Further work must be carried out on histamine metabolism using additional A, B or mixed type MAO inhibitors and different doses of histamine, as the dose of the amine may be an important parameter[34]. However, results of previous work[12,30], strengthened by these data, suggest that type A MAO inhibitors should interfere less with the metabolism of histamine than other MAO inhibitors.

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The effect of pH and methylation on the interaction of deoxycholate with rat liver alcohol dehydrogenase[1]

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Summary. The activation of rat liver alcohol dehydrogenase by deoxycholate depends on the anionic form of the steroid. Methylation of the enzyme protein leads to an increase of both turnover number and K_m for ethanol and to a change in the effect of deoxycholate, which behaves as an inhibitor. It is suggested that the steroid and methylation effects depend on the same basic mechanism, in which one or more Lys groups are involved.

Deoxycholate is a modifier of liver alcohol dehydrogenase and its effect is species-dependent[2-4]. The modification of the rat liver enzyme has been specially studied with reference to its kinetic features[5]. In this case the overall effect is an activation, and the kinetic changes caused by the steroid are very similar to those observed for the horse enzyme after chemical modification of Lys residues[6,7]. The present work aims at elucidating the nature of the enzyme-steroid interaction, by studying the effects of deoxycholate on rat liver alcohol dehydrogenase at different pH's and after methylation of Lys groups.

Materials and methods. Alcohol dehydrogenase was purified from the liver of Sprague-Dawley COBS male albino rats as described previously[2,5]. The assay of the enzyme was carried out at 30 °C by recording the change of optical density at 340 nm with a Gilford 2400 spectrophotometer, in a test mixture with the following composition: a) 50 mM triethanolamine buffer, pH 7.6, 5 mM EDTA, 0.2 mM NADH, 2 mM propionaldehyde; b) 50 mM sodium pyrophosphate buffer, pH 8.5, 1 mM NAD^+, 9 mM ethanol. The assay of the enzyme at different pH values was carried out using a 80 mM sodium phosphate-Tris-glycine buffer. 1 unit (U) of the enzyme corresponds to the enzymatic activity that transforms 1 µmole substrate per min, under the test conditions. Proteins were determined by the absorbance at 280 nm[8]. Methylation of alcohol dehydrogenase with formaldehyde in the presence of sodium borohydride was carried out at 0 °C as described by Means and Feeney[9], in 100 mM pyrophosphate buffer pH 9.0, with a final ratio of 6 µmoles formaldehyde/mg protein. After methylation the sample was supplemented with 0.4 mg/ml dithioerythritol and dialyzed overnight against the same buffer.

Results and discussion. Figure 1 shows the effect of pH on the activity of rat liver alcohol dehydrogenase. The initial reaction rate in the absence of bile acids declines sharply between pH 6.3 and 8.7. The activity in the presence of deoxycholate reaches a maximum between pH 6.9 and 7.2. At higher pH values the enzyme is always activated by deoxycholate, while at lower pH values this activation effect decreases and it is reversed into inhibition below pH 6.5. These data suggest that the activation effect on the enzyme could depend on the anionic form of the deoxy-

cholic acid (pK_a 6.6). Actually, taurodeoxycholic acid (pK_a 1.4) enhances the enzyme activity even at pH lower than 6.5.

In order to investigate whether Lys residues are involved in the interaction between steroid and dehydrogenase, the rat liver enzyme was methylated with formaldehyde in the presence of sodium borohydride and the effect of this chemical modification on the enzyme activity and on the activation by deoxycholate was evaluated. Results are shown in table 1. As reported for horse liver alcohol dehydrogenase[10,11], protein methylation leads to an enhancement of enzyme activity; for rat liver alcohol dehydrogenase a 7-fold increase is observed. The presence of substrates and analogs during the methylation procedure influences the effect of methylation on enzyme activity: both the reduced and oxidized forms of the coenzyme are effective as protective agents, while $NADP^+$ is much less efficient. Protection given by 5'-AMP seems to indicate that such an effect depends on the adenosine moiety of the coenzyme. Ethanol and deoxycholate show a negligible protective effect.

The pH dependence of the methylated enzyme has also been determined, and it does not differ qualitatively from that of the unmodified enzyme. This could suggest that the pKs of the functional groups are not significantly changed by the alkylation procedure.

The effect of deoxycholate on the initial rate of the reaction catalyzed by the methylated enzyme, with ethanol as substrate, has also been investigated. The pattern (figure 2) corresponds to a competitive inhibition by the steroid ($K_i = 90$ μM) and is quite different from that observed for the native enzyme[3,5]. In table 2 the kinetic parameters for methylated and non-methylated enzyme are compared. Clearly methylation increases the turnover number and the K_m for ethanol, as observed for the native enzyme in the presence of deoxycholate. After methylation, the steroid leads to a further increase in the apparent K_m for ethanol, whereas the turnover number is unchanged. The dissociation constant (K_d) with respect to deoxycholate is very similar for both native and methylated alcohol dehydrogenases.

The increase in the apparent K_m for ethanol both for the methylated enzyme and for the enzyme in the presence of deoxycholate is difficult to explain on a kinetic basis, owing to the intrinsic complexity of such kinetic constants. However, this effect could be qualitatively explained, taking into account the structural similarity between C_{27} steroid alcohols - the physiological substrates of liver alcohol dehydrogenase[12,13] - and deoxycholate, that could

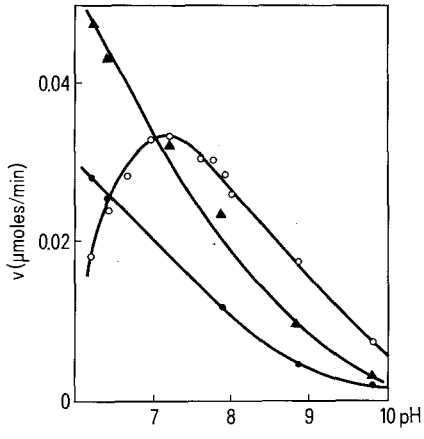

Fig. 1. Effect of pH and bile acids on the activity of rat liver alcohol dehydrogenase. Enzyme activity was assayed at 30 °C, with propionaldehyde as substrate. ●, Control; ○, + 1 mM sodium deoxycholate; ▲, + 1 mM sodium taurodeoxycholate.

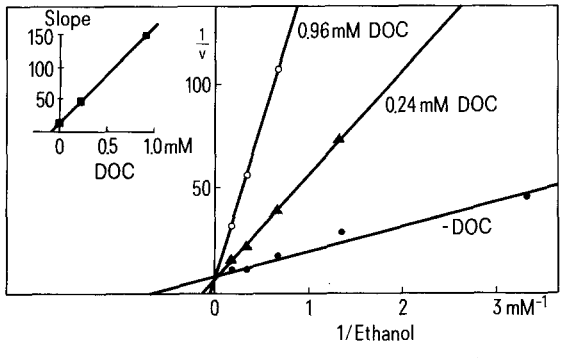

Fig. 2. Inhibition of methylated rat liver alcohol dehydrogenase by sodium deoxycholate (DOC). Test conditions were: 50 mM pyrophosphate buffer, pH 8.5, 1 mM NAD^+, 7.2 μg of methylated and dialyzed enzyme protein, ethanol and DOC as indicated, in a final volume of 1 ml; 30 °C. 1/v is expressed as $min/\Delta E_{340}$. In the inset, the slope values are reported as a function of deoxycholate concentration.

Table 1. Effect of the presence of substrates and analogs during the methylation procedure of rat liver alcohol dehydrogenase

Addition	Units/mg protein	Relative activity
None (control)	0.83	1.00
$HCHO + NaBH_4$	5.90	7.11
$HCHO + NaBH_4$ + 1 mM NADH	0.89	1.07
$HCHO + NaBH_4$ + 1 mM NAD^+	0.83	1.00
$HCHO + NaBH_4$ + 1 mM $NADP^+$	3.04	3.66
$HCHO + NaBH_4$ + 1 mM 5'-AMP	2.45	2.95
$HCHO + NaBH_4$ + 10 mM ethanol	4.21	5.07
$HCHO + NaBH_4$ + 1 mM deoxycholate	3.82	4.60

The enzyme was methylated at 0 °C by formaldehyde and sodium borohydride, in the presence of the compounds indicated. Enzyme activity was assayed with propionaldehyde as substrate, before (control) and 20 min after the 1st addition of formaldehyde.

Table 2. Turnover numbers and kinetic constants for unmodified and methylated rat liver alcohol dehydrogenase, in the presence or absence of sodium deoxycholate (DOC)

	$V_{max}/[E]$ (sec^{-1})	K_m(ethanol) (μM)	K_d(DOC) (μM)
Unmodified enzyme	0.71	342	-
Unmodified enzyme + 1 mM DOC	4.76	3080	82
Methylated enzyme	3.39	1340	-
Methylated enzyme + 1 mM DOC	3.39	20000	90

The data from figure 2 were used to calculate the kinetic parameters for the methylated enzyme. Other data from Hanozet et al.[2] and Simonetta et al.[3]. All measurements were carried out in pyrophosphate buffer, pH 8.5 and 30 °C. [E] is the concentration (per active site) of the enzyme. K_d is the dissociation constant for deoxycholate.

thus compete with ethanol for binding to the same catalytic site.

The increased turnover number in the presence of deoxycholate has been attributed to a modification by this steroid of a rate-limiting conformational change involved in the reaction mechanism of the dehydrogenase[5]. Such an increase is similar to that observed with the enzyme modified by reductive alkylation: since the 2 effects are not additive, they are probably dependent on the same mechanism, in which 1 or more amino groups, protected from methylation by the binding with the coenzyme, could be involved. Therefore the interaction of rat liver alcohol dehydrogenase with deoxycholate and the methylation procedure could promote the same general conformational modification, which seems to be the basic rationale of the observed kinetic changes.

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Co-polymerization of dopa and cysteinyldopa in melanogenesis in vitro[1]

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Summary. Tyrosinase oxidation of dopa in the presence of varying amounts of 5-S-cysteinyldopa led to the production of pigments whose chemical and physical (colour and solubility) properties are intermediate between those of pure eumelanin- and phaeomelanin-type pigments.

Recent advances[3,4] in the chemistry of melanogenesis have revealed that melanin pigmentation of hair, skin and eye results mainly from 2 separated but biogenetically interrelated classes of pigments: the black-brown, insoluble, nitrogenous eumelanins which derive from the enzymic (tyrosinase) oxidation of tyrosine via dopa, and the sulphur-containing alkali-soluble phaeomelanins, ranging from yellow to reddish-brown, which arise by a diversion from the eumelanin pathway through the intervention of cysteine or related sulphydryl compounds[5]. Chemical and biochemical studies provide evidence that a major intermediate in the biosynthesis of phaeomelanins is 5-S-cysteinyldopa (sulphur content: 10.1%) which is formed by the addition of cysteine to enzymically produced dopaquinone. The high content (9–10%) of sulphur in the phaeomelanins isolated from reddish hair from various mammals, including man[6], is quite consistent with the proposed pathway. In many cases, however, no strict borderline exists between eumelanins and phaeomelanins with respect to chemical and physical properties. It is, for example, typical of all eumelanins so far isolated that they contain sulphur in various percentages from 1% to as much as 5%, suggesting a possible intermeshing between eumelanin and phaeomelanin pathways[7].

To test this hypothesis, we have carried out a series of model reactions simulating the intermeshing of the 2 pigmentary pathways in melanogenesis by the enzymic oxidation of dopa in the presence of varying amounts of 5-S-cysteinyldopa (abbreviated as cys-dopa).

Materials and methods. Co-oxidation of dopa and cys-dopa. A mixture of L-dopa and cysdopa[8], the total amount being 0.5 mmoles in various ratios (see table) in 0.05 M sodium phosphate buffer, pH 6.8 (40 ml) was vigorously stirred at 37 °C, under an oxygen current, in the presence of mushroom tyrosinase (4 mg, 2230 units/mg, from Sigma Chemical Co.). After 4 hr the oxidation was stopped by acidification to pH 3.5 with 2M HCl and the mixture was kept at room temperature for 1 h. The resulting precipitate was then collected by centrifugation, washed with 1% acetic acid (3 times), acetone (twice) and dried in vacuo over P_2O_5 overnight.

Degradation with 57% HI[6]. A mixture of the biosynthetic melanin (25 mg) and red phosphorus (5 mg) in 3 ml of freshly distilled 57% HI was refluxed for 24 h. The reaction mixture was then evaporated to dryness under reduced pressure and the residue, taken up in water, was fractionated by chromatography on Whatman 3MM paper (eluents: 1-butanol-acetic acid-water, 60:15:25 and 0.08M HCl) to give 3-hydroxy-4-aminophenylalanine, β-6-(4-hydroxybenzothiazolyl)alanine and 2-methyl-β-6-(4-hydroxybenzothiazolyl)alanine identified by comparison of their chromatographic and spectral (UV) properties with those of authentic samples.

Alkaline fusion[6]. A mixture of the biosynthetic melanin (20 mg), NaOH (100 mg), $Na_2S_2O_4$ (15 mg) and water (0.3 ml) was heated in a platinum crucible at 280–300 °C for 10 min. After cooling, the fused mass was worked up according to the procedure already described[9], and ana-