Quenching of Intrinsic Fluorescence of Sperm Specific LDH by Optical Isomers of Gossypol

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Abstract. Intrinsic fluorescence of LDH-C_4 has been studied in the presence of optical isomers of gossypol The study showed that fluorescence due to tryptophan residues after excitation of LDH at 282 nm is quenched by each gossypol enantiomere in a concentration dependent manner Half of the maximum quench ($Q_{50}\%$) of enzyme occurred with gossypol (−) at $0\,9 \times 10^{-4}$ mol/l and with gossypol (+) at $1\,4 \times 10^{-4}$ mol/l showing a maximum quench (Q_{max}) of 45% and 65% respectively, with a corresponding association constant (K_a) of $1\,0 \times 10^4$ l/mol and $0\,4 \times 10^4$ l/mol Stern-Volmer constant (K_{sv}) inferred that quenching of LDH comprises at least two components with two different K_{sv} values $K_{sv(I)}$ and $K_{sv(II)}$ between LDH-C_4 and gossypol (−) were $1\,97 \times 10^3$ l/mol and $1\,22 \times 10^3$ l/mol, and those between LDH-C_4 and gossypol(+) were $2\,3 \times 10^3$ l/mol and $1\,56 \times 10^3$ l/mol Smaller K_{sv} at higher concentrations of gossypol indicated that some of the tryptophan residues in LDH-C_4 are deeply buried within a hydrophobic environment There was no blue or red shift of LDH-C_4 when interacting with either of the gossypol enantiomeres

Key words: Intrinsic fluorescence — Gossypol (−) Gossypol(+) — Male antifertility agent — Association constant (K_a) and Stern-Volmer constant (K_{sv})

Introduction

Gossypol, a male antifertility agent (National Co-ordination Group on Male antifertility, 1978) is an amber coloured polyphenolic compound present in cotton plant Gossypium (*G herbacium G hirsutum* and *G arboreum*) It is known to be 1 1′,6,6′,7,7′-hexahydroxy-3, 3′-dimethyl-5, 5′-diiso-propyl-2, 2′binaphthalene-8,8′ dialdehyde, and has both phenolic and carbonyl groups which can react with acids or amines Gossypol binds strongly with proteins and forms stable complexes Cater and Lyman (1969) reported that gossypol forms complexes with several free amino acids and that the binding of carbonyl group of gossypol and amino group cross linking between protein chains can make the basis of the effect of gossypol on enzymic reactions (Tanksley et al 1970)

Gossypol is known to inhibit sperm-specific lactate dehydrogenase-C_4 (LDH-C_4) (Stephens et al 1986) which is an unique target for chemical contraception in males and in females after immunization (Goldberg et al 1981, Gupta et al 1994, Gupta and Syal 1997) The relative hydrophobicity of LDH-C_4 (Li et al 1983b) in the loop region facilitates complex formation between gossypol and the coenzyme binding site which explains why LDH-C_4 is most sensitive of the three isozymes of LDH after gossypol treatment *In vitro* studies on crude tissue preparations confirmed the inhibition of LDH C_4 by gossypol and its effect on sperm motility (Montamet et al 1982, Whaley et al 1986) although the inhibitory effect was not specific to LDH-C_4 isozyme (Gupta et al 1988) The inhibition of three isozymes of LDH was of a non-competitive type with respect to pyruvate and lactate, and of a competitive type when NAD and NADH were varied (Gupta et al 1988) However, there are marked differences in the disposition and metabolism between (+) and (−) isomers of gossypol Studies by various investigators showed (+) isomer to be less effective as an antifertility agent as compared to (−) or (±) forms (Waller et al 1983, Kim et al 1985, Lindberg et al 1987) Whaley et al (1984), showed that both (+) and (±) enantiomers of gossypol quench tryptophan fluorescence of human and bovine serum albumin (HSA and BSA) Although the kinetic and antigenic properties of LDH-C_4 have been studied extensively (Gupta et al 1981, Kaumaya et al 1990, 1992, Gupta and Kinsky 1993), fluorescence properties of LDH-C_4 after interacting with gossypol have been so far ignored The primary aim of this study is to describe the interaction of LDH-C_4 with two optical isomers of gossypol following quenching of its intrinsic fluorescence, and to compare the interactive properties of this iso/yme using two enantiomeres as quenchers

Materials and Methods

Animals

Balb/C (H-2^d) strain of male mice from Panjab University Animal House were used for isolation of LDH-C_4

Chemicals

8-(G-amino hexyl) AMP sepharose, Na-lactate, Na pyruvate, NAD, $NADH_2$ and reagents for electrophoresis were obtained from Sigma (St Louis, MO, USA) Gossypol(+) (purity 95 5%) and Gossypol(-) (purity 95 5%) were obtained from WHO, Geneva through courtesy of Dr N R Kalla of the Department LDH-C_4 was prepared in the laboratory (Gupta and Kinsky 1993)

Preparation of LDH-C_4

LDH-C_4 from Balb/C murine testes was prepared according to a method reported earlier (Gupta and Kinsky 1993) Testicular extract obtained at 27,000 × g was

heated at 60 °C for 30 min and adjusted to pH 6 5 in the presence of 0 5 mol/l KH_2PO_4 The enzyme solution was passed through an 8-(G-amino hexyl) AMP-sepharose column pre-equilibrated with 0 5 mol/l KH_2PO_4 (pH 6 5) The column was washed excessively with 50 mmol/l potassium phosphate at pH 6 5, and LDH-C_4 was eluted biospecifically with reduced NAD-pyruvate adduct contained in 10 mmol/l potassium phosphate buffer at pH 6 5 The protein was concentrated in 0 15 mol/l NaCl through Amicon Diflo membrane with cut off point of 10,000 or in Centricon microconcentrators (Centricon-10, Amicon) The homogeneity of C-subunit of LDH was confirmed electrophoretically as shown in Fig 1 The purified enzyme had a specific activity of 67 units/mg protein and compared well with earlier reports (Lee et al 1982, Gupta and Kinsky 1993)

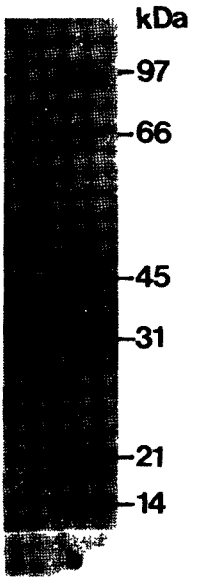

Figure 1. SDS-PAGE of purified LDH-C_4 (30 μg) at 10% acrylamide gel at a current intensity of 30 mA done at 25 °C Lane a – Showing the band of C-subunit of 35 kDa Lane b – Marker Proteins (Low MW kit of Bio Rad)

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out following the method of Laemmli (1970) using 3 5% stacking gel and 10% separating gel at pH 8 3 (running buffer containing Tris, SDS and glycine) Electrophoresis was run on Bio-Rad slab gel electrophoretic apparatus at a current intensity of 30 mA Purified LDH-C_4 (30 μg) and marker proteins (Bio Rad low molecular weight kit) were mixed with sample buffer (10% SDS 2-mercaptoethanol, glycerol and bromophenol blue) and applied over the stacking gel with the help of a micropipette The gels were removed, stained with Coomassie blue R-250, then destained and photographed The band corresponding to the molecular weight of 35 kDa of C-subunit of LDH is shown in Fig 1

Fluorescence measurements

Fluorescence measurements were carried out at 25 ± 2°C on a HITACHI F-3000 Fluorescence Spectrophotometer. For excitation wavelengths, protein was excited at 253 nm, 282 nm, 292 nm maximum fluorescence emission due to tryptophan occurred at 340 nm (λ_{max}) following excitation at 282 nm. Therefore, 282 nm was chosen as the excitation wavelength.

Gossypol solutions were prepared fresh using ethanol as solvent. The fluorescence quenching was corrected empirically for internal absorption by subtraction of the fluorescence shown by gossypol, when excited in absence of LDH-C_4. The true change in fluorescence was obtained using equation (1)

$$sI = sI_{obs} - sI_{int} \tag{1}$$

Where sI is the corrected change in fluorescence, and sI_{obs} and sI_{int} are the values of the observed fluorescence and that due to the internal filter effects respectively. The interaction of LDH-C_4 at 1.45×10^{-6} mol/l concentration with gossypol (LDH-C_4 Gossypol adduct) was monitored by following the change in relative fluorescence intensities and shift in λ_{max} produced by graded concentrations of gossypol as ligand.

Association constant (K_a)

A quenching curve of $Q\%$ against gossypol concentration and corresponding double reciprocal plots and mass action plots were constructed. Mass action plot was used for calculation of association constant K_a, using a procedure as described by Lehrer (1975) (equation 2)

$$K_a = \frac{\beta}{(1-\beta)} \times \frac{1}{[L_f]} \tag{2}$$

in which $\beta = I/Q_{max}$ and $[L_f] = [L] - \beta[L_T]$ where I is the corrected fluorescence intensity, Q_{max} is the maximal fluorescence quenching, $[L_f]$ is the molar equilibrium concentration of the unbound ligand, $[L]$ is the molar constituent concentration of ligand, and $[L_L]$ is the molar concentration of the enzyme expressed on protein basis. Q_{max} was determined by extrapolation of a double reciprocal plot to intercept and assuming the binding stoichiometry to be of the order of 1:1. The value of K_a was obtained from the slope of the plot $\beta/(1-\beta)$ versus $[L_f]$.

Stern Volmer constant (K_{sv})

The quenching reaction between the excited state of an indole ring, M^*, and the ligand, L, is described by equation (3)

$$M^* + L \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} (M^* \quad L) \xrightarrow{k_s} M + Q + s \tag{3}$$

Where (M^* L) is the complex formed by diffusional encounter between M^* and L with rate constant k_d The encounter complex then reacts to dissipate the excited state into heat (s) and ground state of indole ring (M) with rate constant k_i The relationship often employed to describe the collisional quenching process is given by Stern-Volmer equation (4) (Eftink and Ghiron 1976)

$$\frac{I_o}{I} = 1 + K_{sv}[L] \tag{4}$$

where I_o and I are the fluorescence intensities of indole ring at 340 nm, the wavelength of maximum fluorescence (λ_{max}) in the absence and presence of gossypol (L), and K_{sv} is the collisional quenching constant By plotting I_o/I versus concentration of quencher [L], K_{sv} was evaluated from the slope of the line Excitation wavelength for Q_{max} and K_{sv} measurements was set at 280 nm

Results

Binding of gossypol (+) and gossypol (−) quenched the fluorescence of LDH-C_4 in a concentration dependent manner but without any band shift of fluorescence maxima (λ_{max}) Relative intensities plotted against increasing concentrations of gossypol showed Q_{max} for gossypol (−) of 45%, and that for gossypol (+) of 65%, half of Q_{max} i e $Q_{50\%}$ occurred at $0\,9 \times 10^{-4}$ mol/l and $1\,4 \times 10^{-4}$ mol/l with gossypol (−) and (+) respectively (Figs 2*a* 3*a*) Double reciprocal plots (Figs 2*b* 3*b*) indicated that gossypol (+) quenched LDH fluorescence more strongly than gossypol (−) but not as strongly as NAD and NADH (Gupta and Kang 1997) Assuming the binding stoichiometry as $n = 1$, mass action plot (Figs 2*c* and 3*c*) gave a higher value of K_a between LDH and gossypol (−) i e 1×10^1 l/mol as compared to gossypol (+) which had a K_a value of $0\,4 \times 10^4$ l/mol (Table 1) Stern-Volmer constant which depends on gossypol concentration (Figs 2*d*, 3*d*) had a low value of K_{sv} at higher concentrations of gossypol (−) i e $K_{sv(I)} = 1\,97 \times 10^3$ l/mol and $K_{sv(II)} = 1\,22 \times 10^3$ l/mol The corresponding values for gossypol (+) were $K_{sv(I)} = 2\,3 \times 10^3$ l/mol and $K_{sv(II)} = 1\,56 \times 10^3$ l/mol (Table 1) K_a and K_{sv}

Table 1. Apparent K_a and K_{sv} values for LDH-C_4-gossypol interaction

Quencher	K_a ($\times 10^4$ l/mol)	K_{sv} ($\times 10^3$ l/mol)	
		I	II
Gossypol (−)	1 0	1 97	1 22
Gossypol (+)	0 4	2 30	1 56

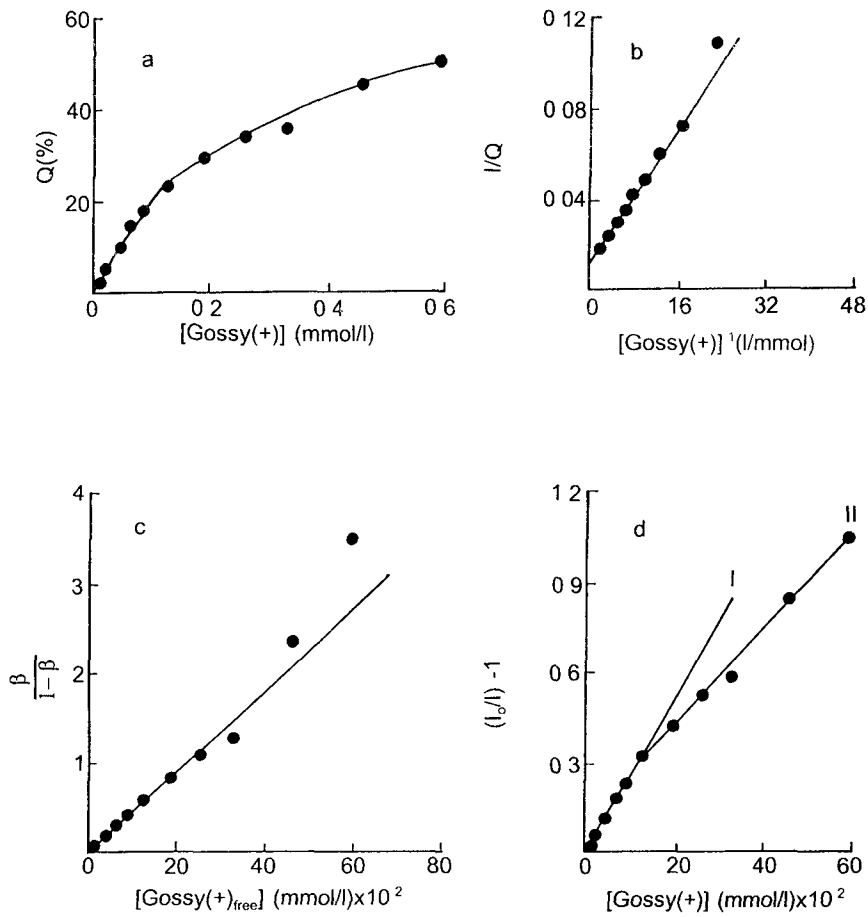

Figure 2. (*a*) Quenching of fluorescence at 340 nm of LDH-C_4 (Q) after excitation at 282 nm at increasing concentrations of gossypol (+) [Gossy (+)] at 25°C (*b*) Double reciprocal plot of LDH-C_4 between $1/Q$ and increasing concentrations of gossypol (+) corresponding to Fig. 2 (a) (*c*) Mass action plot of LDH-C_4 between $\beta/(1-\beta)$ and increasing concentrations of gossypol (+) for determination of K_a (*d*) Stern-Volmer plot of LDH-C_4 between $(I/I_o)-1$ and increasing concentrations of gossypol (+) for determination of K_{sv}

values between LDH-C_4 and gossypol (+) or (−) are higher than between LDH-C_4 and lactate or pyruvate but equal to, or less than between LDH-C_4 and NADH or NAD (Gupta and Kang 1997)

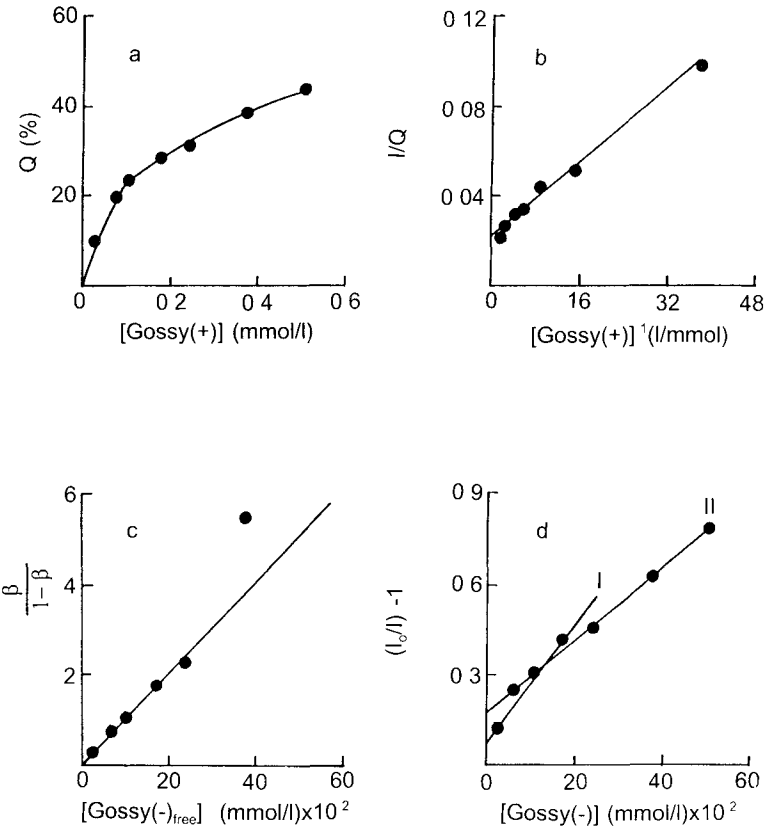

Figure 3. (*a*) Quenching of fluorescence at 340 nm of LDH-C_4 (Q) after excitation at 282 nm at increasing concentrations of gossypol (—) [Gossy (—)] at 25 °C (*b*) Double reciprocal plot of LDH-C_4 between $1/Q$ and increasing concentrations of gossypol (—) corresponding to Fig. 2(*a*) (*c*) Mass action plot of LDH-C_4 between $\beta/(1-\beta)$ and increasing concentrations of gossypol (—) for determination of K_x (*d*) Stern Volmer plot of LDH-C_4 between $(I/I_0) - 1$ and increasing concentrations of gossypol (—) for determination of K_{sv}

Discussion

A strategy often employed in studying the solution structure of proteins is to map out those residues which are exposed versus those which are buried (Kronman and Robbins 1970) Tryptophanyl residues have received considerable attention in topographical studies of protein by fluorescence quenching in the presence of various agents (Lehrer 1971 1975) However in a multiprotein system these studies are usually associated with many complications

LDH-C_4 of mice contains six tryptophanyl residues per subunit (Li et al 1983a,b) Gossypol induces conformational determinants in LDH-C_4 which enhance humoral antibody response leading to a high rate of infertility in allogenic mice (Gupta and Syal 1997), the effect could be related to crosslinking between protein chains caused by gossypol (Tanksley et al 1970) Stern-Volmer plots for LDH-C_4 with gossypol (−) or (+), were either linear or curved showing downward curvature representing single or bicomponent parts of the reaction, similar to some other proteins using a variety of quenchers yielding different K_{sv} values (Teale and Badey 1970, Lehrer 1975, Eftink and Ghiron 1976) Such negative deviations result from fluorescence of certain tryptophans being selectively quenched before others in a given protein At a low concentration of gossypol the slope of the Stern-Volmer plot reflects largely the quenching of the more accessible residue(s) (Eftink and Ghiron 1976) At higher concentrations, the easily quenched fluorescence has been greatly depleted, and tryptophans with lower quenching constants become dominant Selective quenching in this manner can only be detected if the quenching constants for each fraction of the fluorescence are quite different If this is *not the case, the data tend to collapse to give apparently linear plots* Static quenching causes the plots to curve upwards, and, therefore, oppose any negative deviation due to selective quenching (Eftink and Ghiron 1976) The fact that for LDH-C_4 when quenchers were gossypol (−) and (+) the Stern-Volmer plots did indeed curve downwards, indicates that the curvature due to selective quenching overwhelms any positive deviations caused by the static components The heterogeneous fluorescence suggests that some of the residues in this protein like many others (Eftink and Ghiron 1976) are almost completely buried within the structure (Li et al 1983b) and the indole rings of tryptophan(s) located in a hydrophobic environment become accessible due to the change in the conformation of the enzyme brought about by gossypol Since K_a is a measure of chemical affinity between gossypol and the enzyme it suggests that gossypol (−) is more reactive than gossypol (+) with LDH-C_4 Thus the loss of enzyme activity due to gossypol (Gupta et al 1988) is dependent on the nature of optical isomers and explains why gossypol (−) is more effective than gossypol (+) in inducing infertility in male species reported earlier (Waller et al 1983, Kim et al 1985 Lindberg et al 1987)

The ease with which gossypol (+) can quench the fluorescence of tryptophan in LDH seems to indicate that gossypol (+) in comparison to gossypol (−) can diffuse more easily into the interior of the protein, where it can encounter the indole rings of tryptophan shielded by protein segments, and thus increases Q_{max} and diffusional quenching constant (K_{sv}) Since tryptophan-225 in C-subunit of LDH is located at the surface and other tryptophan residues are partially or wholly buried within the molecule (Li et al 1983a,b), it is possible that gossypol (−) is able to quench the fluorescence arising mainly from tryptophan-225 Thus, the greater infertility

effect of gossypol (−) is reflected in its binding affinity (K_a) rather than in K_{sv} or to the extent of Q_{max}

During interaction of gossypol with BSA and HSA fluorescence measurements indicated a high affinity binding site ($K_a = 2.2 \times 10^6$ l/mol) Based on extrinsic CD and difference spectrum measurements one more binding site on BSA for gossypol with $K_a = 2.7 \times 10^3$ was demonstrated (AppuRao 1992) CD and NMR spectra reported by Storm-Hansen et al (1989) implicated the formation of Schiff bases during interactions of amino acids and protein with gossypol On the other hand Cater and Lyman (1969) showed that gossypol forms complexes with several amino acids and helps in crosslinking, and thus affects the enzyme activity (Tanksley et al 1970) Although such studies on LDH-C_4 are not available, yet such possibilities cannot be ruled out

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References

AppuRao A G (1992) A stoichiometric analysis of bovine serum albumin-gossypol interactions A fluorescence quenching study Indian J Biochem Biophys **29,** 179–185

Cater C M Lyman C M (1969) Reactions of gossypol with amino acids and other amino compounds J Amer Oil Chem Soc **46,** 649—653

Eftink M R Ghiron C A (1976) Exposure of tryptophanyl residues in proteins Quantitative determination by fluorescence quenching studies Biochemistry USA **15,** 672 680

Goldberg L Wheat J L Powell J E Stevens V C (1981) Reduction of fertility in female baboons immunized with lactate dehydrogenase-C_4 Fert Steril **35,** 214—217

Gupta G S Goldberg E (1981) Thermodynamic stability of lactic dehydrogenase isozymes from rabbit and guinea pig Indian J Biochem Biophys **18,** 182—186

Gupta G S Kang B P S (1997) LDH-C_4 substrate binary complexes studied by intrinsic fluorescence method Indian J Biochem Biophys (Accepted)

Gupta G S Kinsky R G (1993) Modulation of allo-immune responses *in vivo* and *in vitro* by sperm specific lactate dehydrogenase-C_4 Mol Cell Biochem **125,** 145—151

Gupta G S Syal N S (1997) Immune responses of chemically modified homologous LDH-C_4 and their effect on fertility regulation in mice Amer J Reprod Immunol **37,** 206—211

Gupta G S Kapur S, Kinsky R G (1988) Inhibition kinetics of lactate dehydrogenase isozymes by gossypol acetic acid Biochem Int **17,** 25—34

Gupta G S Malhotra R Sehgal S (1994) Regulation of fertility in female mice after immunization with human sperm specific LDH Role in conception and contraception Indian J Exp Biol **32,** 14—19

Kaumaya P T P Berndt K D Heidorn D B Trewhella J Kezdy F J Goldberg E (1990) Synthesis and biophysical characterization of engineered topographic

immunogenic determinants with alpha alpha topology. Biochemistry USA **29,** 13—23

Kaumaya P. T. P., VanBuskirk A. M , Goldberg E., Pierce S. K. (1992): Design and immunological properties of topographic immunogenic determinants of a protein antigen (LDH-C_4) as vaccines. J. Biol. Chem. **267,** 6338—6346

Kim I. C , Waller D. P., Fong H. H. S (1985): Inhibition of LDH-X by gossypol optical isomers. J. Andrology **6,** 344—347

Kronman M J., Robbins F. M. (1970): Buried and exposed groups in proteins. In: Fine Structure of Proteins and Nucleic Acids (Eds. G.D. Fasman and S.N. Timasheff) Vol. 4, pp 271—416, Marcel Dekker, New York

Laemmli U. K. (1970): Cleavage of structural proteins during the assembly of the head of the bacteriophage T_4. Nature **227,** 680—685

Lee C-Y, Yuan J. H., Goldberg E. (1982): Lactate dehydrogenase isozymes from mouse. Methods Enzymol. **89,** 351—382

Lehrer S S. (1971): Solute perturbation of protein fluorescence. The quenching of tryptophanyl fluorescence of model compound and of lysozyme by iodide ions. Biochemistry USA **10,** 3254—3263.

Lehrer S. S (1975): Perturbations of intrinsic protein fluorescence In Biochemical Fluorescence: Concepts (Eds. R. F. Chen, H. Edelhoch) Vol. 2, pp 515—544, Marcel Dekker, New York.

Li S S L., Fitch W. M., Pan Y C F , Sharief F. S. (1983a). Evolutionary relationships of vertebrate lactate dehydrogenase isozymes A_4 (Muscle), B_4 (Heart), and C_4 (Testis) J. Biol. Chem. **258,** 7029—7032

Li S. S. L., Feldmann R. J., Okabe M., Pan Y. C. F. (1983b) Molecular features and immuniological properties of lactate dehydrogenase C_4 isozymes from mouse and rat testis J. Biol. Chem **258,** 7017—7028

Lindberg M. C., Naqvi R. H., Mathn S. A., Zhou R. H., Bialy G., Blye R. D (1987): Comparative antifertility effects of gossypol enantiomers in male hamsters. Int. J. Androl. **10,** 1013—1022

Montamet E E., Burgos C., Burgos N. M. G., Rovai L. E., Blanco A., Segura E. L. (1982) Inhibitory action of gossypol on enzymes and growth of trypanosoma cruzi. Science **218,** 288—289

National coordination group on male antifertility (1978): Chinese Med J. **4,** 417—420

Stephens D. T., Whaley K. J , Klinkow N. M., Goh P., Hoskins D. D. (1986): Kinetic characteristics of the inhibition of purified cynomolgus monkey lactate dehydrogenase isozyme by gossypol. J. Andrology **7,** 367—377

Storm-Hansen T., Carnett C., Jaroszowski J W (1989) Interaction of gossypol with amino acids and peptides as a model of enzyme inhibition. Int. J. Peptide Prot Res **34,** 306—310

Tanksley T. D., Neumann H., Lyman C M., Pace C. N., Prescott J. M. (1970): Inhibition of pepsinogen activation by gossypol. J. Biol. Chem. **245,** 6456—6461

Teale F. W J., Badey R A. (1970): Depolarization of the intrinsic and extrinsic fluorescence of pepsinogen and pepsin. Biochem. J. **116,** 341—348

Waller D. P., Bunyaprophatsara N., Martin A , Vournazos C. J., Ahmed M. M., Soejarto D D., Cordel G. A., Fong H. H. S , Russel L. D., Malone O. P. (1983) Effect of (+) gossypol on fertility in male hamster. J Andrology **4,** 276—282

Whaley K. J., Sampath D. S., BalaRam P. (1984): Optically active gossypol as a CD probe of intereactions with serum albumin. Biochim. Biophys. Acta **801,** 127—130

Whaley, K J , Stephens D T Klimkow, N M Hoskins, D D (1986) Monkey lactate dehydrogenase-C_4 as a model for the interaction of enzyme with gossypol Contraception **33,** 605—616

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