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INHIBITION STUDIES ON CHICKEN MUSCLE ALDOSE REDUCTASE

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Abstract—Several compounds that are known to inhibit mammalian aldose reductases were examined for their effects on chicken muscle aldose reductase (EC 1.1.1.21). Sorbinil was the most effective compound tested. Alrestatin and phenobarbital were effective inhibitors of the enzyme although their IC_{50} values were 10-fold more than that of Sorbinil. Indomethacin, diphenylhydantoin, phenacetin, and valproate were also inhibitors of chicken muscle aldose reductase but were less effective. These compounds are all non-competitive inhibitors with respect to substrate. Menadione bisulfite, a water-soluble analog of Vitamin K_3 which is a substrate for carbonyl reductase but not aldose reductase, was a competitive inhibitor of chicken aldose reductase with respect to substrate. This observation is discussed with reference to the possible treatment of muscular dystrophy with specific inhibitors of aldose reductases.

Aldose reductase belongs to a group of monomeric NADPH-dependent aldo/keto reductases (EC 1.1.1.21) that catalyze the reduction of a wide range of aromatic and hydroxy-aliphatic aldehydes [1, 2]. Although the exact physiological roles of these enzymes are unknown, aldose reductase functions as the first enzyme in the polyol pathway which converts glucose into sorbitol and then to fructose [3]. The accumulation of sorbitol in several tissues is a cause of some of the clinical complications of diabetes [4]. Many of these pathological changes can be overcome by administering compounds that inhibit aldose reductase [5–9]. Sorbitol also accumulates in the muscle of patients with Duchenne muscular dystrophy [10], and there is a 13-fold increase in the activity of aldose reductase in dystrophic muscle compared with normal muscle [11]. This effect can be diminished by 3,3′-tetramethyleneglutarate, a known inhibitor of aldose reductase [11].

The enzymic activity of prostaglandin E 9-ketoreductase increases in chicken dystrophic breast muscle [12]. However, the enzyme responsible for this activity is not specific for 9-ketoprostaglandins, and there is considerable evidence that it is a member of the group of monomeric NADPH-dependent aldo/keto reductases [13, 14]. With the confusion that still exists over the nomenclature of the monomeric aldo/keto reductases, there is a strong possibility that it was aldose reducase that was assayed as prostaglandin E 9-ketoreductase in the muscle of the normal and dystrophic chickens [12]. Support for this hypothesis comes from the work of Bernado *et al.* [15] who purified an enzyme from chicken muscle that catalyses the NADPH-linked reduction of α-hydroxycarbonyls. Although this enzyme has many properties in common with mammalian aldose reductases [1–3, 16, 17], it was called L-glycol dehydrogenase [15]. One of the observations that was made during the purification of the L-glycol dehydrogenase from chicken muscle [15] and also during the purification of prostaglandin E 9-ketoreductase from chicken heart [18] was that there is a natural inhibitor of these enzymes present in these tissues. The increase in prostaglandin E 9-ketoreductase activity that is associated with chicken muscular dystrophy is not due to the presence of more enzyme, but rather to a decrease in the amount of an inhibitor present in this tissue [12]. We have shown recently that there is only one monomeric NADPH-dependent aldo/keto reductase present in chicken breast muscle [19]. This enzyme has sufficient properties in common with chicken kidney aldose reductase [20] and mammalian muscle aldose reductases [16, 17] to be considered an aldose reductase. In addition, the chicken muscle aldose reductase shares many features with L-glycol dehydrogenase [15]. It is most probable that a single enzyme is responsible for these activities and the reduction of 9-ketoprostaglandins.

One of the characteristics of dystrophic chickens is their inability to right themselves when placed on their backs. Clinical trials with normal and dystrophic chickens showed that administration of diphenylhydantoin [21–25] or indomethacin [25] produces significant results with a restoration of righting ability in treated dystrophic birds. Although the mode of action of these drugs has not been established, it is generally thought that they act by altering ion flux across the plasma membrane [21, 25]. Diphenylhydantoin and indomethacin are inhibitors of aldose reductase [17]. Therefore, an alternative explanation for the mode of action of these drugs is that they act by inhibiting the chicken muscle aldose reductase and thus prevent the conversion of glucose into sorbitol. To test this possibility, we have examined the effect of some mammalian aldose reductase inhibitors on purified chicken muscle aldose reductase. From these studies we hope to develop a potent inhibitor of this enzyme and to test it *in vivo* with genetically dystrophic chickens.

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MATERIALS

All chemicals, unless otherwise stated, were purchased from the Sigma Chemical Co., St. Louis, MO. Dr. T. G. Flynn provided Sorbinil (Pfizer) and Alrestatin (Ayerst). Chicken muscle aldose reductase was purified from male White Leghorn chickens of approximately 8 weeks of age as previously described [19]. The enzyme preparation had a specific activity of 0.9 μmole of pyridine-3-aldehyde reduced per min per mg protein. On electrophoresis on polyacrylamide gels under denaturing conditions there was one major band and a minor band which corresponded to a slightly lower molecular weight.

EXPERIMENTAL PROCEDURES AND RESULTS

Aldo/keto reductases evolve rapidly [26, 27]. It was of interest, therefore, to determine the potency of some mammalian aldose reductase inhibitors towards the chicken enzyme. The reaction mixture contained 30 mM DL-glyceraldehyde as substrate, 160 μM NADPH, and 0.1 M phosphate buffer, pH 7.0, in a final volume of 3 ml [17]. The assay was initiated by the addition of enzyme. The activity was determined by measuring the decrease in absorbance at 340 nm. Table 1 presents a comparison of the effects of some drugs on the activities of chicken and pig [17] muscle aldose reductases. The results are expressed as percent inhibition taking the control value as 0 percent.

The nature of the inhibition of these compounds with chicken aldose reductase was investigated by measuring the enzymic activity in the presence of fixed amounts of an inhibitor and varying the substrate concentration. The substrate in this case was pyridine-3-aldehyde. This compound was chosen as it is a good substrate for aldose reductase, aldehyde reductase, and carbonyl reductase [28, 29]. Thus, it was possible to compare the effects of the inhibitors on the active sites of these monomeric oxidoreductases using the same substrate in each case. The reaction mixture also contained 160 μM NADPH and 0.1 M phosphate buffer, pH 7.0. Assays were performed at 25° and initiated by the addition of enzyme. The results were analysed using a nonlinear regression curve fitting package [ROSFIT] which has been specifically designed for enzyme kinetic analysis with a Hewlett–Packard HP 85 microcomputer [30]. The data were fit to models for competitive inhibition, classical noncompetitive inhibition, and uncompetitive inhibition. The models were discriminated by comparing the residual mean square errors for each. At least three concentrations of inhibitor and five concentrations of substrate were used in each analysis. All of the compounds were noncompetitive inhibitors of chicken muscle aldose reductase with respect to the substrate. This is shown in Fig. 1 using Lineweaver–Burk plots for the sake of illustration. Inhibition constants for these compounds are given in Table 2.

One of the characteristics that can be used to differentiate among the different types of monomeric aldo/keto reductases is their ability to catalyze the reduction of menadione [28]. In the course of determining the type of aldo/keto reductase that is present in chicken breast muscle, it was found that a water-soluble analog of menadione, menadione bisulfite,

Table 1. Effects of inhibitors on the enzymatic activities of chicken and pig muscle aldose reductases

Inhibitor	Concn (mM)	Percent inhibition	
		Chicken*	Pig†
None		0	0
Sorbinil‡	0.01	35	55
	0.1	74	74
Alrestatin§	0.01	14	54
	0.1	46	73
Phenobarbital‖	0.1	43	8
	1.0	50	19
Indomethacin¶	0.1	13	67
	1.0	37	
Diphenylhydantoin**	0.1	27	48
	1.0	31	
Valproate††	0.1	21	8
	1.0	23	27
Phenacetin‡‡	0.1	13	
	1.0	17	

* Results are the average of three experiments.
† From Cromlish and Flynn [17].
‡ *d*-6-Fluorospiro-(chroman-4,4′-imidazolidine-2,5′-dione).
§ 1,3-Dixo-1-H-benze-(de)-isoquinoline(3-H)-acetic acid.
‖ 5-Ethyl-5-phenylbarbituric acid.
¶ 1-(*p*-Chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid.
** 5,5-Diphenyl-2,4-imidazolidinedione.
†† 2-Propylpentanoic acid.
‡‡ *N*-(4-Ethoxyphenyl)-acetamide.

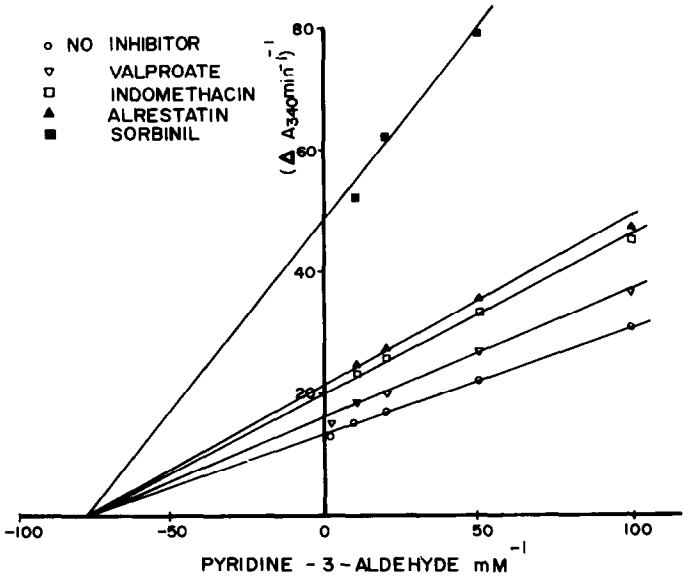

Fig. 1. Double-reciprocal plots of chicken muscle aldose reductase in the absence and presence of inhibitors. Key: no inhibitor (○), 0.1 mM Sorbinil (■), 0.1 mM Alrestatin (▲), 1.0 mM indomethacin (□), and 1.0 mM valproate (▽).

Table 2. Inhibition constants for various drugs with chicken muscle aldose reductase

Inhibitor	Type of inhibition	K_i^* (mM)
Sorbinil	Noncompetitive	0.037 ± 0.001
Alrestatin	Noncompetitive	0.151 ± 0.005
Phenobarbital	Noncompetitive	2.76 ± 0.64
Indomethacin	Noncompetitive	1.95 ± 0.06
Diphenylhydantoin	Noncompetitive	3.08 ± 0.51
Phenacetin	Noncompetitive	3.42 ± 0.17
Valproate	Noncompetitive	4.80 ± 0.24

* The K_i ± S.D. for each compound was determined using a non-linear regression curve fitting program (ROSFIT) [30].

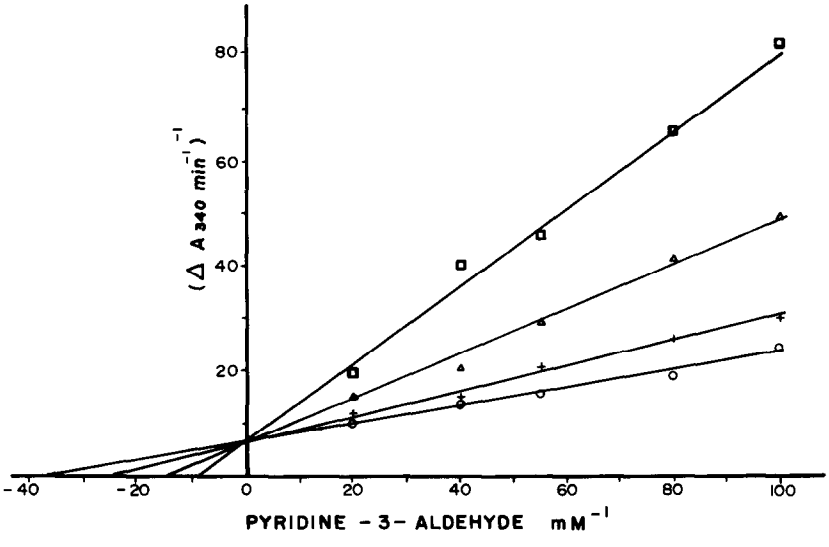

Fig. 2. Double-reciprocal plot of inhibition of chicken aldose muscle aldose reductase by menadione bisulfite. Key: no inhibitor (○), 0.1 mM inhibitor (+), 0.5 mM inhibitor (△), and 1.0 mM inhibitor (□).

was not reduced in the presence of the enzyme and NADPH. To our surprise however, this compound acted as an inhibitor of the aldose reductase. This was investigated further as described above. Figure 2 illustrates the result that was obtained. In contrast to the other inhibitors that were tested, menadione bisulfite was a competitive inhibitor of chicken aldose reductase with respect to substrate, and had an inhibition constant of 0.025 ± 0.004 mM.

DISCUSSION

Several inhibitors of mammalian aldose reductases have been reported, and it is hoped that at least some of them may prove useful in combating the side-effects of diabetes [5–9, 31]. If aldose reductase is also responsible for some of the clinical symptoms of muscular dystrophy, then these drugs should provide a chemotherapeutic means for alleviating some of the problems that this disease causes. The genetically dystrophic chicken [32] is a useful animal model system for testing this hypothesis. It was decided to investigate the potency of some known mammalian aldose reductase inhibitors with purified chicken muscle aldose reductase before testing their efficiency *in vivo* with genetically dystrophic chickens.

Sorbinil was found to be the most effective inhibitor of chicken aldose reductase, and its IC_{50} was similar to that for pig muscle aldose reductase [17]. The other compounds were at least an order of magnitude less potent. The observation that Alrestatin was 10-fold less inhibitory towards the chicken enzyme than towards the pig enzyme was surprising as this drug has been used clinically with diabetics [33]. Indomethacin and diphenylhydantoin were also less effective with the chicken enzyme but phenobarbital had a greater effect on chicken muscle aldose reductase than on the pig enzyme. Valproate, at a concentration of 1 mM, totally inhibits the aldehyde reductase of rat brain, but at this concentration of valproate there is negligible loss of activity of the rat brain aldose reductase [34]. It has been proposed that this differential sensitivity to valproate be used to distinguish between different forms of the monomeric aldo/keto reductases [28]. However, both the chicken and the pig muscle reductases were inhibited by approximately 25% in the presence of 1 mM valproate (Table 1). This emphasizes the point that aldo/keto reductases are evolving rapidly [26, 27], and should serve as a warning against extrapolating from species to species with results obtained with non-specific inhibitors of aldose reductase.

The finding that these compounds are noncompetitive inhibitors with respect to substrate for chicken aldose reductase was not unexpected as this has been observed with human aldose reductase [5, 8]. In addition, these compounds are not specific for aldose reductases and they have been shown to be noncompetitive inhibitors of aldehyde reductases [35–37], carbonyl reductase [38] and both succinic semialdehyde dehydrogenase and γ-aminobutyric acid (GABA) aminotransferase [39]. This undoubtedly poses a problem for their use as therapeutic agents and indicates the need for drugs that are specific for aldose reductases. The best approach will be to search for compounds that are directed towards the active site of aldose reductases.

Menadione bisulfite behaved as a competitive inhibitor of chicken muscle aldose reductase. As far as we are aware, this is the first report of a competitive inhibitor with respect to substrate of an aldose reductase. This result is most encouraging as it may reveal the path to the heart of the active site of the enzyme. In terms of therapeutic value, menadione is a substrate for carbonyl reductase [38] so will not interfere with the physiological role of this enzyme. The effect of menadione or its analogs on aldehyde reductase has not been investigated yet. Moreover, menadione is a synthetic form of Vitamin K and is known as Vitamin K_3 [40]. As the elevated levels of aldose reductase in the breast muscle of dystrophic chickens are the result of a decrease in the concentration of a natural inhibitor [12], it is possible that the disease may be partially the result of a vitamin deficiency. This result also raises the possibility that some of the problems associated with hereditary muscular dystrophy may be overcome by a dietary regime, or, if this fails, by the administration of a drug structurally related to menadione.

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