Mechanistic Studies with Solubilized Rat Liver Steroid 5α-Reductase: Elucidation of the Kinetic Mechanism

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ABSTRACT: A solubilized preparation of steroid 5α-reductase (EC 1.3.1.30) from rat liver has been used in studies focused toward an understanding of the kinetic mechanism associated with enzyme catalysis. From the results of analyses with product and dead-end inhibitors, a preferentially ordered binding of substrates and release of products from the surface of the enzyme is proposed. The observations from these experiments were identical with those using the steroid 5α-reductase activity associated with rat liver microsomes. The primary isotope effects on steady-state kinetic parameters when [4*S*-^{2}H]NADPH was used also were consistent with an ordered kinetic mechanism. Normal isotope effects were observed for all three kinetic parameters (V_m/K_m for both testosterone and NADPH and V_m) at all substrate concentrations used experimentally. Upon extrapolation to infinite concentration of testosterone, the isotope effect on V_m/K_m for NADPH approached unity, indicating that the nicotinamide dinucleotide phosphate is the first substrate binding to and the second product released from the enzyme. The isotope effects on V_m/K_m for testosterone at infinite concentration of cofactor and on V_m were 3.8 ± 0.5 and 3.3 ± 0.4, respectively. Data from the pH profiles of these three steady-state parameters and the inhibition constants ($1/K_i$) of competitive inhibitors versus both substrates indicate that the binding of nicotinamide dinucleotide phosphate involves coordination of its anionic 2′-phosphate to a protonated enzyme-associated base with an apparent pK near 8.0. From these results, relative limits have been placed on several of the internal rate constants used to describe the ordered mechanism of the rat liver steroid 5α-reductase.

In recent years, considerable efforts have been focused on understanding the role of androgens as potential causal agents for tissue-specific disorders (Mooradian et al., 1987; Imperato-McGinley et al., 1986; Toscano, 1986). From numerous studies the concept has evolved that 5α-dihydrotestosterone (DHT)[1], rather than its metabolic precursor testosterone (T), acts as the principal androgenic mediator within certain organs including male accessory glands and skin (Imperato-McGinley et al., 1986; Vermorken et al., 1980). Consequently, inhibition of the enzyme that catalyzes the conversion of T to DHT, Δ^4-3-oxo-steroid 5α-reductase [EC 1.3.1.30; steroid 5α-reductase (SR)] has been proposed as a means of attenuating or preventing pharmacological disorders associated with these tissues. At least one of these compounds is currently undergoing investigation in man as a palliative treatment for benign prostatic hypertrophy (Vermeulen et al., 1989). Our interests in modulating pathways of steroid biosynthesis have led us to the identification of a novel class of inhibitors, 17β-*N*,*N*-dialkylcarbamoyl-3-androstene-3-carboxylic acids (steroidal acrylates), of human prostatic SR (Metcalf et al., 1989). A mode of interaction between enzyme and steroidal arcylate involving the formation of an enzyme–NADP$^+$–inhibitor dead-end complex has been proposed from initial studies with microsomal-associated SR from both human (Metcalf et al., 1989) and rat prostate (Levy et al., 1989).

Despite this increasing interest in SR as a therapeutic target, the enzyme remains poorly characterized. A number of factors have contributed to the lack of success in enzyme purification, including the relatively low levels of activity in target organs, the inefficiency of solubilization procedures, and activity instability following solubilization. Hence, the current understanding of enzyme catalysis has orginated primarily from studies with microsomal-associated SR activities.

Concerns with mechanistic implications drawn from particulate-associated enzyme activity have prompted an investigation toward identifying a preparation of SR that could be better characterized kinetically, particularly with respect to a more complete appreciation of the kinetic mechanism and pH dependence of enzyme catalysis. Since its intrinsic level of SR is several hundred fold greater than that in androgenic tissues (Liang et al., 1983), we have chosen to study SR solubilized from rat liver microsomes as a model system. Concurrently, the interactions between SR and the steroidal acrylates leading to enzyme inhibition have been investigated with this same enzyme source (Levy et al., 1990).

In this paper, we describe our studies designed to elucidate mechanistic characteristics of the solubilized rat liver steroid 5α-reductase. On the basis of the data from inhibition experiments and the concentration dependence of the primary isotope effect upon hydride transfer, an ordered kinetic mechanism is proposed. Results from studies of the pH dependence upon steady-state kinetic parameters are fit within this mechanistic scheme.

Experimental Procedures

Materials

[4-^{14}C]Testosterone (55–57 mCi/mmol), [1,2-^{3}H]DHT (51.6 Ci/mmol), and [4-^{14}C]DHT (57 mCi/mmol) were purchased from Amersham Corp. or New England Nuclear (NEN). Econosolve II and Aquasol 2 were obtained from NEN. [1-^{2}H]Glucose (98%) and [^{2}H]H_2O (98%) were

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[1] Abbreviations: T, testosterone; DHT, 5α-dihydrotestosterone; SR, Δ^4-3-oxo-steroid 5α-reductase (EC 1.3.1.30); thio-NADP$^+$, thionicotinamide adenine dinucleotide phosphate; MK-906, 17β-[*N*-(2-methyl-2-propyl)carbamoyl]-4-aza-5α-androst-1-en-3-one; NADPD, (4*S*)-[4-^{2}H]NADPH; DTT, dithiothreitol; 3-AADP, 3-aminopyridine adenine dinucleotide phosphate.

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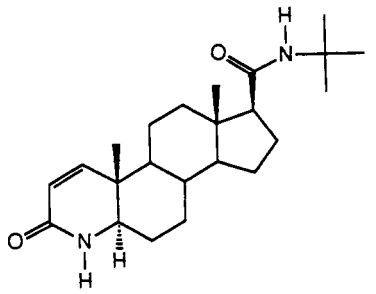
purchased from MSD Isotopes. 17β-[*N*-(2-Methyl-2-propyl)carbamoyl]-4-aza-5α-androst-1-en-3-one (MK-906)



MK-906

was synthesized by a previously reported procedure (Rasmusson et al., 1986). Glycerol was obtained from LKB (Pharmacia). Emulgen 913 was the gift of Kao-Atras Co. (Tokyo). All Sepharose- and Sephadex-based chromatographic supports were purchased from Pharmacia. DEAE-cellulose was obtained from Whatman. Other chemicals and enzymes were purchased from Sigma Chemical Co. or Aldrich Chemicals. Protein concentrations were estimated by the method of Bradford (1976) using the Bio-Rad protein dye assay with bovine serum albumin as the protein standard. Radioactivity was determined with either a Beckman LS-5801 scintillation counter calibrated to disintegrations per minute (dpm) with Beckman standards or with a System 2000 Bioscan imaging scanner (Bioscan, Washington, DC). Single-wavelength UV/vis spectral changes were monitored on a Gilford 260 spectrophotometer. Multiple-wavelength spectra were recorded with a Hewlett-Packard 8450A spectrophotometer with a diode array detector. Analyses of enzyme reactions were performed on prechanneled silica TLC plates containing a preabsorbing region (Si250F-PA, Baker).

Methods

Preparation of Microsomes from Rat Liver. Male (Sprague-Dawley) rats were fasted overnight to decrease glycogen levels prior to removal of the livers. The livers were rinsed in buffer A [20 mM sodium phosphate, pH 6.5, 0.3 M sucrose, and 1 mM dithiothreitol (DTT)] and frozen at −80 °C until used for the preparation of microsomes. Except as noted, all procedures involving enzyme preparation and manipulations were conducted at 0–4 °C.

Minced rat livers (30–60 g) were rinsed and homogenized in a minimal volume of buffer A with either a Brinkmann Polytron or a Waring blender. The solution was centrifuged at 10000*g* for 30 min (RC-5 Sorvall centrifuge); the pellet was discarded. The supernatant was filtered through cheesecloth and centrifuged at 100000*g* (Beckman L8-M ultracentrifuge) for 60 min. The microsomal pellet was washed by resuspension in 20 volumes of buffer A with a Dounce glass hand homogenizer and the suspension centrifuged at 100000*g*. The pellet was suspended in buffer A to a concentration of 10–20 mg of protein/mL; the microsomal solutions were stored at −80 °C until used for enzyme activity assays or preparation of solubilized SR.

Solubilization of SR from Rat Liver Microsomes. Rat liver microsomes were suspended in extraction buffer at a final concentration of 0.5–1.0 mg of protein/mL. The standard extraction buffer consisted of 100 mM sodium citrate, 100 mM potassium chloride, 1.0 mM DDT, 0.1% Lubrol PX, 20% glycerol, and 0.1 mM NADPH adjusted to pH 7.5 (buffer B). Following vigorous mixing, the solution was incubated on ice for 30 min and then centrifuged at 100000*g* for 60 min. Aliquots (0.5–1.0 mL) of the supernatant, which contained greater than 80% of the microsomal enzyme activity, could be stored at −80 °C with no loss of enzyme activity for several months. The enzyme also could be stored for several days in the extraction buffer at 4 °C with minimal loss of enzyme activity.

SR Activity Assay. SR activity was determined by following the conversion of testosterone (T) to 5α-reduced steroids, taken as the sum of 5α-dihydrotestosterone (DHT) and androstanediol (ADIOL). [^{14}C]Testosterone in ethanol was deposited in test tubes and concentrated to dryness in a Savant Speed-Vac evaporator. Buffer and NADPH were added, and each tube was equilibrated to assay temperature. Except as noted for individual experiments, a cofactor regenerating system ($NADP^+ \rightarrow NADPH$) consisting of 1 mM glucose 6-phosphate and 0.5 unit/mL glucose-6-phosphate dehydrogenase was included in each assay. The reaction was initiated by addition of an aliquot of enzyme preparation to a volume of 0.5 mL in 20 mM sodium phosphate, pH 6.5 or 7.5 as indicated in individual experiments. Concentrated enzyme solutions were serially diluted into buffer A so that the consumption of substrate was less than 20% in all assays. In general, assays with solubilized liver SR were conducted at 30 °C, while those with microsomal associated enzyme were incubated at 37 °C. Following incubation for a maximum of 40 min, the reaction was stopped by addition of 4 mL of ethyl acetate containing 0.2 μmol of T, DHT, ADIOL, and androstenedione each as carriers and markers. After separation from the aqueous layer, the organic solvent was removed by evaporation in the Savant evaporator and the residue applied to silica gel TLC plates in a total volume of 40 μL of chloroform/methanol (1:1). The plates were developed twice with chloroform/acetone (9:1), and the relative content of radiolabel in the substrate (T) and the products (DHT plus ADIOL) was determined for each lane by using a Bioscan imaging scanner. The final amount of Lubrol in the assay tubes did not exceed 0.002%, a concentration that did not affect enzyme activity. With 1.0 μM T and 200 μM NADPH at 37 °C and pH 6.5 typical specific activities of the microsomal associated and the solubilized SRs were 5.0 and 6.2 $nmol \cdot (min \cdot mg)^{-1}$, respectively; for the solubilized enzyme at 30 °C, the specific activity was 3.6 $nmol \cdot (min \cdot mg)^{-1}$.

Inhibition Studies. Potential steroidal inhibitors in ethanol were added to the assay tubes with substrate (T), and the contents were evaporated to dryness. Nicotinamide analogues were added along with the incubation buffer. All other procedures were the same as outlined for the SR activity assay. Inhibition constants ($K_{i,app}$) were estimated by Dixon analysis (Dixon, 1953) with initial substrate concentrations of 1.0 μM T and 25 or 200 μM NADPH. With the steroidal inhibitors, the cofactor regenerating system was included to eliminate inhibition by $NADP^+$; no regenerating system was added in the product or dead-end inhibition studies with the nicotinamide dinucleotides. More complete analyses were performed as described below.

Deuterium Isotope Effects upon Hydride Transfer. The NADPH and NADPD [(4*S*)-[4-^{2}H]NADPH] used for determination of isotope effects were prepared enzymatically by using [1-^{1}H]- or [1-^{2}H]-α-D-glucose, $NADP^+$, hexokinase, Mg-ATP, and glucose-6-phosphate dehydrogenase according to the procedure of Velera et al. (1987). The reduced cofactors were purified by using a variation of the previously described ion-exchange (DEAE-cellulose) procedure (Pastore & Friedkin, 1961) in which ammonium bicarbonate replaced NaCl in the column elutions (Ryerson et al., 1982). Product fractions were combined, frozen, and lyophilized in the dark to

remove solvent and NH_4HCO_3. The samples of isolated product were greater than 90% reduced as determined by the final 340 nm/260 nm absorbance ratios (Windholz et al., 1983). Samples of reduced cofactors were stored desiccated at −20 °C in the dark.

Experiments to measure the isotope effects upon the SR-catalyzed reduction of T were conducted with the enzymatically prepared cofactors. For these experiments, the solubilized SR was prepared with buffer B as described above except that $NADP^+$ replaced the NADPH to prevent any eventual dilution of NADPD. All assays contained a cofactor regenerating system of 450 μM Mg-ATP, hexokinase (0.15 unit), glucose-6-phosphate dehydrogenase (0.20 unit), and 550 μM [1-^{1}H]- or [1-^{2}H]glucose to eliminate potential product inhibition by $NADP^+$. Cofactor concentrations were corrected for the carry-over from the extraction buffer. Assays were incubated at 37 °C in 0.1 M sodium phosphate buffer, pH 7.0; concentrations of T and NADP(H)(D) varied from 0.3 to 3.0 μM and from 1 to 50 μM, respectively. Concentrated solubilized enzyme solutions were diluted with the incubation buffer immediately before each experiment and kept on ice prior to initiation of reaction; no loss of activity was observed for the diluted enzyme over the course of the experiments. Sets of data were obtained at varying concentrations of one substrate and fit to the Michaelis–Menten equation (eq 1). The inverses of the apparent kinetic parameters (V_m and V_m/K_m) were then evaluated as a linear function of the reciprocal of the second substrate concentration. Enzymatically prepared NADPH was shown to be kinetically equivalent to that obtained commercially.

Dependence of Kinetic Parameters on pH. Enzyme activities were determined in constant ionic strength (μ = 0.02 M) buffer solutions consisting of succinic acid, imidazole, and diethanolamine (Ellis & Morrison, 1982) as previously described (Brandt & Levy, 1989). The effects of pH upon initial velocity kinetic parameters were determined with T as the variable substrate (0.4–2.0 μM) at a constant concentration of NADPH (25 or 200 μM) and with variable NADPH concentrations (4–50 μM) at nonvarying levels of T (1.0 μM). All activities were normalized to the amount of protein used in the assays. Initial velocity data were fitted to eq 1; the pH dependence upon V_m and V_m/K_m was analyzed by the BELL program (Cleland, 1979). Experiments in deuterated solutions ([^{2}H]H_2O) utilized the same protocols as described above; solution acidities determined by a glass electrode were corrected by the addition of 0.4 pK unit (Jencks, 1969).

Effects of pH upon the potency of competitive inhibitors (MK-906 versus T and $NADP^+$ versus NADPH) were conducted as described above except that the concentrations of both T and NADPH were held constant. Apparent inhibition constants were determined (Dixon, 1953) at each pH and evaluated by the BELL program.

Analysis of Data. Data from initial velocity, product inhibition, dead-end inhibition, and pH studies were fit to appropriate rate equations with the programs described by Cleland (1977, 1979). In the figures, displayed points are experimental values and the curves are computer-calculated best fits. Linear data evaluations were fit to the equation $y = mx + b$ by using the LINE program. Kinetic studies involving the variation of one substrate were fit to a hyperbolic function, eq 1, with the HYPER program. Initial velocity data obtained upon varying both substrates were evaluated by eq 2 or 3 using the SEQUEN or EQORD programs, respectively. Inhibition experiments were evaluated by the linear competitive (COMP), noncompetitive (NONCOMP), and uncompetitive (UNCOMP) models (eqs 4–6, respectively). Data for pH profiles

$$v = V_mA/(K_a + A) \quad (1)$$

$$v = V_mAB/[(K_{ia}K_b + K_aB + K_bA + AB)] \quad (2)$$

$$v = V_mAB/[(K_{ia}K_b + K_bA + AB)] \quad (3)$$

$$v = V_mA/[K_a(1 + I/K_{is}) + A] \quad (4)$$

$$v = V_mA/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (5)$$

$$v = V_mA/[K_a + A(1 + I/K_{ii})] \quad (6)$$

of parameters (y) that decreased with a slope of 1 or −1 at both low (K_1) and high (K_2) pH were fitted by eq 7 (BELL).

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (7)$$

The statistical criteria suggested by Cleland (1979) were employed in determining the best fit of model to the experimental data.

RESULTS

Characteristics of Microsomal and Solubilized Rat Liver SR. The SR activity associated with rat liver microsomes was efficiently extracted by the combined components of buffer B. Omission of any of these constituents decreased the yields of SR activity relative to the yields obtained with the complete buffer B. For example, recovery of enzyme activity decreased in the absence of citrate (~86% of the activity obtained with buffer B), Lubrol PX (~1%), potassium chloride (70–85%), and NADPH (9–15%). The protein concentrations of solutions prepared with 0.05–0.1% Lubrol PX or 0.1–0.5% Emulgen 913 were comparable; however, the specific activity for SR extracted with Lubrol was higher by approximately 30%. Emulgen 913 subsequently was shown to inhibit SR activity. When the NADPH was replaced by $NADP^+$, the solubilized SR had a specific activity that was decreased by approximately 70%. The latter preparation could be stored for up to 4 months at −80 °C, after which time appreciable loss of enzyme activity was observed.

Once solubilized, enzyme activity was reasonably stable even when diluted 100 times with 20 mM phosphate, pH 6.6, containing 1 μM NADPH; greater than 80% enzyme activity remained after storage of such diluted enzyme solutions at 4° C for 3 days. The presence of cofactor has been shown to be of primary importance to maintenance of enzyme activity. When assayed at pH 6–7.5, enzyme activity was shown to be linear with both protein concentration and time for greater than 60 min. Consequently, all enzyme assays have been assumed to approximate initial velocity conditions.

Attempts at further fractionation of the protein components in these solubilized preparations met with minimal success. A number of alternative separation techniques were attempted, including chromatographies with ion-exchange (DEAE-cellulose, DEAE-Sephadex, QAE-Sephadex), hydrophobic (phenyl-Sepharose, hexyl-Sepharose, butyl-Sepharose, aminohexyl-Sepharose), and ligand-dye affinity (Red-Sepharose, Blue Sepharose, $NADP^+$ agarose) supports. For most of these alternatives, minimal enzyme activity was recovered following chromatography. Upon gel filtration (Sephadex G-25 and G-50), the recovered SR showed no increase in specific activity.

Initial Velocity Evaluation of SR Activity. Initial velocity data obtained upon variation of NADPH and T concentrations with SR solubilized by using buffer B were best fit to a sequential model (eq 2). From this evaluation, maximum velocity [V_m = 6.8 ± 0.9 nmol·(min·mg)$^{-1}$] and apparent kinetic constants for NADPH[2] (K_a = 2.5 ± 0.5 μM, K_{ia} = 1.1 ± 0.3

Table I. Summary of the Inhibition Patterns for Rat Liver Steroid 5α-Reductase

inhibitor[a]	variable substrate	enzyme and conditions[b]	kinetic pattern[c]	inhibition constants[c] (μM)
$NADP^+$	NADPH	M, pH 6.6	intersecting (C)	$K_{is} = 35 \pm 3$
$NADP^+$	NADPH	S, pH 7.5	intersecting (C)	$K_{is} = 5 \pm 1$
$NADP^+$	testosterone	M, pH 6.6	intersecting (NC)	$K_{is} = 13 \pm 1$; $K_{ii} = 40 \pm 5$
$NADP^+$	testosterone	S, pH 7.5	intersecting (NC)	$K_{is} = 34 \pm 2$; $K_{ii} = 32 \pm 3$
thio-$NADP^+$	NADPH	M, pH 6.6	intersecting (C)	$K_{is} = 0.21 \pm 0.03$
thio-$NADP^+$	NADPH	S, pH 7.5	intersecting (C)	$K_{is} = 0.7 \pm 0.1$
thio-$NADP^+$	testosterone	M, pH 6.6	intersecting (NC)	$K_{is} = 15 \pm 3$; $K_{ii} = 31 \pm 6$
thio-$NADP^+$	testosterone	S, pH 7.5	intersecting (NC)	$K_{is} = 6 \pm 1$; $K_{ii} = 12 \pm 1$
3-AADP	NADPH	S, pH 7.5	intersecting (C)	$K_{is} = 4.1 \pm 0.4$
MK-906	NADPH	M, pH 6.6	parallel (UC)	$K_{ii} = 0.013 \pm 0.002$
MK-906	NADPH	S, pH 7.5	parallel (UC)	$K_{ii} = 0.010 \pm 0.002$
MK-906	testosterone	M, pH 6.6	intersecting (C)	$K_{is} = 0.006 \pm 0.002$
MK-906	testosterone	S, pH 7.5	intersecting (C)	$K_{is} = 0.005 \pm 0.001$

[a] A cofactor regenerating system, as described under Methods, was included in the studies with MK-906. The concentrations of nonvariable substrate were 1 μM T and 25 μM NADPH. [b] Enzyme source is microsomal (M) or solubilized (S) rat liver SR. Further details of experimental conditions are outlined in the text. [c] The kinetic patterns were analyzed by using the FORTRAN programs by Cleland (1977); the best fits of data to linear competitive (C), noncompetitive (NC), or uncompetitive (UC) models are indicated. Inhibition constants are derived from the computer analyses of experimental data.

μM) and T ($K_b = 1.3 \pm 0.3$ μM) were estimated. With the microsomes as a source of SR activity, the initial velocity data fit equally well to both the sequential and equilibrium ordered models. For reference, typical results of a fit to eq 2 for the sequential model found $V_m = 5.2 \pm 0.4$ nmol·(min·mg)$^{-1}$, $K_a = 4.0 \pm 0.9$ μM, $K_{ia} = 4.8 \pm 1.2$ μM, and $K_b = 1.9 \pm 0.3$ μM and to eq 3 for the equilibrium ordered mechanism gave $V_m = 4.2 \pm 0.3$ nmol·(min·mg)$^{-1}$, $K_{ia} = 15.8 \pm 3.0$ μM, and $K_b = 1.2 \pm 0.2$ μM.

Upon variation of NADPD and T, initial velocity data with solubilized rat liver SR best conformed to the equilibrium ordered model [$V_m = 1.3 \pm 0.1$ nmol·(min·mg)$^{-1}$, $K_{ia} = 6.0 \pm 1.5$ μM, and $K_b = 1.4 \pm 0.3$ μM]. The initial velocity pattern with enzymatically prepared NADPH was sequential; the kinetic constants were comparable to those with commercially obtained NADPH above: $V_m = 6.3 \pm 0.5$ nmol·(min·mg)$^{-1}$, $K_a = 3.5 \pm 0.6$ μM, $K_{ia} = 1.9 \pm 0.4$ μM, and $K_b = 2.1 \pm 0.5$ μM.

Attempts to reverse the direction of SR catalysis with [^{3}H]- or [^{14}C]DHT in the presence of $NADP^+$ were unproductive; no radiolabeled T could be detected as product in these experiments over pH range 5.0–8.5.

Dead-End Inhibition Analyses. Several structural analogues and components of NADPH were investigated as potential inhibitors of enzyme activity to be used in mechanistic studies. The product of the SR reaction, $NADP^+$, and thionicotinamide adenine dinucleotide phosphate (thio-$NADP^+$) were shown to be inhibitors of the microsomal associated enzyme ($K_{i,app} = 35 \pm 3$ and 20 ± 5 μM, respectively); both these compounds and 3-aminopyridine adenine dinucleotide phosphate (3-AADP) also inhibited the solubilized enzyme activity (see below). NAD^+ demonstrated no inhibitory ability, while NADH was neither a substrate nor an inhibitor for the rat liver SR. A number of other compounds also were evaluated; at concentrations of 1–500 μM, neither ATP-ribose, 2′:3′-cyclic $NADP^+$, 3′-$NADP^+$, deamino-NADP(H), β-nicotinamide ribose monophosphate, dihydronicotinamide ribose monophosphate, 1,N^6-etheno-$NADP^+$, nor β-$NADP^+$ dialdehyde (periodate oxidized β-nicotinamide adenine dinucleotide phosphate) had any inhibitory effect on SR activity. It should be noted that the nucleotides with a 2′-phosphate ($NADP^+$, thio-$NADP^+$, and 3-AADP) interacted most strongly with SR.

[2] For convenience, NADPH and T are represented as substrates A and B, respectively, throughout the paper. No mechanistic implications should be drawn from this assignment except as implied from symmetry arguments (eq 3) or as indicated within the text.

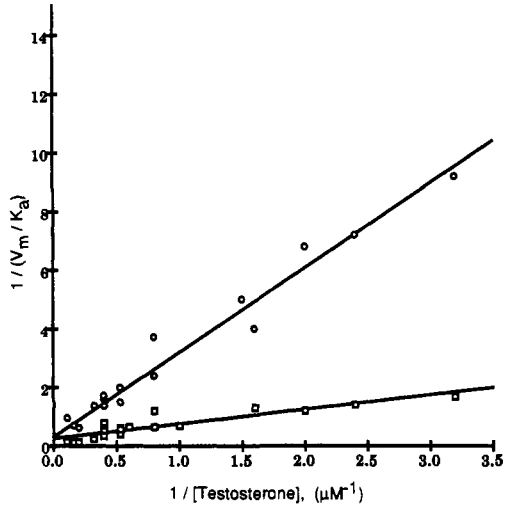


FIGURE 1: Isotope effect on V_m/K_m upon deuterium substitution of the nicotinamide (4*S*)-hydrogen with solubilized SR. Steady-state kinetic parameters were determined with NADPH (□) and NADPD (○) by using 0.4 and 2.0 μg of solubilized protein per assay, respectively, as described in the text. The curves represent values of $(V_m/K_a)^{-1}$ [(min·mg·μM)/nmol] vs the reciprocal of the concentration of T (μM^{-1}). Values of 5.2, 4.9, 4.3, 3.4, and 2.5 for $(V_m/K_a)_H/(V_m/K_a)_D$ were observed at 0.33, 0.5, 1.0, 2.0, and 4.0 μM T, respectively; the curves converge to a common point on the ordinate, demonstrating no isotope effect [$(V_m/K_a)_H/(V_m/K_a)_D \sim 1$] at infinite concentration of T.

The product inhibition patterns of both solubilized and microsomal rat liver SR with $NADP^+$ versus NADPH were best evaluated with eq 4, representing a linear competitive inhibition model (Table I). While limited by its solubility, no inhibition by DHT could be observed, even in the presence of exogenous $NADP^+$. With both thio-$NADP^+$ and 3-AADP, linear competitive inhibition versus NADPH also was observed. The dead-end inhibition patterns of thio-$NADP^+$ versus the second substrate, T, conformed to an intersecting (linear noncompetitive) pattern represented by eq 5. As a steroidal dead-end inhibitor, MK-906 demonstrated an intersecting (linear competitive) inhibition pattern versus T and a parallel (linear uncompetitive) pattern versus NADPH. These inhibition patterns, as summarized in Table I, would be consistent with a sequential kinetic mechanism (Segel, 1975) which is ordered whereby NADPH is the first substrate binding to the surface of enzyme and $NADP^+$ is the second product released following catalysis.

Steady-State Isotope Effects with (4S)-[4-^{2}H]NADPH. The steady-state isotope effects upon transfer of hydride to

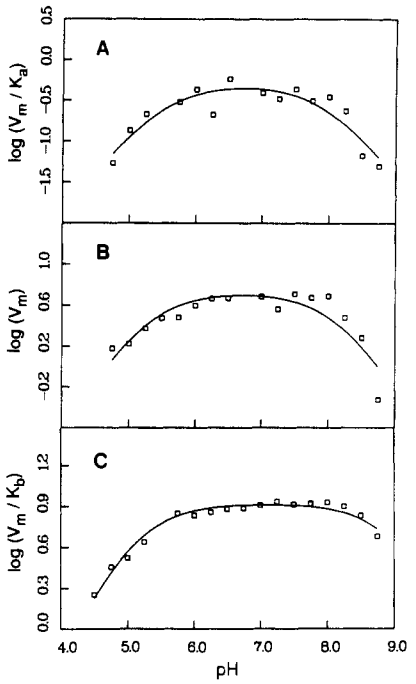


FIGURE 2: Variation of steady-state initial velocity parameters of solubilized SR with pH. Kinetic parameters of V_m/K_m and V_m with either NADPH (6–50 μM) or testosterone (0.4–2.0 μM) as the variable substrate were determined throughout the indicated pH range in a constant ionic strength buffer (μ = 0.02). All activities were normalized to the protein content (2.2 μg) in the assays conducted at pH 7.5. Values obtained for V_m/K_a [nmol·(min·μM)$^{-1}$] (panel A) and V_m [nmol·min^{-1}] (panel B) upon variation of NADPH concentrations were evaluated at 1 μM T. The parameter V_m/K_b [nmol·(min·μM)$^{-1}$] was determined with T as the variable substrate (panel C) at 1.0 mM NADPH. All assays included the NADPH-regenerating system. A curve similar to that in panel C was obtained at lower concentration (0.2 mM) of NADPH. The curves in all three panels were fitted to eq 7 (BELL). Inflection points are summarized under Results.

T were determined with reduced cofactors that were prepared enzymatically. Initial studies with NADPD were conducted over a range of T concentrations; all parameters were compared to reactions with similarly prepared NADPH. As depicted by the difference in slopes of the curves in Figure 1, the isotope effect of hydride transfer on V_m/K_m for NADPH decreases upon increasing concentrations of T.[3] Most importantly, the value of the isotope effect for NADPH disappeared upon extrapolation to infinite steroid concentration (Figure 1); this ratio, $(V_m/K_a)_H/(V_m/K_a)_D$, at infinite concentration of steroid was found to be 1.1 ± 0.2. The isotope effect on V_m/K_m for T also decreased, although less dramatically; with increasing concentrations of this substrate $(V_m/K_b)_H/(V_m/K_b)_D$ declined from 4.3 to 4.2 to 3.9 at 1, 4, and 10 μM, respectively. Upon extrapolation to infinite concentration of NADP(H)(D) a normal isotope effect of 3.8 ± 0.5 was obtained for $(V_m/K_b)_H/(V_m/K_b)_D$. Similarly, the isotope effect on V_m ranged from 4.2 to 3.5 on increasing T from 0.33 to 4.0 μM; upon extrapolation to infinite steroid concentration the effect on maximal velocity, $(V_m)_H/(V_m)_D$, was 3.3 ± 0.4.

pH Dependence of Kinetic Parameters. Studies on the pH dependence of V_m, V_m/K_a, and V_m/K_b were initiated to determine the importance of ionizable groups in binding and catalysis of solubilized rat liver SR. As demonstrated in Figure 2A, two ionizable groups (pK_1 = 5.6 ± 0.3 and pK_2 = 7.9 ± 0.2) were observed in the V_m/K_a profile for NADPH. Similar breaks at pK_1 = 5.3 ± 0.2 and pK_2 = 8.1 ± 0.2 are observed in the pH curve upon V_m (Figure 2B). With T as the variable substrate, the entire profile is less responsive to changes in pH; two ionizable events (pK_1 = 5.0 ± 0.2 and pK_2 = 8.9 ± 0.3), depicted in Figure 2C for V_m/K_b, are found on both V_m and V_m/K_m at saturating levels (0.2 and 1.0 mM) of NADPH. All data sets were evaluated by the BELL program, where each inflection represents a single protonation/deprotonation event. No loss of enzyme activity was observed upon preincubation in the extreme pH ranges followed by dilution and assay for enzyme activity at pH 7.5. Decreased enzyme activity at extreme pH values consequently does not result from irreversible loss of enzyme activity. No significant solvent effect was observed upon the ionization events of these steady-state parameters when determined in deuterated buffers; within the errors of the individual experiments, the results from such profiles were indistinguishable from those summarized above (data not shown).

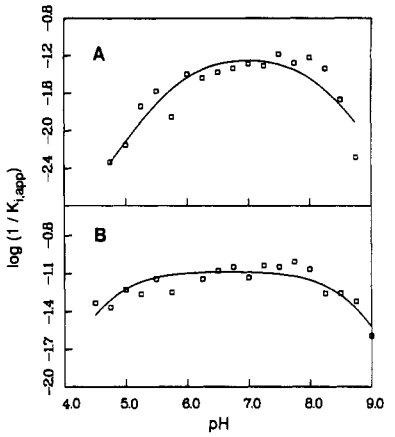


FIGURE 3: pH dependence of the inhibition constants for competitive inhibitors of SR substrates. Inhibition constants ($K_{i,app}$) were estimated by Dixon analysis (1953) at substrate concentrations of 1.0 μM T and 25 μM NADPH. The NADPH-regenerating system was included for assays including MK-906 but omitted in the NADP$^+$ experiments; both inhibitors remained competitive at the pH extremes versus their respective substrate analogues. The concentration ranges employed for NADP$^+$ and MK-906 were 0–100 μM and 0–12 nM, respectively. The curve presented in (A), log ($1/K_{i,app}$) (μM^{-1}) vs pH, with NADP$^+$ as inhibitor, was fit to eq 7 (BELL), demonstrating inflection points at pH 5.9 and 8.1. Data from inhibition by MK-906, shown by the log $1/K_{i,app}$ (nM^{-1}) vs pH plot in (B), also were fit to eq 7 to yield inflection points at pH 4.6 and 8.8.

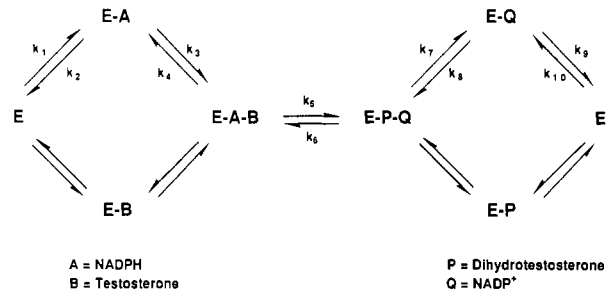
The pH profiles for inhibition of solubilized SR by NADP$^+$ and MK-906 (Figure 3A,B) are similar to those for their respective competitive substrates. The two inflections determined with each inhibitor, as estimated by the BELL program, were pK_1 = 5.9 ± 0.2 and pK_2 = 8.1 ± 0.2 for NADP$^+$ and pK_1 = 4.6 ± 0.3 and pK_2 = 8.8 ± 0.1 for MK-906. Since each compound is a reversible inhibitor of SR, these ionization events must represent the true ionization constants associated with binding of cofactor and steroid.

Discussion

The solubilization of SR activity has been reported from several tissues including porcine testes (Watkins et al., 1988), rat epididymis (Scheer & Robaire, 1983), human (Houston et al., 1985) and rat (Moore & Wilson, 1974) prostate, rat hypothalamus (Bertics & Karavolas, 1984), and rat liver (Liang et al., 1983; Graef et al., 1978; Ichihara & Tanaka,

[3] Isotope effects were calculated as the ratios of kinetic parameters determined with NADPH to those measured with NADPD; symbols for the respective kinetic constants are represented by right subscripted H and D. More complete theoretical discussions applicable to nicotinamide dinucleotide dependent bisubstrate enzymes have been given by Klinman (1977, 1978) and Cleland (1982).

Scheme I



1987a,b). From a review of the conditions employed in these extractions, an efficient and reproducible procedure for the solubilization of rat liver SR has been devised. From our experiments, it seems that all of the components in the solubilization buffer are critical to achieve high recovery of enzyme activity from microsomes. After solubilization, the enzyme solutions can be substantially diluted into a neutral buffer without loss of SR activity so long as the solution contains a minimum concentration (~50 nM) of cofactor.

Although not exhaustive, attempts at fractionation of the solubilized enzyme preparation proved inconsistent. Upon duplication of a procedure for the partial purification of SR from rat liver (Ichihara & Tanaka, 1987a), only a small fraction of total enzyme activity was recovered with no accompanying increase in specific activity. While enzyme activity could be recovered by ion-exchange chromatography, we found the yields to be meager. As was anticipated for such an integral membrane protein, enzyme activity was retained by hydrophobic supports. Unfortunately, no conditions could be identified, including variations in ionic strength, alterations of detergent concentration, and inclusion of chaotropic agents, to reliably elute enzyme activity. In these approaches toward purification of the liver SR, minimal attempt was made to maintain any artificial membrane environment (Ichihara & Tanaka, 1987b; Cooke & Robaire, 1987) or to consider potential regulatory factors (Le Goff et al., 1988) that might influence enzyme activity. Inclusion of these variables could prove imperative toward the successful purification of SR.

Enzyme purity notwithstanding, the solubilized rat liver SR conformed well to kinetic evaluation. Enzyme activity was sufficiently stable such that a consistent understanding of the kinetic mechanism and the effects upon variation of pH could be gleaned. Product and dead-end inhibition studies with the solubilized SR were undertaken to determine whether the binding of substrates to enzyme leading to catalysis was ordered or random (Scheme I). Inhibition patterns with thio-$NADP^+$ and MK-906 are consistent with the preferentially ordered binding of substrates to the surface of both microsomal and solubilized SR. Since $NADP^+$ is a competitive inhibitor versus NADPH, both must associate with the same enzyme form (E); consequently, the release of products is also most likely to be ordered according to the top pathway in Scheme I. Considering that DHT does not decrease enzyme activity, even in the presence of sufficient exogenous $NADP^+$ to cause 20–50% SR inhibition, release of the first product must be fast relative to its reassociation to the binary complex of E–$NADP^+$ ($k_7 \gg k_8$, Scheme I).

In this context, the conversion of the sequential initial velocity pattern with NADPH as substrate to equilibrium ordered with NADPD is of particular interest. Implicit in this change in pattern is that K_a becomes small compared to K_{ia} so the sequential model (eq 2) is reduced to that for equilibrium ordered (eq 3) upon substitution of the 4*S*-hydrogen with deuterium. Thus, the heavy atom substitution causes the maximal enzyme velocity to decrease such that the rate of product formation becomes significantly less than the dissociation rate (k_2) of the enzyme and the first substrate bound (E–A). Most likely, this change results from suppression of the unimolecular rate constant for hydride transfer (k_5), thereby slowing the interconversion of internal complexes. Since the first substrate bound to a bisubstrate enzyme is often slow to dissociate (Cleland, 1977), it might be inferred that SR has a relatively slow intrinsic catalytic rate constant.

It has been demonstrated with rat liver SR that the C-5 hydrogen of DHT originates from the 4*S*-position of the nicotinamide ring (Abul-Hajj, 1972). By analogy to nicotinamide adenine dinucleotide dependent dehydrogenases (Klinman, 1972; Bush et al., 1973), comparisons of steady-state kinetic constants (V_m, V_m/K_a, and V_m/K_b) to those obtained with (4*S*)-[4-^{2}H]NADPH should display primary isotope effects. Experimentally, normal isotope effects were observed for the three parameters at all substrate concentrations employed. The magnitude of the isotope effect on V_m/K_m for NADPH, calculated as the ratio $(V_m/K_a)_H/(V_m/K_a)_D$, consistently decreased with increasing concentration of T and disappeared upon extrapolation to infinite steroid concentration. For a system following an ordered binding of substrates to enzyme, the association of the first substrate (A) at infinite concentration of the second (B) would require that addition of B to the E–A complex be fast and essentially irreversible; hence, the steady-state parameter V_m/K_a becomes equivalent to the rate of addition of cofactor to enzyme and would not be affected by the isotropic substitution (Klinman, 1978). Thus, ablation of the isotope effect on V_m/K_a is consistent with the preferentially ordered binding of substrates as proposed above.[3]

Each V_m/K_m term is a measure of the conversion of free substrate (A or B) to products up to the first irreversible kinetic step; in contrast, V_m determines the conversion of bound substrates to free products and can include steps subsequent to those observed upon V_m/K_m (Klinman, 1977; Cleland, 1982). For the scenario wherein the release of both products from the enzyme is fast, the observed isotope effect on V_m would reflect an intrinsic value. Under such conditions, the isotope effect on V_m would be expected to be the same as that on V_m/K_b. However, since the results with the solubilized SR would indicate that $(V_m)_H/(V_m)_D < (V_m/K_b)_H/(V_m/K_b)_D$ (3.3 ± 0.4 and 3.8 ± 0.5, respectively), it is unlikely that $(V_m)_H/(V_m)_D$ is equivalent to an intrinsic effect (Cleland, 1982). These results can be rationalized if a partially rate determining event follows the first irreversible step—either chemical conversion (k_5) or release of the first product (k_7)—which limits V_m/K_b. Since there is no product inhibition by DHT and there is a substantial isotope effect on V_m, k_7 is probably faster than both k_5 and k_8. Consequently, dissociation of the second product is the most likely additional factor limiting V_m.

For enzymes that proceed by an ordered kinetic mechanism, the dependence of V_m/K_m on pH for the first substrate binding will show ionizations which influence the bimolecular rate constant for the combination of enzyme (E) and that substrate (A). The functionalities responsible for the breaks in the pH profile can be associated with either enzyme or substrate. In this regard, it must be recognized that the more basic of the two ionizable groups of NADPH, which has pK values of 3.9 and 6.1 (Windholz et al., 1983), is comparable to the pK_1 found in the V_m/K_m profile for NADPH (pK_1 = 5.6 ± 0.3). $NADP^+$, a competitive inhibitor of NADPH, has the same intrinsic ionization constants and shows a similar pH profile

($pK_1 = 5.9 \pm 0.2$) for SR inhibition. For a freely reversible inhibitor, any observed ionization in the pH profile should represent the true pK of the titrated functionality. Thus, the group with p$K \sim 5.7$ that requires deprotonation for binding of cofactor is the 2′-phosphate of the nicotinamide dinucleotide phosphate. Strengthening this conclusion is the observation that cofactor analogues lacking the 2′-phosphate, such as NADH, NAD^+, and 3′-$NADP^+$, do not demonstrate any kinetic interaction with the solubilized rat liver SR, while compounds having this functionality, specifically $NADP^+$, thio-$NADP^+$, and 3-AADP, are efficient inhibitors. In the same two pH profiles of SR with NADPH and $NADP^+$, protonation of a group with a constant near 8.0 also is required for binding. Presumably, this event originates from an enzyme-associated group that stabilizes the E–NADPH and E–$NADP^+$ complexes through coordination to the anionic 2′-phosphate. The importance of the 2′-phosphate group in determining the specificity of nucleotide binding has been demonstrated in other enzyme systems, as in pig heart $NADP^+$-dependent isocitrate dehydrogenase, where, by use of ^{31}P NMR, it has been demonstrated that the pK of this group is much lower within the enzyme complexes than in free solution (Mas & Colman, 1984).

The analogous pH profiles for steroidal substrate and inhibitor also reveal two important ionizations. With both T and MK-906, the group with the higher acidity ($pK_1 = 5.0 \pm 0.2$ and 4.6 ± 0.3, respectively) must be deprotonated, while the second ($pK_2 = 8.9 \pm 0.3$ and 8.8 ± 0.1, respectively) must be protonated for binding of steroid and catalysis. These ionizations could represent the same groups as proposed for the binding of the nicotinamide dinucleotides. The observed shifts to more extreme pH are an anticipated result of the kinetic order whereupon binding of steroid, the ionizable functionalities which influence the binding of nicotinamide dinucleotide phosphate, would become less accessible to the solvent (Cleland, 1977). Any commitment to catalysis upon formation of productive complexes with substrates or any degree of "stickiness" on the part of the steroidal inhibitor, MK-906, would also push the observed ionizations to more extreme pH from those determined in the cofactor profiles.

The preferentially ordered kinetic mechanism for the solubilized rat liver SR, deduced from inhibition analyses and primary isotope effects, is conserved with the enzyme which is bound within the microsomes (see Table I for comparison of dead-end plots). By analogy, the majority of proposed kinetic mechanisms for microsomal associated SR from other sources similarly are ordered (Campbell & Karavolas, 1989; Levy et al., 1989; Houston et al., 1987). While differences in affinity of SR from various sources for common ligands (Rasmusson et al., 1986) implicate the existence of unidentified factors that influence the binding energetics, many features of the kinetic mechanisms of these isozymes would seem to be conserved. Consequently, the detailed mechanistic principles found with the solubilized rat liver SR should prove to be integral toward a better, and possibly more universal, understanding of the interactions between SR, substrates, and inhibitor molecules.

Acknowledgments

We thank Dr. D. A. Holt for the preparation of MK-906 and Dr. B. W. Metcalf for continued support during the course of this work.

Supplementary Material Available

Dead-end inhibition patterns of MK-906 versus both T and NADPH with solubilized rat liver SR (Figure S-1) (2 pages). Ordering information is given on any current masthead page.

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Inhibition of Rat Liver Steroid 5α-Reductase by 3-Androstene-3-carboxylic Acids: Mechanism of Enzyme–Inhibitor Interaction

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ABSTRACT: The interactions of a series of newly discovered inhibitors of Δ^4-3-oxo-steroid 5α-reductase (SR; EC 1.3.1.30), the 3-androstene-3-carboxylic acids (steroidal acrylates), have been studied by using a solubilized rat liver enzyme preparation. As exemplified by one member of this series, 17β-[*N*,*N*-diisopropylcarbamoyl)androst-3,5-diene-3-carboxylic acid (**1a**), the dead-end inhibition patterns of selected compounds in this class are best evaluated by a linear uncompetitive kinetic model versus either substrate, testosterone (T) or NADPH. These results were interpreted within the context of the preferentially ordered kinetic mechanism for rat liver SR to arise from the association of inhibitor to the binary complex of enzyme and $NADP^+$. This proposed inhibition mechanism was supported by data from double-inhibition experiments implicating the synergistic binding of steroidal acrylate and $NADP^+$ to SR. Further evidence for the preferential formation of this ternary complex was obtained from filtration binding assays with [^{3}H]-**1a**, where radioligand association to protein was greatly enhanced in the presence of $NADP^+$. The amount of [^{3}H]-**1a** binding to protein was proportional to the specific activity of SR in the enzyme preparations, and the estimated dissociation constant from binding data by Scatchard analysis (K_d = 25 nM) was comparable to the inhibition constants estimated for SR activity (K_i = 12–26 nM). From the pH profile for inhibition of the solubilized liver SR with **1a**, it is proposed that the anion of the steroidal acrylate (pK_1 = 4.7 ± 0.2) is the active inhibitory species, coordinating to a protonated active site functionality (pK_2 = 7.5 ± 0.1). On the basis of data from similar experiments with structural analogues of **1a**, the determinants for binding recognition and inhibitory potency are compared to structural features of the putative enzyme-bound intermediate states. These compounds represent a potential therapeutic alternative in the treatment of 5α-dihydrotestosterone specific androgen dependent disease states.

Since the publication of papers describing the phenotypic characteristics of humans deficient in 5α-dihydrotestosterone (DHT)[1] (Imperato-McGinley et al., 1974; Walsh et al., 1974) ample evidence has accumulated supporting the proposal that this product of testosterone (T) metabolism is the principal androgen for the trophic growth and support of the prostate (Imperato-McGinley et al., 1979) and function of the sebacious gland (Sansone & Reisner, 1971). In addition, unusually high levels of DHT have been correlated with diseases such as benign prostatic hypertrophy (BPH) (Geller et al., 1976; Wilkin et al., 1980), acne (Sansone & Reisner, 1971), male pattern baldness (Bingham & Shaw, 1973), and female hirsutism (Kuttenn et al., 1977). Furthermore, recent results with pharmacological models have provided support for the hypothesis of DHT as a potent, tissue-specific androgen (Brooks et al., 1986; Blohm et al., 1986; Rittmaster et al., 1987). From this proposal of androgen action has evolved the concept that administration of specific antagonists of DHT could pose as effective therapy for these metabolic disorders. One approach toward this goal would be through the blockade of DHT biosynthesis. Here, the most attractive target is Δ^4-3-oxo-steroid 5α-reductase [steroid 5α-reductase (SR); EC 1.3.1.30], the NADPH-dependent enzyme that converts T into DHT.

Several classes of SR inhibitors with varying potency have been described. Those compounds that have been best characterized both in vitro and in DHT responsive model systems include the 6-methylene derivatives of progesterone and testosterone (Kadohama et al., 1983; Petrow et al., 1983) and the 3-keto-4-diazo-5α-dihydro steroids (Blohm et al.,

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[1] Abbreviations: T, testosterone; DHT, 5α-dihydrotestosterone; SR, Δ^4-3-oxo-steroid 5α-reductase (EC 1.3.1.30); MK-906, 17β-[*N*-(2-methyl-2-propyl)carbamoyl]-4-aza-5α-androst-1-en-3-one; **1a**, 17β-(*N*,*N*-diisopropylcarbamoyl)androst-3,5-diene-3-carboxylic acid; **2a**, 17β-[*N*-(2-methyl-2-propyl)carbamoyl]androst-3,5-diene-3-carboxylic acid; dpm, disintegrations per minute.

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