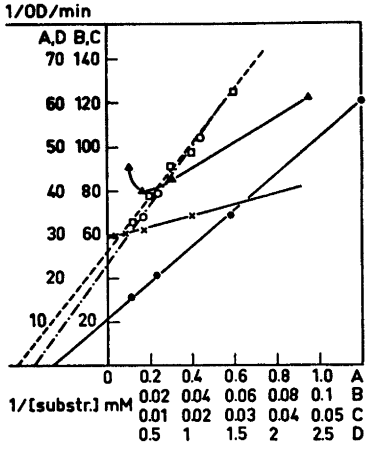

Fig. 6. Double reciprocal plots of RLADH with various substrates.

The cuvette contained 0.45 mM of NAD^+ in a total volume of 3 ml 0.1 M glycine buffer (pH = 10.0) with varying amounts of substrate at 23.5°C.

A □ ethanol, conc. varying from 1.67 to 8.33 mM;
B ▲ isopropanol, conc. varying from 10.5 to 90.0 mM;
C × butanol, conc. varying from 49.8 to 498.0 mM;
D ○ cyclohexanol, conc. varying from 0.84 to 2.3 mM;
D ● cyclohexanone, conc. varying from 0.057 to 0.344 mM; in this case the cuvette contained 0.175 mM NADH in a total volume of 3 ml 0.1 M phosphate buffer (pH = 7.0).

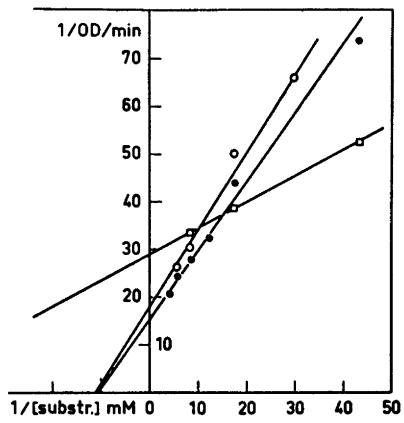

Fig. 7. Double reciprocal plots of RLADH with various substrates. The cuvette contained 0.175 mM NADH in a total volume of 0.1 M phosphate buffer (pH = 7.0), with varying amounts of substrate at 23.5°C. ○ 5β-DHT, conc. varying from 0.023 to 0.23 mM (enzyme prepared with DTE); ● 5β-DHT, conc. varying from 0.023 to 0.23 mM (without DTE); – 5β-A-3β-OH, conc. varying from 0.023 to 0.23 mM; in this case the cuvette contained 0.45 mM of NAD^+ in a total volume of 0.1 M glycine buffer (pH = 10.0).

Table 5. Effects of DTE on the kinetic values of RLADH.

Substrate	K_m (mM)		V_{max} (μM min^{-1} U^{-1})	
	Without DTE	With DTE	Without DTE	With DTE
Ethanol	2.13	2.31	6.03	10.5
5β-DHT	0.117	0.091	7.6	10.4

The decrease in activity of RLADH was parallelled by a corresponding change in sulfhydryl groups, determined by titrating the enzyme with PCMS.[11] These titrations were made on enzyme prepared with and without stabilizing agents.

DISCUSSION

The instability of RLADH during all stages of purification, especially in the pure state (Fig. 2) was a very difficult problem in the present study. It had been previously suggested[1] that the presence of proteolytic enzymes liberated during the preparation might be responsible for the sensitivity of the enzyme to acidic pH and heat treatment. From the results obtained here, however, it seems more likely that the instability is due to oxidation of SH groups in the enzyme. A PCMS titration showed a reduction in the number of SH groups which corresponded to the decrease in activity; this, plus the effect of DTE in stabilizing the enzyme, confirms the latter suggestion.

Because of the instability of the enzyme, various stabilizing agents were tried. A comparison of specific activity and yield between preparations with and without 5 % ethanol reveals little difference up to the first column. After the Sephadex G 100 step, however, these values doubled when ethanol was present. Similar results were obtained with DTE or ME, but the yield was considerably lower than in the case of 5 % ethanol.

The effects of increasing the concentration of DTE in a sample of pure RLADH which had lost some of its activity were investigated (Table 3). It was noticed that 2 – 4 mg of DTE/ml was necessary for stabilizing the enzyme for a period up to 14 days and for recovery of previously lost activity. The apparent decrease in ethanol activity at higher concentrations of DTE was due to interference of excess DTE, since after dialysis the ethanol activity increased to its original level. The presence of DTE did not interfere with the estimation of steroid activity.

The ratio of steroid activity to ethanol activity also varied with the method of purification. This ratio was about 1.2 – 1.8 in preparations without stabilizing agents, whereas after purification in the presence of ethanol it was about 1.0 – 1.2 and with DTE higher than 1.8.

The purification of RLADH was best followed by means of starch gel electrophoresis. Fig. 3 shows electrophoreograms of samples from four different stages of purification. The protein staining shows stepwise elimination of con-

taminants. In every case four isoenzyme bands were seen after activity staining; in Sample IV protein staining showed only the bands corresponding to these isoenzymes.

The extensive changes in the electrophoretic picture caused by the presence of DTE (Figs. 4 and 5), together with the effect of DTE as a stabilizing agent, might well indicate that the different enzyme forms are interchangeable and created by secondary chemical reactions.

It was found that RLADH has a greater activity toward steroid than toward ethanol. The K_m value for ethanol was found to be 2.13 mM, which is higher than the reported value [1,14] of 0.4 – 0.5 mM. Also, a value of 0.0185 mM for 5β-A-3β-OH as steroid substrate was obtained, which is higher than the reported value for 3β-OHCA (0.002 mM). The discrepancy between these values can be explained by the instability and varying purities of the RLADH preparations used. In spite of these differences it should be noted that the same trend for the K_m values was observed in both studies. Though the K_m value of 2.13 mM for ethanol with RLADH differed from a reported value of about 0.5 mM for horse liver alcohol dehydrogenase (HLADH), the K_m values for NAD^+, of 0.108 mM with RLADH and 0.145 mM with HLADH were found to be remarkably similar.[15] The use of DTE did not change the kinetic values markedly (Table 5). Isopropanol and butanol were found to be poor substrates for RLADH, whereas cyclohexanol and 5β-A-3β-OH proved to be better substrates than ethanol.

The reductive action of this enzyme was studied with cyclohexanone and 5β-DHT as substrates. It is very significant to note that cyclohexanol and cyclohexanone (Fig. 6) gave similar K_m values, and 5β-A-3β-OH seems to be a better substrate than 5β-DHT (Table 4 and Fig. 7). From these reults it could be suggested that an alcohol substrate having a cyclic structure might prove to be the most favorable substrate for RLADH. The stereospecificity for 5β-steroids previously found [14] was again confirmed.

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