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In Vitro Dechlorination of a 4,6-Dichloro-$\Delta^{4,6}$-keto Steroid*

D. E. Maynard,† O. Gurny, M. Carson, R. A. LeMahieu, M. A. Schwartz, M. K. Taylor, and R. W. Kierstead

ABSTRACT: Investigation of the *in vitro* metabolism of 4,6-dichloro-17α-hydroxypregna-4,6-diene-3,20-dione acetate (I) showed that in addition to the expected 4,6-dichloro-3-hydroxy derivatives IIIa and IIIb, the 6-monochloro compound II and the corresponding 6-monochloro-3-hydroxy compounds IVa and IVb were formed. Although proportions varied, the qualitative distribution of the metabolites was the same whether a 9000*g* rat liver microsomal preparation or the 105,000*g* supernatant fraction was used. After the metabolites had been characterized, attempts were made to identify the factor or factors responsible for the unique 4-dechlorination of I.

The effects of various substituents on the *in vitro* and *in vivo* metabolism of compounds possessing the basic progesterone nucleus have been widely studied. It is well known that the usual metabolic fate of progesterone in mammalian systems follows the path of Δ^4-3-keto, and 20-keto reduction as well as several ring hydroxylations, depending on species and tissue (Dorfman and Ungar, 1965).

There have been exceptions to this pattern, such as that reported by Ungar *et al.* (1957) where rat liver tissue was shown to be capable of reducing Δ^4-3-ketones to Δ^4-3-ols. Particularly germane to the present work was the report by Ringold *et al.* (1964) that substitution by halogen at C-2, C-4, or C-6 enhanced both rate and yield of Δ^4-3-ol formation from the corresponding Δ^4-3-ones. Conversely, these investigators found methyl substitution at these positions to severely retard reduction, and they offered cogent arguments for the electronic destabilization of the Δ^4-3-keto group by electronegative substitution, with consequent stabilization of the proposed transition state for reduction. It is of interest that the metabolism of 4-chlorotestosterone by human liver slices also produced the Δ^4-3-hydroxy derivative (Starka *et al.*, 1969).

An investigation by Cooke and Vallance (1965) of the metabolism of megestrol acetate[1] by rat and rabbit liver preparations clearly demonstrated the retardation of metabolism by modification of the progesterone skeleton. It can be inferred from this study that introduction of a 6,7-double bond may be partially responsible for the absence of 6-methyl hydroxylation, a conversion reported earlier (Castegnaro and Sala, 1962; Helmreich and Huseby, 1962) to be the major pathway of medroxyprogesterone acetate metabolism in humans.

The only chloro-substituted C_{21}-progestational steroid to have been widely used is chlormadinone acetate (II) (Ringold *et al.*, 1959; Brückner *et al.*, 1961). Its metabolism in women has been investigated (Bermudez *et al.*, 1968), but the identity of its metabolites has not been reported.

The 4-dechlorination undergone by the 4,6-dichloro steroid I in our experiments is without precedent in biological systems. It should be mentioned, however, that a similar reaction, the reductive dechlorination of DDT to DDD, has been observed in several systems (Klein *et al.*, 1964; Miskus *et al.*, 1965; Bunyan *et al.*, 1966). It was shown by Bunyan *et al.* (1966) that this dechlorination was enhanced under anerobic conditions by the presence of GSH. The present work devotes considerable attention to this tripeptide as a possible participant in the unique 4-dechlorination of I.

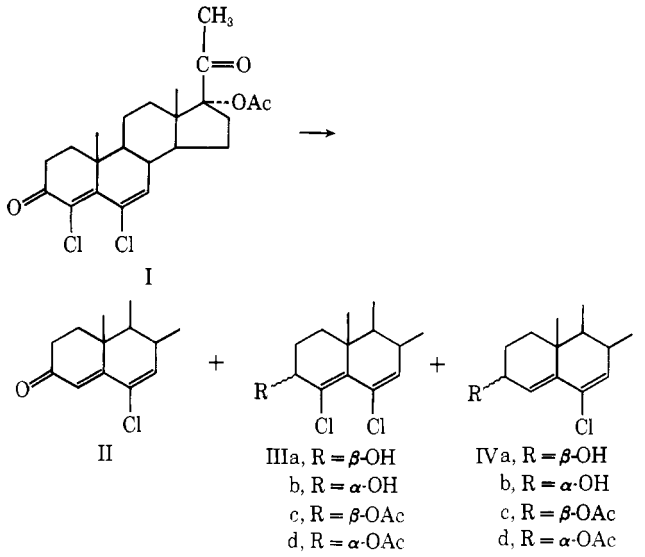
Experimental Section

Synthesis of 4,6-Dichloro-3β,17α-dihydroxypregna-4,6-dien-20-one 17-Acetate (IIIa). A solution of 2.000 g (4.5 mmoles) of I (Kierstead *et al.*, 1970) in 20 ml of anhydrous tetrahydrofuran was added dropwise over a 15-min period to 3.280 g (13.68 mmoles) of lithium aluminum tri-*tert*-butoxyhydride in 20 ml of anhydrous tetrahydrofuran under a nitrogen atmosphere. After stirring at room temperature for 2 hr, 20 ml of acetone was added followed by 150 ml of 10% acetic acid in water. The mixture was extracted with two 75-ml portions of chloroform; the organic phases were

* From the Departments of Chemical Research and Clinical Pharmacology, Hoffmann-La Roche Inc., Nutley, New Jersey 07110. *Received August 19, 1970.*

† To whom correspondence should be addressed.

[1] Trivial names and abbreviations used are: megestrol acetate, 17α-hydroxy-6-methylpregna-4,6-diene-3,20-dione acetate; medroxyprogesterone acetate, 17α-hydroxy-6α-methylpregn-4-ene-3,20-dione acetate; chlormadinone acetate, 6-chloro-17α-hydroxypregna-4,6-diene-3,20-dione acetate; DDT, 1,1-bis-(*p*-chlorophenyl)-2,2,2,-trichloroethane; DDD, 1,1-bis(p-chlorophenyl)-2,2-dichloroethane; GSH, reduced glutathione; GSSG, oxidized glutathione; NEM, *N*-ethylmaleimide; SKF-525A, 2-diethylamino-2,2-diphenylvalerate hydrochloride; Glc-6-P, glucose 6-phosphate.



combined, washed with 5% sodium bicarbonate, dried over $MgSO_4$, and concentrated under reduced pressure. The crude solid was crystallized from acetone–hexane to give IIIa (1.696 g, 85% yield): mp 255–257°; λ_{max}^{EtOH} 256 mμ (ε 21,000); $[\alpha]_D^{25}$ +26.1° (in $CHCl_3$, *c* 1.0%). *Anal.* Calcd for $C_{23}H_{30}O_4Cl_2$: C, 62.58; H, 6.85; Cl, 16.07. Found: C, 62.63; H, 6.72; Cl, 16.23.

Synthesis of 4,6-Dichloro-3β,17α-dihydroxypregna-4,6-dien-20-one Diacetate (IIIc). A mixture of 1.000 g of IIIa, 10 ml of acetic anhydride, and 10 ml of pyridine was left at room temperature for 18 hr. The excess reagents were removed on the oil pump and the residue was diluted with xylene and again concentrated to dryness. Crystallization from methanol gave IIIc (0.908 g, 83% yield): mp 218–220°; λ_{max}^{EtOH} 257 mμ (ε 18,850); $[\alpha]_D^{25}$ 24.2° (in $CHCl_3$, *c* 1.1%. *Anal.* Calcd for $C_{23}H_{30}Cl_2O_4$: C, 62.11; H, 6.67; Cl, 14.67. Found: C, 62.29; H, 6.66; Cl, 14.62.

Tissue Preparation. Livers from 150- to 200-g male Charles River rats were homogenized in 2 volumes of ice-cold 0.15 M KCl. Centrifugation at 9000*g* for 20 min yielded a supernatant microsomal fraction. A second centrifugation of the 9000*g* material for 60 min at 105,000*g* produced a nonparticulate supernatant fraction.

Aerobic Incubation. Each flask contained 6 μmoles of NADP, 3.4 μmoles of ATP, 400 μmoles of KCl, 20 μmoles of $MgCl_2$, and 14 μmoles of glucose 6-phosphate in a final volume of 4.8 ml of 0.1 M potassium phosphate buffer, pH 7.4 (Conney *et al.*, 1960). Enzyme equivalent to 300 mg of liver was added, followed after a 5-min preincubation by approximately 400 μg of substrate in 0.2 ml of propylene glycol–acetone (9:1). Samples were incubated at 37° for 60 min in a Dubnoff metabolic shaker.

Anaerobic Incubation. Each flask contained 1.2 μmoles of NADP, 3 μmoles of riboflavin, and 200 μmoles of nicotinamide in 7.5 ml of 0.1 M phosphate buffer, pH 7.4 (Fouts and Brodie, 1957). After addition of enzyme equivalent to 660 mg of tissue, followed by substrate as described above, the samples were incubated at 37° for 1 hr under nitrogen. Incubations were terminated by extracting twice with ethyl acetate. The combined extracts were dried under nitrogen, then taken up in acetone for thin-layer chromatography.

Separation and Quantitation of Metabolites. Samples were applied to silica gel G plates containing a fluorescent indicator (Mallinckrodt 7106). Each plate was developed for 1 hr in benzene–methanol (98:2), dried, then developed in the same direction in benzene–methanol (95:5) for an additional hour. Authentic samples of I, II, IIIa, and IVa were run as location standards.

After elution with chloroform–ethanol (1:1), the samples were dried under nitrogen, then redissolved in absolute ethanol for determination of ultraviolet spectra. The absorption maxima (mμ) and extinction coefficients used for quantitation were as follows: I, 298 (17,200); II, 284 (22,000); IIIa, 256 (21,100); IVa, 244 (22,800); V (R = H), 296 (20,700); V (R = CH_3), 301 (19,750); V (R = F), 298 (19,250); VI (R = F), 283 (24,580); VII (R = H), 251 (21,350); VII (R = CH_3), 252 (20,700); VII (R = F), 251 (23,100); VIII (R = F), 240 (22,250).

All spectral data were obtained with a Coleman-Hitachi EPS-3T recording spectrophotometer.

Gas–Liquid Chromatography. A Hewlett-Packard Model 402 instrument equipped with dual 4 ft × 4 mm glass U-columns and flame ionization detectors was used. A packing of 3.8% UC-W98 (methylsilicone) on 80–100-mesh Diatoport S gave satisfactory separations of both free and derivatized metabolites. Standard operating parameters were: column temperature, 240°; injection block, 265°; flame detector, 270°; gas flow rates (ml/min) helium 60, hydrogen 40, air 300.

Acetate derivatives were prepared by treating metabolites with excess pyridine–acetic anhydride (2:1) at room temperature overnight; the reagents were then removed under nitrogen. Trimethylsilyl ethers were formed by treatment with *N,O*-bis(trimethylsilyl)acetamide (Chambaz and Horning, 1967), and chromatographed directly.

Metabolite Oxidations. As standard procedure, the 3-hydroxy metabolite was dissolved in 0.5 ml of chloroform in a glass-stoppered centrifuge tube. MnO_2 (50 mg) (Winthrop Laboratories, New York, N. Y.) and a small magnetic stirring bar were added, and the reaction mixture was stirred continuously for 24 hr. After filtration and evaporation, the product was dissolved in absolute ethanol for identification and quantitation by ultraviolet spectroscopy. Comparison of the gas–liquid chromatography retention times with those of authentic compounds substantiated the identification.

Thin-Film Dialysis. The techniques and equipment described by Craig (1967) were used with only minor modification. No alteration was made in the Visking dialysis tubing. The dialysate was first changed in the morning after overnight dialysis at 5°, then was changed three more times at approximately 30-min intervals. Assuming equilibrium was attained each time, more than 90% of any freely dialyzable substance should have been removed.

Enzyme Purification. The procedure of Boyland and Chasseaud (1968) was used to obtain a rat liver enzyme fraction capable of adding glutathione to a variety of α,β-unsaturated carbonyl compounds. This and other liver preparations were assayed for protein (Sutherland *et al.*, 1949), oxidized and reduced glutathione (Klotzch and Bergmeyer, 1965), and total thiol (Ellman, 1954).

Results

Separation of the incubation extracts by thin-layer chromatography usually showed the presence of four compounds detectable by ultraviolet absorption. Occasionally, the least polar of these, later identified as unchanged substrate I, could not be isolated.

The availability of authentic samples of II, IIIa, and IIIc, as well as IVa and IVc (Le Febvre and Gaudry, 1965), greatly simplified the task of identifying the other three materials

TABLE I: Comparison of Metabolite Distribution During *in Vitro* Studies of Microsomal and Supernatant Rat Liver Fractions.

Enzymes	Incubation Conditions	% Distribution of Total Metabolites[a] I	II	IIIa + IIIb	IVa + IVb
9000*g*	Aerobic	9	38	53	0
	Anaerobic	0	82	4	14
105,000*g*	Aerobic	4.5	71	20	4.3
	Anaerobic	0	77	13.5	9.5

[a] Total recoveries varied from 65 to 75%.

TABLE II: Distribution of Metabolites after *in Vitro* Anaerobic Incubation of Various Chlorosteroids with Rat Liver Supernatant Fraction.

Substrate	% Distribution of Total Metabolites[a] I	II	III[b]	IV[b]
I	0	77	13.5	9.5
II	0	80	0	20
IIIa	0	83	8	9

[a] Total recovery varied from 65 to 83%. [b] No resolution of epimers was attempted.

recovered from incubation. Table I compares the distribution of the metabolites under both aerobic and anaerobic conditions in experiments where either 9000*g* or 105,000*g* rat liver fractions were employed.

During gas–liquid chromatography of the 3-hydroxy metabolite fractions, which appeared homogeneous on thin-layer chromatography, a partial resolution into two components was seen. It was thought that this might be due to the presence of 3α- as well as 3β-hydroxy isomers in the sample. This was confirmed *via* two different approaches. Gas–liquid chromatography of the underivatized metabolite fractions showed that one component had a relative retention time equal to the authentic 3β-OH isomer (IIIa or IVa). On acetylation, resolution was greatly improved, one component again exhibiting a relative retention time equal to that of the authentic 3β-acetate (IIIc or IVc). Similar results were obtained on gas–liquid chromatographic analysis of the corresponding trimethylsilyl ethers.

When the presumed 3-hydroxy metabolite mixture was oxidized by manganese dioxide, the sole product was the corresponding 3-keto compound (I or II), as identified by its ultraviolet spectrum and comparative gas–liquid chromatography.

It can also be seen in Table I that the 9000*g* supernatant contains both substantial 3-keto reducing activity and dechlorinating activity. In this fraction, aerobic conditions unexpectedly produced a dominance of 3-keto reduction. Anaerobically, however, dechlorination predominated in this microsomal fraction. When the 105,000*g* liver fraction was employed, dechlorination of I to II was the preferred pathway under both incubation conditions.

Further studies of the conversion of I into II were accomplished with the 105,000*g* supernatant under anaerobic conditions. When it was found that nicotinamide and riboflavin had no effect on the yield of II, these compounds were omitted from the incubation medium. When endogenous NADPH was not supplemented, the 3-hydroxy metabolites IIIa and IVa, previously found in small amounts (Table I) were no longer detected.

Although the absence of supplemented NADPH also greatly reduced the total recovery of steroid from the incubation, the proportion of dechlorinating activity in the system was undiminished. The significance of this finding is discussed elsewhere in this paper.

The competition between 3-keto reduction and 4-dechlorination was further studied by anaerobic incubation of I, II, or III with the 105,000*g* liver system. The metabolite distributions are shown in Table II.

It initially seemed that I could be converted into IV *via* II or III, or both. However, the high yield of II from IIIa, plus the detection of a small amount of I from IIIa at "zero time," reinforces the impression that oxidation to I, followed by dechlorination to II and subsequent reduction to IV is the favored pathway. It is not possible to rule out the direct conversion of III into IV by these experiments.

The *in vitro* metabolism of several 6-substituted 4-chloro steroids was investigated in an attempt to assess the relative effects of 6-substitution on 4-dechlorination. The results (Table III), together with those for I (Table I), suggest that a halogen or other electronegative substituent at C-6 is required for substrate dechlorination to compete successfully with 3-keto reduction.

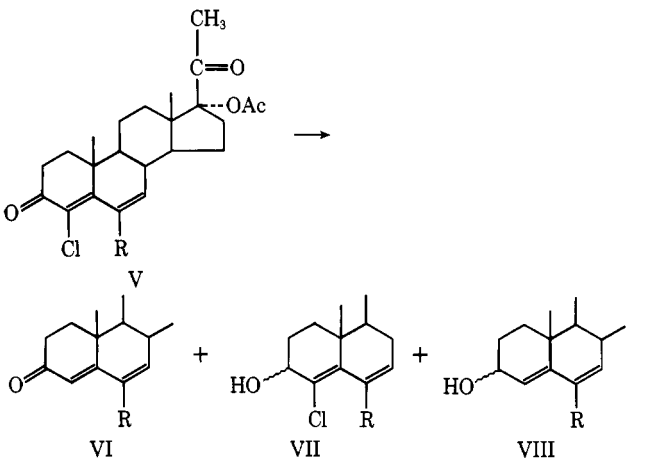


Table IV outlines the results of experiments where several enzyme inhibitors were employed.

The inhibition seen in the presence of NEM and iodoacetamide suggest that a sulfhydryl group is involved in the dechlorination reaction. Neither reagent inhibits 3-keto reduction, as is evident from the increased formation of IIIa and IIIb. A subsequent experiment using dehydroascorbic acid showed no inhibition of dechlorination by this oxidizing agent. However, when the enzyme had been partially denatured by heat or low pH, dehydroascorbic acid became a most effective inhibitor of the dechlorination reaction (Table V).

It was of interest to study the physicochemical aspects of the dechlorination factor present in the rat liver supernatant

TABLE III: Metabolite Distribution During *in Vitro* Anaerobic Incubation of Some 6-Substituted 4-Chloro Steroids with Rat Liver Supernatant Fraction.

Substituent	% Recovery	% Distribution of Total Metabolites V	VI	VII	VIII
R = H[a]	65	20	0	80	0
R = CH_3[b]	47	0	0	100	0
R = F[b]	40	0	40	42	18

[a] Brückner *et al.* (1961). [b] LeMahieu *et al.* (1971).

fraction. Repetitive thin-film dialysis of the 105,000*g* enzyme showed that no appreciable dechlorinating activity traversed the membrane, and conversely, no measurable loss of activity in the retentate was seen. An experiment in which the native enzyme was placed in a boiling-water bath for 15 min unexpectedly resulted in only partial loss of dechlorinating activity.

Theoretical consideration of the mechanism of dechlorination of I at this point centered on indirect displacement of the C-4 chlorine *via* hydride transfer (NADH or NADPH) to C-7. When incubated with I in phosphate buffer under the usual conditions, however, the nicotinamide-adenine dinucleotides were found devoid of dechlorinating activity *per se*, in the absence of liver supernatant fraction.

The ubiquitous tripeptide, glutathione, was then considered as a possible dechlorinating agent. Several attempts were made to labilize the liver dechlorinating factor to dialysis by variation of pH, but under conditions where free GSH was readily dialyzable, only minimal amounts of dechlorinating activity passed through the membrane.

The only procedure to successfully precipitate a major portion of the dechlorinating factor was denaturation with four volumes of acetone. As a result of this, and its dialysis behavior, the factor was tentatively considered a protein, though the heat stability was unusually great for an ordinary enzyme.

Assay of GSH in the freshly prepared rat liver 105,000*g* supernatant showed that approximately 4–6 μmoles of the reduced tripeptide were present in those experiments which resulted (Table I) in complete *in vitro* metabolism of I and good recovery of the 6-monochloro steroid II.

It could be shown, however, that in pH 7.4 phosphate buffer, with *no* enzyme present, that a tenfold molar excess of GSH could only effect about 60% dechlorination. Moreover, it appeared that a water-soluble intermediate formed, because recovery in the organic extract was significantly lower than when enzyme was involved and additional amounts of steroid could be recovered from the aqueous phase if it were treated with additional GSH.

Table VI shows that augmentation of the GSH–steroid reaction mixture with enzymic generating systems for GSH as well as NADPH could almost match the yield of II seen with native enzyme, albeit GSH was still present in great excess over the measured biological concentrations.

In another attempt to define the nature of the biological dechlorinating factor, the rat liver glutathione *S*-alkenyl transferases (Boyland and Chasseaud, 1968) were studied, using I as substrate. Table VII describes a typical experiment wherein incubation was carried out at pH 6.5 to minimize

TABLE IV: Effects of Enzyme Inhibitors on *in Vitro* Dechlorination.

Inhibitor (M)	% Distribution of Total Products I	II	IIIa + IIIb	IVa + IVb
None	0	88	4	8
NEM, 10^{-3}	8	17	69	6
NEM, 10^{-4}	0	85	4	11
Iodoacetamide, 10^{-3}	0	0	100	0
$HgCl_2$, 10^{-3}	100	0	0	0
KCN, 10^{-3}	0	85	6	9
1,2-Naphthoquinone, 10^{-3}	0	100		
SKF-525A, 10^{-3}	0	80	12	8

TABLE V: Effect of Dehydroascorbic Acid on *in Vitro* Dechlorination.

Incubation Conditions	% Distribution of Total Products[a] I	II	IIIa + IIIb	IVa + IVb
Control	0	85	3	12
+ 100 μmoles of dehydroascorbic acid	0	92	3	5
pH 1 (30 min), pH 7.4	10	90	0	0
pH 1 (30 min), pH 7.4, + 100 μmoles of dehydroascorbic acid	90	10	0	0

[a] Total recovery varied from 60 to 75%.

TABLE VI: Enzymic Enhancement of GSH-Mediated Dechlorination.

Incubation Conditions[a]	% Recovery	% of Total Metabolites I	II
GSH (10 μmoles)	59	37	63
GSH (10 μmoles), NADPH (4 μmoles), GSSG reductase	76	15	85
GSSG reductase	91	100	0
GSH (10 μmoles), NADPH (4 μmoles), GSSG reductase, Glc-6-P, Glc-6-P-dehydrogenase	81	12	88

[a] Per micromole of I.

TABLE VII: Enzymic *vs.* Chemical Dechlorination of I.

Incubation[a]	% Recovery	% Distribution of Products	
		I	II
Purified GSH transferase, GSH (10 μmoles), pH 6.5	30	19	81
GSH only (10 μmoles), pH 6.5	74	93	7

[a] Per micromole of I.

the chemical reaction of GSH and I. This artificial system was capable of dechlorinating I but at greatly reduced efficiency and in the face of very low recovery of steroid.

Discussion

In addition to uncovering a unique reaction, 4-dechlorination, these studies also demonstrate the existence of interesting competing pathways of steroid metabolism. The apparent equilibrium of the C-3 oxidoreduction in the dichloro steroids is displaced toward II, even under reducing conditions. This may be due to the inherent nature of the reductase(s) involved, or, more likely, is a result of a mass-action effect caused by the very facile dechlorination of I. Maximum steady-state levels of II are reached very quickly when I is incubated with the rat liver preparation.

For these reasons, it may be proposed that, on anaerobic incubation, III is first oxidized to I, which is then converted into II, rather than the reaction sequence III→IV→II. The observation that II yields only small amounts of IV, while III is almost wholly converted into II, favors this hypothesis, The small amounts of I detected at zero time during incubation of III offers confirmatory evidence. Thus, the small amount of IV obtained by incubation of III probably arises *via* III→I→II→IV, not by III→IV.

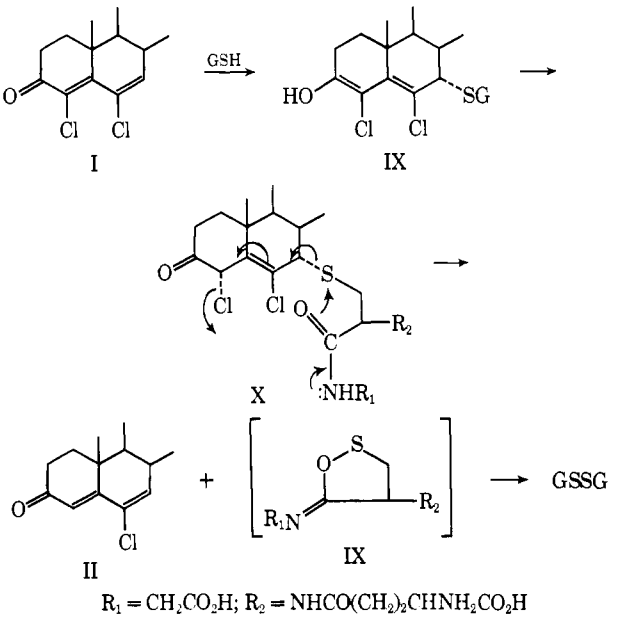
While inhibition studies with *N*-ethylmaleimide and iodoacetamide suggest the participation of a sulfhydryl group in dechlorination, and dialysis studies suggest that a protein is involved, the precise nature of this reaction is still undefined. The fact that GSH is capable alone of effecting the 4-dechlorination of I at physiological pH may be an important clue to the identity of the factor. However, the amount of GSH required for the chemical dechlorination far exceeds the quantity actually assayed in typical tissue preparations used for *in vitro* studies.

On the other hand, it was shown that supplementation of the GSH–steroid system with glutathione reductase and an NADPH-generating system allows formation of 6-monochloro steroid to approach that seen with liver enzyme, though this artificial system required supranormal proportions of the sulfhydryl compound to achieve good steroid recovery. The effectiveness of this system also suggests that the by-product of GSH-mediated 4-dechlorination is oxidized glutathione.

Although glutathione, due to its relatively high concentration in liver, is a logical candidate as a cofactor, it is not the only natural sulfhydryl compound capable of dechlorinating I under our *in vitro* conditions. LeMahieu *et al.* (1971) report that cysteine, coenzyme A, and *dl*-α-dihydrolipoic acid will also participate in this reaction.

It was stated earlier that omission of NADPH or a generating system for this coenzyme from incubation with the rat liver system caused not only disappearance of 3-hydroxy metabolites, but also decreased recovery of II and/or substrate, I. A similar NADPH-dependent reduction of C-2, C-4, or C-6 halogenated Δ^4-3-keto steroids to Δ^4-3-ols was reported by Ringold *et al.* (1964) who attributed it to the saturated 3-keto reductases of rat liver.

A mechanism proposed by LeMahieu *et al.* (1971) for the GSH-mediated dechlorination of I seems a sound starting point for consideration of the mechanism of the biological dechlorination.



The initial step involves conjugate addition of GSH to C-7 of I. Protonation of the resultant enol IX at C-4 followed by intramolecular decomposition of the intermediate X yields II, hydrogen chloride, and XI. Hydrolysis of XI and disproportionation of the resulting sulfenic acid (Lüttringhaus and Schneider, 1964) yields oxidized glutathione (GSSG).

In the *in vitro* incubations with rat liver supernatant, the 4-dechlorination reaction competes with reduction of the 3-ketone. Varying the C-6 substituent (Table III) results in greatly increased proportions of 3-hydroxy metabolites. The electron-withdrawing 6-chloro substituent of I would be expected to facilitate nucleophilic attack of GSH at C-7. Electron density at C-7 in V (R = H or CH_3) will be greater than in I and consequently attack by GSH will be retarded. In these cases, reduction of the 3-ketone is observed with no competing 4-dechlorination. Although the inductive effect of fluorine is stronger than that of chlorine, the fluorine substituent on an aromatic ring is electron releasing in overall character (De La Mare and Ridd, 1959). Thus, the electron density at C-7 of V (R = F) will be greater than in I, although probably less than when the C-6 substituent is hydrogen or methyl. In this case 4-dechlorination is slightly favored over reduction of the 3-ketone (Table III). It should also be noted that the activating influence of the γ halogen may be to facilitate coordination or hydrogen bonding, rather than to alter the electrophilicity of C-7.

It can be concluded from the present study that the *in vitro* dechlorination reaction (a) involves some biological sulfhydryl compound (possibly glutathione), (b) is a complex reaction,

which may involve an enzyme (or enzymes) or which may be merely mediated by some protein-borne thiol group quite apart from any enzymically active site, and (c) occurs by some rate-limiting reaction wherein a water-soluble steroid intermediate is formed.

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