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PURIFICATION AND PROPERTIES OF ALDOSE REDUCTASES FROM THE PLACENTA AND THE SEMINAL VESICLE OF THE SHEEP

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SUMMARY

NADP-dependent aldose reductase has been purified 35-fold from ovine placenta and 70-fold from ovine seminal vesicles.

The purified placental enzyme still showed two precipitation arcs when tested by immunodiffusion and immunoelectrophoresis. The seminal vesicle preparation appeared to be homogeneous, using the same procedures. There was no immunological cross reaction between the enzymes purified from the two sexes of the same species.

The kinetic behaviour of the two enzymes were very similar, with high reactivity towards aldehydes as substrates, but low reactivity towards the corresponding alcohols. With aldehydes as substrates both enzymes showed marked deviation from linearity in the Lineweaver–Burk plot, indicating substrate activation.

INTRODUCTION

In foetal blood, glucose is the main free hexose in Carnivora and Rodentia, while fructose dominates in Ungulata and Cetacea[1]. In many species, including man, free fructose is the main or only hexose of seminal plasma[2]. The mechanism of formation of free fructose has been clarified mainly by HERS[3]. In sheep seminal vesicles free fructose is derived from free glucose with the sugar alcohol sorbitol as intermediate[3]. Two enzymes are involved in the reaction, a NADP-dependent aldose reductase (alditol:NADP oxidoreductase, EC 1.1.1.21) which catalyses the reduction of glucose to sorbitol, and a NAD-dependent ketose reductase (L-iditol:NAD oxidoreductase, EC 1.1.1.14) catalysing the oxidation of sorbitol to fructose.

As regards the formation of foetal blood fructose, HERS[4] also showed that aldose reductase activity is high in placental tissue of sheep, while the oxidation of sorbitol probably takes place by the action of ketose reductase of the foetal liver. BRITTON, HUGGETT AND NIXON[5] have later presented strong evidence indicating that also oxidation of sorbitol takes place in the placenta.

The studies of HERS[4] which were based on crude enzyme preparations from

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sheep tissues indicated that the enzyme responsible for the reduction of glucose to sorbitol was even more active with glyceraldehyde as substrate. This was confirmed by VELLE AND ENGEL[6] using more purified enzymes of bovine origin. As part of a comparative study of polyol dehydrogenases in placenta and seminal vesicle, the present paper deals with the properties of the purified enzymes from sheep tissues.

MATERIALS AND METHODS

Tissue

Placenta foetalis was collected during the lambing season, immediately frozen, kept at −20°, and processed as soon as possible.

When a sufficient amount of tissue had been obtained, it was thawed and the cotyledons and intercotyledonary connective tissue were separated. Since cotyledonar tissue in preliminary tests were shown to be the richest source of the enzyme, these were used in the following investigations.

Seminal vesicles were obtained from apparently normal, mature wethers at slaughter, chilled and processed within 2 h of slaughter.

Reagents

$NADP^+$ and NADPH were obtained from Boehringer und Soehne, Mannheim, DL-glyceraldehyde from Fluka, DL-lactaldehyde was supplied by Dr. O. Z. Sellinger of Tulane University, New Orleans, La., U.S.A. Propanediol was obtained from Fluka, Basel, Switzerland and glycerol from Merck, Darmstadt, Germany.

The glyceraldehyde and lactaldehyde solutions were kept frozen, and were dedimerized just prior to use by heating at 85° for 5 min; they were then kept at 37° during enzyme assays. Glucose and galactose solutions were prepared immediately before use as substrates.

DEAE–Sephadex 50, coarse, new bead form (Pharmacia, Uppsala, Sweden) was pretreated according to the instructions given by the manufacturers.

In the immunization experiments Bacto adjuvant complete Freund from Difco Laboratories, Detroit, U.S.A., was used.

Methods

Protein measurements were made by absorbance reading in a Zeiss M 4 Q III spectrophotometer at 280 mμ in quartz cuvettes of 1-cm light path.

Enzyme activity was measured spectrophotometrically at 340 mμ in glass cuvettes of 1-cm light path. Since the enzymes in question display considerably higher activity towards lactaldehyde and glyceraldehyde than towards sorbitol, the low molecular weight substrates were used in most assays. In standard assays the solutions to be tested were 0.1 M in Tris–HCl (pH 7.5), 16.7 mM in glyceraldehyde and 0.167 mM in NADPH when aldehydes were used as substrates. Final volume, 3 ml. With propanediol as substrate, the standard assay system was 0.1 M in glycine–NaOH (pH 10), 1 M in propanediol, and 0.167 mM in $NADP^+$, in a final volume of 3.0 ml. With aldehydes as substrate the reaction was initiated with substrate. With alcohols as substrate the reaction was initiated with coenzyme. The readings were started 20 sec after the final addition of reagent, and the reaction was followed by readings each 10-sec

intervals for 1 min. Reaction rates were determined from the extrapolated values of the three first readings, the change in absorbance per min being calculated.

One unit of enzyme activity is defined as the amount of protein catalysing the transformation of 1 μmole of substrate per min at 37° under the conditions specified above. Specific activity is given in units per mg of protein.

Purification of the enzymes

The procedures followed for the enzyme purification were generally those described by VELLE AND ENGEL[6]. The description of this part of the work may therefore be dealt with summarily.

The cotyledons and the seminal vesicles were homogenized in an Ato-Mix blender for 2 min at maximal speed in a medium 1 mM in EDTA, 10 mM in nicotinamide, 1 mM in cysteine hydrochloride and 30 mM in sodium bicarbonate. The samples were centrifuged at 2500 $\times$ *g* for 20 min, and the supernatant fluid then stirred for 1 h with 0.1 vol. of 0.1 M $CaCl_2$ to precipitate the microsomes.

The centrifugation was repeated at 2500 $\times$ *g* for 20 min and the supernatant fluid brought to 50% saturation by addition of solid ammonium sulphate. After centrifugation at 4000 $\times$ *g* for 15 min and discarding the precipitate, the supernatant fluid was brought to 80% saturation by addition of solid ammonium sulphate. The precipitate thus formed was collected by centrifugation at 4000 $\times$ *g*, and dissolved in a small amount of a 5 mM Tris–phosphate buffer (pH 7.5). Desalting of the protein solution was carried out by repeated dialysis against the buffer to be used in the experiments that followed.

DEAE–Sephadex and hydroxyl apatite chromatography. The dialysed protein solution in amounts of approx. 0.5 g (30–40 ml) was applied to a column of DEAE–Sephadex, 30 cm high and 2.7 cm in diameter, previously equilibrated with 5 mM Tris–phosphate buffer (pH 7.5).

The buffer concentration was kept constant and the elution from the column was carried out by stepwise addition of NaCl to the buffer.

VELLE AND ENGEL[6] eluted the active fraction from bovine tissues with the buffer made 0.15 M in NaCl. In the present experiments the active fractions were eluted with a buffer made 0.2 M in NaCl. A typical elution pattern for the enzyme from seminal vesicle is shown in Fig. 1.

In preliminary experiments it was shown that desalting was not necessary before chromatography on hydroxylapatite. The active fraction from the DEAE–Sephadex was therefore transferred directly to the column of hydroxylapatite in all subsequent experiments.

The preparation of the hydroxylapatite column and the elution were carried out as previously described[6]. The elution patterns were very similar for the enzymes from the two tissues. Fig. 2 illustrates the pattern for the seminal vesicle enzyme. A summary of the purification achieved by these methods is given in Table I. For the seminal vesicle enzyme the total yield of activity appeared to increase during the last purification step. The reason for this is not known.

PROPERTIES OF THE ENZYMES

Stability. Neither of the enzymes is stable over long periods when tissues are

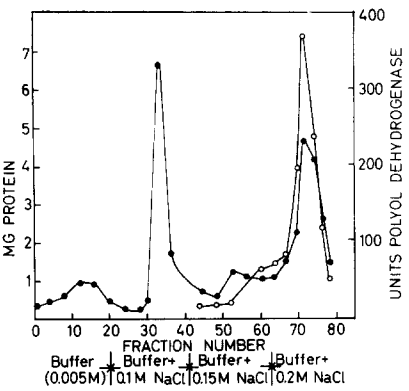


Fig. 1. Chromatography of seminal vesicle extract on DEAE-Sephadex. For experimental details, see text. Substrate in enzyme assay was DL-glyceraldehyde. ●—●, protein, spectrophotometrically determined; ○—○, units polyol dehydrogenase. Each fraction, 10 ml. Flow rate, approx. 1.2 ml/min.

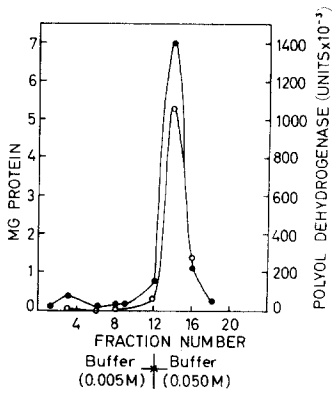


Fig. 2. Chromatography of seminal vesicle extract on hydroxylapatite. Each fraction contained 4 ml. Substrate in enzyme assay was DL-glyceraldehyde. ●—●, protein, spectrophotometrically determined; ○---○, units polyol dehydrogenase.

kept in the frozen state. In placental tissue kept at −20°, all enzyme activity had disappeared after 2 months.

The enzymes are stable after chromatography on DEAE-Sephadex when kept frozen. After chromatography on hydroxylapatite the stability is reduced, and repeated freezing and thawing leads to marked loss of activity.

Heating to 56° for 2 min causes about 95% loss of the enzyme activity in the

TABLE I

PURIFICATION OF PLACENTAL AND SEMINAL VESICLE ENZYMES

	Placental enzyme				*Seminal vesicle enzyme*			
	Total protein (mg)	*Spec. activity units* $\times 10^{-3}$	*Total units* $\times 10^{-3}$	*Yield (%)*	*Total protein (mg)*	*Spec. activity units* $\times 10^{-3}$	*Total units* $\times 10^{-3}$	*Yield (%)*
Original supernatant fluid after $CaCl_2$ precipitation	21 900	4.68	102 500	100	9660	4.44	42 800	100
50–80% $(NH_4)_2SO_4$ fraction	8 100	6.42	52 000	50.7	194	26.81	5 200	12.2
DEAE-Sephadex column eluate	322	83.8	26 900	26.2	44.3	91.3	4 060	9.5
Hydroxylapatite column eluate	99.5	163.5	16 250	15.9	25.1	315	7 900	18.4

The substrate used in enzyme assay was DL-glyceraldehyde, conditions are given in the text.

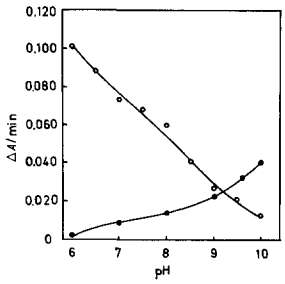


Fig. 3. pH-dependence of the placental enzyme. Assay systems as described in text. ○—○, Substrate DL-glyceraldehyde, coenzyme NADPH ●—●, substrate DL-1,2-propanediol, coenzyme $NADP^+$.

absence of coenzyme. Similar treatment in the presence of coenzyme causes a 90% loss of activity.

pH-dependence. Both the seminal vesicle enzyme and the placental enzyme display activity over a wide pH range. This is in accordance with the findings for the bovine enzymes[6].

The buffers used were phosphate (0.05 M) in the pH range 6.0–8.0, Tris–HCl (0.1 M) in the pH range 7.5–9.0, and glycine–NaOH (0.1 M) in the pH range 8.5–10.

As shown in Fig. 3 the enzymes displayed the highest activity at pH 6 with glyceraldehyde as substrate. With propanediol as substrate the highest activity was found at pH 10.

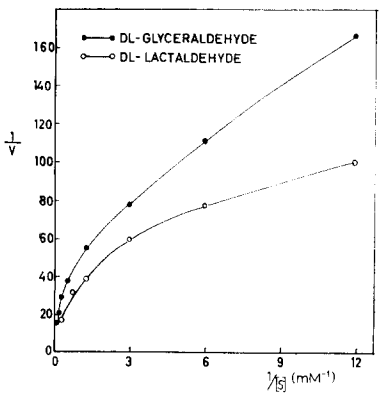
Kinetic studies. Both enzymes showed higher activity with aldehydes as substrate than with alcohols. Both glyceraldehyde and lactaldehyde were good substrates, while glucose and galactose showed relatively low reactivity (Table II).

TABLE II

SPECIFIC ACTIVITIES OF PURIFIED ENZYMES FROM OVINE PLACENTA AND SEMINAL VESICLE

Substrate	*Substrate concn. (M)*	*Placenta units* $\times 10^{-3}$	*Seminal vesicle units* $\times 10^{-3}$
DL-Lactaldehyde	0.0167	159	100
DL-Glyceraldehyde	0.0167	146	104
D-Glucose	0.333	14.2	8
D-Galactose	0.333	15.8	6.4
DL-1,2-Propanediol	1	35.8	13
Glycerol	1	10.4	5.5

None of the enzymes followed the Michaelis–Menten equation when the aldehydes were used as substrates. The double reciprocal plots for the kinetics (Figs. 4 and 5) of aldehyde reduction covering a 10 000-fold concentration range show clear-cut deviations from linearity. Determination of K_m values for the aldehydes were therefore not possible. To test the possibility of product activation, the enzymes were preincubated with 0.1 M propanediol in the standard assay system for 5 min before the reaction was initiated, using aldehyde and reduced NADP as substrates. This procedure gave no increase in enzyme activity.



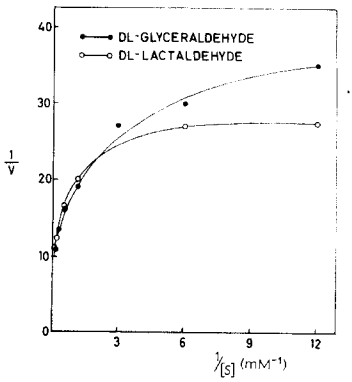


Fig. 4. Lineweaver–Burk plots for the placental enzyme with DL-glyceraldehyde and DL-lactaldehyde as substrate. The ordinate is the reciprocal of change in absorbance at 340 mμ/min, and the abscissa is the reciprocal of substrate concentration. Experimental conditions as described under *Methods*.

Fig. 5. Lineweaver–Burk plots for the seminal vesicle enzyme using DL-glyceraldehyde and D-lactaldehyde as substrates. Labelling as in Fig. 4.

Another possibility was that the enzyme was activated by $NADP^+$ formed during the reaction. However, preincubation of the enzymes with $NADP^+$ followed by addition of NADPH and glyceraldehyde as substrate resulted in a 5% decrease in reaction rate.

With alcohols as substrate and a coenzyme concentration of 0.167 mM, both enzymes followed the Michaelis–Menten equation. For propanediol and glycerol the K_m values were 0.7 and 1.4 M, respectively, when placental enzyme was used. For the seminal vesicle enzyme the values were 0.8 and 1.2 M, respectively.

With glyceraldehyde as substrate at a concentration of 16.7 mM the K_m for NADPH with the placental enzyme was found to be $1.7 \cdot 10^{-4}$ M and with the seminal vesicle enzyme $3.2 \cdot 10^{-4}$ M.

The two most active substrates found for the two enzymes were lactaldehyde and glyceraldehyde. Of the alcohols tested, propanediol was the most reactive, followed by glycerol (Table II). These observations are in accordance with previously observed properties of the bovine enzymes[6]. For the bovine enzymes the ratio of the specific activities of the placental to the seminal vesicle enzyme was of the order of 2:1 for the aldehydes as substrates and 5:1 for the alcohols as substrates. For the ovine enzymes tested under the same conditions the corresponding ratios were found to be of the order of 1.5:1 and 2:1. These ratios were not dependent upon the specific activities of the enzyme preparations used.

Inhibition experiments

Heavy metals. Both enzymes were sensitive to heavy metal ions, even in low concentrations. The seminal vesicle enzyme seemed to be more sensitive to the metal ions than the placental enzyme. Table III shows a summary of the effect of heavy metals. Cu^{2+} showed the largest inhibitory effect. Inhibition was less when the enzymes were preincubated with coenzyme. VELLE AND ENGEL[6] found that Mg^{2+} and Fe^{3+}

TABLE III

EFFECT OF HEAVY METAL IONS ON ENZYME ACTIVITY

Experimental conditions: A, 0.1 M in Tris–HCl buffer (pH 7.5), 0.167 mM $NADP^+$, 16.7 mM in glyceraldehyde. Total volume 3 ml. The samples were preincubated for 5 min before initiation with glyceraldehyde. B, The same experimental conditions as in A but the reaction was initiated with coenzyme. The results are given in % inhibition.

Metal ions	*Concentration*	*Placental enzyme*		*Seminal vesicle enzyme*	
		A	*B*	*A*	*B*
Mg^{2+}	10^{-3} M	8.2%	18.2%	13.2%	32.4%
Ca^{2+}	10^{-4} M	33.3%	82.7%	27.8%	85.1%
Cu^{2+}	10^{-5} M	22.2%	98 %	43.7%	100 %
Zn^{2+}	10^{-5} M	14.8%	20.3%	16.2%	26 %
Fe^{3+}	10^{-4} M	0 %	39.1%	0 %	36.4%

markedly stimulated the activity of the bovine seminal vesicle enzyme, but this could not be shown for the ovine enzymes.

Sulfate. HERS[4] claimed that sulfate ions in a concentration of 0.4 M caused a marked stimulation of aldose reductase activity of ovine placental homogenates. The effect of sulfate on the purified enzymes seemed to depend on the experimental conditions. In Table IV the influence of sulfate on enzyme activity is shown. Both enzymes were activated when glyceraldehyde was used as substrate, while the enzyme activity was depressed when propanediol was the substrate.

Urea. The enzymes were preincubated with urea in varying concentrations for 30 min, but no activation was obtained. Instead there was an increasing inhibition of enzyme activity with increasing urea concentration and a concentration of 2.5 M in urea resulted in 75% inhibition.

TABLE IV

THE INFLUENCE OF SULFATE ON ENZYME ACTIVITY

Experimental conditions: The test solution was 0.1 M in Tris–HCl (pH 7.5), 0.167 mM in NADPH, 16.7 mM in glyceraldehyde. Total volume 3 ml. The samples were preincubated for 5 min at 37° and the reaction was initiated with substrate. With propanediol as substrate the test solution was 1 M in propanediol, 0.1 M in glycine–NaOH (pH 10), 0.167 mM in $NADP^+$. Total volume 3 ml. The reaction was initiated with coenzyme.

Sulfate concn. (M)	*% Change in activity*			
	Placental enzyme		*Seminal vesicle enzyme*	
	Glyceraldehyde as substrate	*Propanediol as substrate*	*Glyceraldehyde as substrate*	*Propanediol as substrate*
0.1	46.5	−19.5	15	− 6
0.2	71.–	−35.9	27	−32
0.3	94.–	− 7.–	27	−28
0.4	104.–	−44.7	17.5	−52
0.5	109.–	— –	24.5	−54
0.6	109.–	−57.9	32.5	—
0.7	110.–	—	43.2	—
0.8	—	−75.8	42.–	—
0.9	—	−73.7	42.–	—
1.0	132.–	—	50.–	−52

Parachloromercuribenzoate. The placental and the seminal vesicle enzymes are both extremely sensitive to reagents that bind sulfhydryl groups (Table V). Rapid and almost complete inhibition was produced by 10^{-6} M parachloromercuribenzoate. Activity could partly be restored for the seminal vesicle enzyme by addition of cysteine, indicating the necessity of sulfhydryl groups for enzyme activity.

TABLE V

INACTIVATION OF ENZYMES WITH PARACHLOROMERCURIBENZOATE AND REACTIVATION BY CYSTEINE

Experimental conditions: The samples were preincubated for 5 min at pH 7.5 under conditions as given under *Methods*. A, Preincubation without coenzyme; B, preincubation with coenzyme (0.167 mM). The reaction was initiated by substrate (DL-glyceraldehyde) in a final concentration of 16.7 mM.

Conditions	*Placental enzyme*	*Seminal vesicle enzyme*	
	A	*A*	*B*
No inhibitor	0	0	0
10^{-6} M *p*-chloromercuribenzoate	66.8	100	82
3 min after addition of cysteine (10^{-3} M)	66.8	66	92

Immunological studies

Rabbits were injected with preparations of purified enzymes. No immunity was obtained when the enzymes alone in amounts of the order of 1.2 mg protein were injected intravenously every third day for 3 weeks.

5 ml complete Freunds adjuvant was mixed with 5 ml of enzyme solution (1.2 mg protein) and the solution was injected in a single dose subcutaneously. The rabbits were bled after 3 weeks, and the serum tested by the agar gel double-diffusion method *ad modum* Ouchterlony and the immuno-electrophoretic technique. Electrophoresis was carried out at 100 V and 4 mA for 1 h.

The placental enzyme showed two distinctly separated precipitation arcs when subjected to gel diffusion and also after immunoelectrophoresis. When the enzyme was inactivated by heat treatment (at 56° for 5 min) prior to immunoassay by the two methods, only one arc was observed. Furthermore, when heat-inactivated enzyme preparation was used as antigen and its anti-serum used in the testing of the unheated enzyme preparation, only one precipitation arc was found. It was therefore probable that the precipitation arc which disappeared following heat treatment of the enzyme preparation was due to the enzyme.

To further test this possibility. antiserum against unheated and heated enzyme preparations were tested for inhibitory effect in the standard assay system using glyceraldehyde as substrate. Antiserum (0.5 ml) against unheated antigen completely abolished enzyme activity. Antiserum (0.5 ml) against heat-treated antigen gave only slight inhibition (about 25%). Controls run with normal serum added gave no inhibition. It may therefore be concluded that heat treatment at 56° leads to complete inactivation of the enzyme and at the same time to a marked alteration of its antigenic properties.

This observation indicates the possibility of using antiserum against heat-treated enzyme preparations in further purification of the placental aldose reductase.

The purified seminal vesicle enzyme showed precipitation arc in the gel diffusion

test as well as after immunoelectrophoresis. No precipitation arc was produced when the enzyme preparation was inactivated at 56° for 5 min prior to testing.

Antiserum against the placental enzyme gave no reaction with the seminal vesicle enzyme and *vice versa*.

DISCUSSION

NADP-dependent polyol dehydrogenases present in ovine placenta and seminal vesicle have been purified to a significant degree in the present investigation, about 35-fold for the placental enzyme and about 70-fold for the seminal vesicle enzyme. These degrees of purification are of the same order of magnitude as previously reported for the corresponding bovine enzymes[6].

The enzymes from female and male sheep showed closely similar properties when subjected to ammonium sulfate fractionation, and chromatography on DEAE–Sephadex as well as hydroxylapatite columns. In these procedures they also behaved very similarly to the enzymes of bovine origin. Also the pH dependence was closely similar for the ovine and bovine enzymes.

As regards substrate specificity, the enzymes purified from the two animal species showed the same general picture, exhibiting activity towards lactaldehyde as well as glyceraldehyde and glucose. In his experiments with crude preparations of sheep tissues HERS[4] observed much higher reaction rates with glyceraldehyde than with glucose as substrate. The same was found to be true for the more purified preparations used in the present investigation. Although it cannot be entirely ruled out that the reduction of glyceraldehyde may be catalysed by an enzyme different from that acting on glucose, the apparent homogeneity of the protein fraction obtained from the seminal vesicle speaks against this possibility. Furthermore dehydrogenases acting on polyols are generally considered to possess relatively low substrate specificity. It therefore seems reasonable to assume that the enzymes reported on in this paper are identical with the aldose reductase (alditol:NADP oxidoreductase, EC 1.1.1.21) studied by HERS. The final conclusion on this point must, however, await the isolation of the enzymes in an unequivocally pure state.

The kinetics of the ovine and bovine enzymes were also very similar. Thus for the placental enzymes K_m values of 0.8 and 1.4 M were found for propanediol and glycerol, respectively, when the bovine enzyme was studied, while for the ovine enzyme the corresponding figures were 0.7 and 1.4 M.

The nonlinearity in the double reciprocal plot of velocity against substrate concentration observed for the bovine enzymes[6] was also found for the ovine enzymes (Figs. 4 and 5). This type of kinetic behaviour indicates substrate activation. This explanation is supported by the lack of further activation when either glycerol or $NADP^+$ was added to the reaction mixture before initiating the reaction with glyceraldehyde and NADPH. The phenomenon of substrate activation has so far been found for few enzymes using natural substrates.

DALZIEL AND DICKINSON[7] found substrate activation of liver alcohol dehydrogenase in the presence of saturating coenzyme concentrations when cyclohexanol was used as substrate. Their explanation is the formation of an abortive complex, enzyme–product coenzyme–substrate, from which the product coenzyme dissociates more rapidly than from the normal enzyme–product–coenzyme complex. The kinetic

behaviour of the enzymes reported here seems to fit this theory. Thus the enzymes approached normal initial-rate behaviour when tested in systems with decreasing coenzyme concentration. The inhibition caused by addition of product coenzyme before initiating the reaction aldehyde → alcohol further strengthens the assumption of substrate activation.

Alternatively the kinetic findings might indicate the presence of two enzymes with different Michaelis constants. However, the results of the immunological investigations gave no indication of the presence of more than one protein in the purified seminal vesicle fraction. Moreover, in the placental fraction, although two proteins were present, heat treatment caused the disappearance of only one of the zones, concomitant with the disappearance of enzyme activity, indicating the presence of only one enzyme in this preparation, too.

Both enzymes gave good immunological response, and for both enzymes it was shown that heat treatment at 56° caused inactivation as well as partial or complete destruction of antigenic properties. In spite of the great similarity in chromatographic and kinetic behaviour, the ovine enzymes purified from placenta and from seminal vesicles were however antigenically completely different.

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