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Studies on the Mechanism of Action of the Δ^5-3-Ketosteroid Isomerase from Rat Adrenal Small Particles*

Nancy L. Oleinick† and Seymour B. Koritz

ABSTRACT: Isomerization of androst-5-ene-3,17-dione has been carried out in a deuterated medium under conditions of (a) enzyme catalysis by a rat adrenal microsomal fraction activated by oxidized diphosphopyridine nucleotide (DPN^+); (b) nonenzymatic reaction at pH 7.0; (c) acid catalysis; and (d) base catalysis. In all cases, incorporation of deuterium into the 6β position of the product is found. Incorporation at C-4 of the product is also found for all conditions except acid catalysis. A similarity in mechanism between alkaline isomerization and catalysis by the rat adrenal enzyme is suggested, and the proposed intermediate is the conjugate enolate anion. A profound deuterium isotope effect is seen in the enzymatic isomerization, and this is decreased in the presence of DPN^+ or reduced DPN (DPNH).

The mechanism of action of Δ^5-3-ketosteroid isomerase was initially investigated with the enzyme from *Pseudomonas testosteroni* by Talalay and Wang (1955), Kawahara and Talalay (1960), and Wang *et al.* (1963). When the reaction was conducted in deuterium-labeled water, they found no incorporation of label into the Δ^4-3-ketone product, in contrast to the acid- and base-catalyzed reactions, from which they concluded that the enzymatic isomerization proceeds *via* direct transfer of a hydrogen atom from C-4 to C-6 of the steroid nucleus. Malhotra and Ringold (1965) have prepared various C-4- and C-6-deuterated substrates and have shown that the bacterial enzyme catalyzes a stereospecific intramolecular transfer of a proton from the axial 4β to the axial 6β position. The rate-determining step is loss of the 4β proton. Based on the lack of deuterium incorporation at C-4, the proposed intermediate is a $\Delta^{3,5}$-dienol rather than the enolate anion, in common with the acid-catalyzed reaction.

In direct contrast, Werbin and Chaikoff (1963, 1964) have indicated that the mammalian adrenal isomerase must catalyze the reaction by another mechanism which involves loss of the 4β proton to the medium. Thus, when guinea pigs were fed a mixture of cholesterol-4-^{14}C and stereospecifically labeled cholesterol-4β-^{3}H, the ^{3}H:^{14}C ratio was greatly reduced in the urinary cortisol and 6β-hydroxycortisol. Furthermore, when these two labeled cholesterols were incubated with a mitochondrial acetone powder from beef adrenals, the tritium label was retained in the pregnenolone formed but lost in progesterone. This is strong evidence that the loss of the 4β-^{3}H occurred at the isomerase step.

It seemed reasonable that if the reaction catalyzed by the mammalian isomerase were carried out in a D_2O medium, deuterium should be found in the 6 position of the product, and that additional information concerning a mechanism could be obtained by this more direct approach. The authors have now isomerized Δ^5-AND[1] under the following conditions: (1) enzyme catalysis by a rat adrenal microsomal fraction activated by DPN^+ (Oleinick and Koritz, 1966); (2) nonenzymatic isomerization at pH 7.0; (3) acid catalysis; and (4) base catalysis. Application of the infrared absorption spectra for the various possible deuterium-labeled androstenediones (Malhotra and Ringold, 1964) has allowed us to detect similarities between the enzymatic catalysis and the base-catalyzed reaction.

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† This report is from the Ph.D. dissertation of Nancy L. Oleinick, predoctorate fellow at the University of Pittsburgh. Present address: Department of Biochemistry, Western Reserve University, School of Medicine, Cleveland, Ohio.

[1] Abbreviations: Δ^5-AND, androst-5-ene-3,17-dione; Δ^4-AND, androst-4-ene-3,17-dione; DPN^+, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; BSA, bovine serum albumin.

Experimental Section

Preparation of Reagents. (1) Rat adrenal microsomal fraction (pellet 3) was prepared. Approximately 120 rats were sacrificed to provide 5000-mg wet weight of adrenals. The pellet 3 was prepared as previously described (Oleinick and Koritz, 1966), and in addition was washed once with 0.154 M KCl in D_2O (99.84%) by resuspension and recentrifugation at 100,000*g* for 60 min. The washed pellet was finally homogenized to 50 ml of 0.154 M KCl in D_2O. (2) Potassium phosphate buffer (0.3 M), pH 7.0, was prepared by dissolving an appropriate amount of KD_2PO_4 (99.5 mole % D) in D_2O (99.84% D) and neutralizing with KOD (40% in D_2O; 98% D). All of the deuterium-labeled compounds were purchased from Bio-Rad Laboratories, Richmond, Calif. (3) DPN (0.01 M) was prepared in 0.3 M phosphate buffer, pH 7.0. (4) Δ^5-AND was synthesized and assayed as described (Oleinick and Koritz, 1966). It was added to the incubation flask in dioxane (6 mg/2.5 ml of dioxane). (5) Δ^4-AND (Sigma Chemical Co., St. Louis, Mo.) was also added in dioxane solution of the same concentration.

Enzymatic Isomerization of Δ^5-AND. The following were combined in a 500-ml erlenmeyer flask: 65 ml of 0.154 M KCl, 10 ml of 0.3 M phosphate buffer, pH 7.0, 25 ml of 0.01 M DPN^+, 25 ml of pellet 3 preparation, and 30 mg of Δ^5-AND (assay: 93% Δ^5, 7% Δ^4) in 12.5 ml of dioxane. All reagents were in D_2O as described above. Another flask was prepared identical with the first except for the addition of 30 mg of Δ^4-AND in 12.5 ml of dioxane in place of Δ^5-AND. This served as a product–enolization control. The isomerization reaction in flask no. 1 was followed by periodically extracting an aliquot into dichloromethane and reading at 240 mμ. After 3 hr at 37°, there was little absorbancy change, and 75% of the substrate had been converted.

Nonenzymatic Isomerization at pH 7.0. The incubation mixture contained 54 ml of 0.154 M KCl, 6.0 ml of 0.3 M phosphate buffer, pH 7.0, 15 ml of 0.01 M DPN^+, and 6 mg of Δ^5-AND (assay: 93% Δ^5, 7% Δ^4) in 2.5 ml of dioxane. Incubation was for 3 hr at 37°, at which time only an additional 7.1% of the substrate had isomerized. In the extraction and purification procedure to be described, both Δ^4-AND and unreacted Δ^5-AND were isolated.

Acid Isomerization. Two flasks were prepared, both containing 24.4 ml of D_2O and 0.6 ml of DCl (38% in D_2O). To the first were added 12 mg of Δ^5-AND (assay: 97.3% Δ^5, 2.7% Δ^4) in 5 ml of dioxane; to the second, an equal amount of Δ^4-AND. The reaction at room temperature was complete in 25 min. Both flasks were diluted with 50 ml of H_2O prior to extraction.

Base Isomerization. Two flasks were prepared, both containing 24.9 ml of D_2O and 0.1 ml of KOD (40% in D_2O). To the first was added 12 mg of Δ^5-AND as described for acid isomerization; to the second, Δ^4-AND. After 15 min at room temperature, there was no further increase in 240-mμ absorption. Since the absorbancy of the isomerization flask indicated an incomplete reaction, it was apparent that some decomposition of the substrate had occurred. H_2O (50 ml) was added to each flask prior to extraction.

Isolation and Characterization of Δ^5- and Δ^4-AND. Each incubation mixture was extracted in 40–50-ml portions with five 10-ml portions of dichloromethane. The combined extracts (50 ml) were reduced to one-half volume, washed with three 5-ml portions of H_2O, and dried with anhydrous sodium sulfate. Assay of the combined extracts for each incubation revealed that no further isomerization had occurred during the extraction procedure.

Δ^4-AND, Δ^5-AND, and other trace substances could be efficiently separated from one another by thin layer chromatography on silica gel G, employing one of two solvent systems (Chang, 1964): in system 1 (stationary phase: ethylene glycol–methanol, 30:70; mobile phase: 2.5% dichloromethane in methylcyclohexane), R_F Δ^4-AND 0.57, and R_F Δ^5-AND 0.90; in system 2 (hexane–ethyl acetate, 1:1), R_F Δ^4-AND 0.59. The Δ^4-AND could be detected under ultraviolet light, while the Δ^5-AND could be detected in the same manner if allowed to isomerize overnight on the plate after chromatographic separation. The absence of tailing in the Δ^5-AND band indicated that negligible isomerization had taken place during the chromatography (20 min).

Each band was extracted separately with dichloromethane or warm methanol. When system 1 was employed, the extracts were washed with water to remove residual ethylene glycol, and a trace of yellow color was eliminated by passage of the sample through a 0.5 × 10 cm column of alumina deactivated by 20% its weight of water and elution with $CHCl_3$–isooctane (3:7) (Nes *et al.*, 1963). All samples were finally recrystallized once from acetone–ligroin.

Total deuterium was determined by analysis.[2] Infrared absorption spectra were taken on a Beckman IR-9 spectrophotometer (KBr pellet). The position of deuterium label was determined by the C–D stretching frequencies (Malhotra and Ringold, 1964): C-6β, 2140 cm^{-1}; C-6α, 2190 cm^{-1}; C-4, 2255 cm^{-1}; C-2β, 2140 cm^{-1}; and C-2,2, 2140 and 2220 cm^{-1}. Although accurate quantitation of deuterium incorporation at the various positions is not possible from the infrared bands, areas within the observed bands were estimated with a planimeter and are presented in Table I based on the area of the 2140-cm^{-1} band within each experiment as 1.

KOH Equilibration. The possibility exists that, at high pH values, deuterium can be introduced into enolizable positions. To remove such deuterium, a portion of the Δ^4-AND from KOD isomerization and enolization was individually dissolved in 2.9 ml of dioxane and combined with 12.5 ml of 0.3 N KOH for 15 min at room temperature. The reaction mixtures

[2] We wish to thank Dr. Allen M. Gold, Columbia University, New York, N. Y., for the deuterium analyses and Dr. Gerald Carlson, Mellon Institute, Pittsburgh, Pa., for the infrared spectra.

TABLE I: Deuterium Incorporation into Androstenedione during Isomerization and Enolization.[a]

Substrate	Conditions	Total Deuterium (atoms/ molecule)	Infrared Bands (cm^{-1}) 2140	2255
			Relative Areas	
Δ^5-AND	Enzymatic isomerization	1.68	1	0.9
Δ^4-AND	Enzyme control	0.0847	*e*	*e*
Δ^5-AND	Nonenzymatic isomerization, pH 7.0			
	Δ^5-AND recovered	0.176	*b*	
	Δ^4-AND formed	1.44	1	1
Δ^5-AND	DCl isomerization	1.17	1[c]	0.2
Δ^4-AND	DCl enolization	0.0209	*e*	*e*
Δ^5-AND	KOD isomerization			
	Δ^4-AND formed	3.26	1	0.3
	Δ^4-AND KOH equilibrated	2.53	1	0.5
Δ^4-AND	KOD enolization			
	Δ^4-AND recovered	1.52	1[d]	*e*
	Δ^4-AND KOH equilibrated	0.678	1[d]	*e*

[a] All samples were treated as described in the Experimental Section. Total deuterium analyses for isomerization reactions are corrected for Δ^4-AND initially present, *i.e.*, they represent atoms of deuterium incorporated per molecule of Δ^5-AND isomerized. [b] The infrared spectrum of this sample was not determined. [c] Total area of peak at 2140 cm^{-1} and slight shoulder at 2190 cm^{-1}. [d] Total area includes peak at 2140 cm^{-1} and shoulders at 2190 and 2220 cm^{-1}. [e] No absorbancy was found.

were then diluted with 50 ml of H_2O and extracted as described above, and the Δ^4-AND separated in the hexane–ethyl acetate thin layer chromatography system. The final products were recrystallized once from acetone–ligroin.

Results

The data in Table I present a summary of the infrared bands and total deuterium incorporation found for the various isomerization and enolization conditions. Under all isomerization conditions, deuterium is incorporated into the 6β position. Incorporation at C-6 is to be expected for any nonenzymatic isomerization. It is also found for the enzyme-catalyzed reaction, as anticipated from the results of Werbin and Chaikoff (1963, 1964). Such findings indicate that the mechanisms of the bacterial and mammalian enzymes are indeed different.

It was initially surprising, however, that incorporation of deuterium was also found at C-4 of Δ^4-AND when the isomerization was conducted at pH 7.0 in the presence or absence of the enzyme. Incubation of Δ^4-AND under neutral conditions demonstrated that the additional deuterium did not arise from further enolization of the product. It is to be noted that after the 3-hr incubation, beginning with substrate containing 7% Δ^4-AND, a total of 75% Δ^4-AND was isolated from the enzymatic incubation flask, while only 14.1% Δ^4-AND was isolated from the spontaneous isomerization flask. Although the incorporation patterns of the two samples are similar, isomerization in the presence of enzyme is obviously faster than in its absence, and the incorporation cannot be accounted for strictly on the basis of the nonenzymatic reaction.

The absence of C-4 proton exchange during catalysis by the enzyme from *P. testosteroni* suggested to Malhotra and Ringold (1965) that the isomerization mechanism was similar to that of acid catalysis, which involves a $\Delta^{3,5}$-dienol intermediate. Our finding of deuterium in the C-4 position suggested that the mammalian enzyme may catalyze a reaction more similar to alkaline isomerization. In this respect, it has been found that the enolate anion, generated by strong base, undergoes preferential protonation at C-4 (Ringold and Malhotra, 1962). Accordingly, isomerization of Δ^5-AND in DCl and KOD were carried out. As may be seen from the data in Table I, when the CD bands for C-4 deuterium (2255 cm^{-1}) were compared to those for C-6β (C-2β) deuterium (2140c m^{-1}), within a given experiment, only a small amount of C-4 deuterium was found for the acid-catalyzed reaction, as has also been found by Malhotra and Ringold (Malhotra and Ringold, 1965), while a larger amount was found for the base-catalyzed reaction. Incubation of Δ^4-AND under identical conditions revealed again that the C-4 deuterium was not a result of additional enolization of the product.

The large deuterium incorporation for KOD isomerization and enolization and the presence of a band at 2140 cm^{-1} for KOD enolization demanded further inquiry. Malhotra and Ringold (1964) showed con-

clusively that base enolization of a Δ^4-3-ketone results in exclusive incorporation at C-2, which gives a CD stretching band at 2140 cm^{-1}, identical with that of 6β-D, and a band at 2220 cm^{-1} for 2α deuterium, if a second atom enters the molecule. Furthermore, Talalay and Wang (1955) also obtained close to four atoms of deuterium in Δ^4-AND during base isomerization. The use of testosterone by Malhotra and Ringold (1964) eliminated the possibility of extra enolization due to a 17-ketone. Thus, it was necessary to establish that isomerization in base produced C-4 and C-6β deuterium, and that further deuterium incorporation (at C-2 and, presumably, C-16) was due to enolization of the 3- and 17-ketones.

Since it is known (Malhotra and Ringold, 1964) that alkaline enolization of testosterone results in proton exchange exclusively at C-2, any deuterium incorporated *via* Δ^4-3-ketone enolization during the KOD-catalyzed reaction should be largely removed by subjecting that product to enolization in KOH, while deuterium at C-4 or -6 should be unaffected. As shown in Table I, during a 15-min equilibration in KOH, about 0.8 atom of deuterium was removed from KOD-isomerized and KOD-enolized products, causing an increase in the infrared band at 2255 cm^{-1} relative to that at 2140 cm^{-1}, a result consistent with the removal of enolizable deuterium which causes absorption at 2140 cm^{-1}. In view of the observations that base-enolizable deuterium cannot be present at C-4 and C-6 (Malhotra and Ringold, 1964) and that the same amount of deuterium is removed from the KOD-isomerized and KOD-enolized products during the 15-min equilibration in KOH, it is apparent that the value of 1.52 atoms of deuterium found for KOD enolization of Δ^4-AND represents an adequate control for deuterium introduced into the Δ^4-AND product of KOD isomerization. Then, the difference between these values, 3.26 − 1.52 = 1.74, is a reasonable estimate of deuterium incorporation due to isomerization alone. Presumably, a longer equilibration time would have resulted in a larger removal of deuterium from enolizable positions and a further increase in the size of the 2255-cm^{-1} band relative to that at 2140 cm^{-1}. KOH equilibration of the DCl-isomerized product produced essentially no decrease in the amount of deuterium incorporated.

Enzymatic isomerization of about 24 mg of Δ^5-AND in D_2O required 3 hr. Although undoubtedly enzyme inactivation occurred during the incubation, previous control experiments had shown that initially there was enough enzyme present to isomerize the entire added 30 mg of Δ^5-AND in 15 min, if the reaction were carried out in a protonated, rather than a deuterated, medium. A profound isotope effect was indicated.

The extent of the isotope effect may be seen from the data in Figure 1. The reaction is faster in H_2O than in D_2O by a factor of 3–4 when pyridine nucleotide is present, but by a factor of 9.7 in the absence of pyridine nucleotide. It is also to be noted that the shapes of the curves are different; *i.e.*, while a linear decline in activity is seen with increasing D_2O content in the absence of pyridine nucleotide, in its presence the decrease in velocity is slow at low D_2O content and increases at high D_2O content.

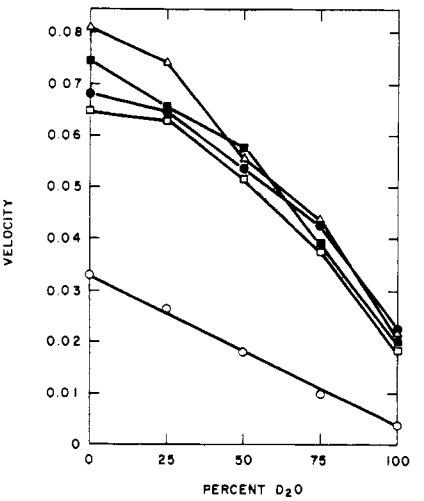


FIGURE 1: Variation in velocity of the isomerase reaction with D_2O content of the medium. The incubation mixtures contained in 20-ml beakers 90 μmoles of potassium phosphate buffer, pH 7.0, 0.5 mg of BSA, various amounts of DPN^+ or DPNH and the pellet 3 suspension as indicated below, 0.175 μmole of Δ^5-AND in 0.05 ml of methanol, and 0.154 M KCl to a final volume of 2.0 ml. Two complete sets of reagents were prepared, one in H_2O and one in 99.84 mole % D_2O, and various amounts of each set were added to each beaker to obtain the proper reagent concentration and D_2O content. Incubation was carried out in a Dubnoff metabolic shaking incubator at 37° for 10 min, after which a 1.5-ml aliquot was extracted with 1.5 ml of methylene chloride. The organic layer was read at 240 mμ, the absorption maximum of Δ^4-AND, in a Beckman DU spectrophotometer. Velocity is expressed as micromoles of Δ^5-AND isomerized in 10 min and is corrected for nonenzymatic isomerization. All values are initial velocities. "% D_2O" means volume per cent of 2.0-ml total incubation mixture which was composed of reactants prepared in 99.84% D_2O. "100% D_2O" means that all reactants were prepared in 99.84% D_2O except Δ^5-AND, which was added in 0.05 ml of methanol. Five conditions were employed: ○ = 0.3 ml of pellet 3, no DPN^+ or DPNH; ● = 0.1 ml of pellet 3 + 10^{-6} M DPNH; □ = 0.05 ml of pellet 3 + 10^{-4} M DPNH; ■ = 0.05 ml of pellet 3 + 10^{-5} M DPN^+; Δ = 0.03 ml of pellet 3 + 10^{-3} M DPN^+. It was necessary to adjust the pellet 3 concentration for different amounts of DPN^+ and DPNH to keep the velocities within the range measurable by the assay.

Discussion

It is apparent that the mammalian adrenal isomerase

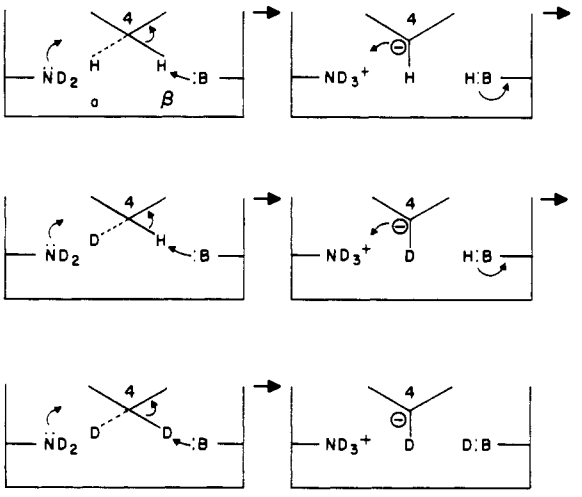
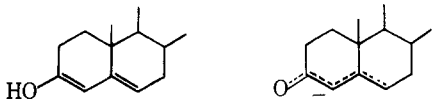


FIGURE 2. A proposed mechanism for the enzymatic deprotonation–reprotonation at C-4.

catalyzes the isomerization reaction *via* a mechanism different from that reported for the enzyme from *P. testosteroni* (Wang *et al.*, 1963; Malhotra and Ringold, 1965). As expected from the results of Werbin and Chaikoff (1963, 1964), incorporation of deuterium from the medium at the 6β position of the steroid nucleus is found. C-6β deuterium is found under all conditions of isomerization employed in this study: enzyme catalysis at pH 7.0, nonenzymatic isomerization at pH 7.0, acid catalysis in D_2O–DCl, and base catalysis in D_2O–KOD. It is not found with the enzyme from the bacterial source (Malhotra and Ringold, 1965).

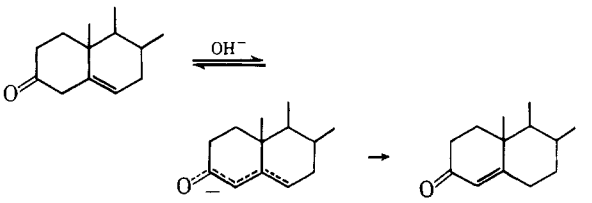
A further, and initially surprising, difference from the bacterial enzyme was the incorporation of additional deuterium at C-4. Deuterium in the C-4 position is seen in all cases except acid catalysis, under which conditions only a very weak band appears in the infrared at 2255 cm^{-1}.

Malhotra and Ringold (1965) have clearly demonstrated that protonation of the neutral $\Delta^{3,5}$-dienol

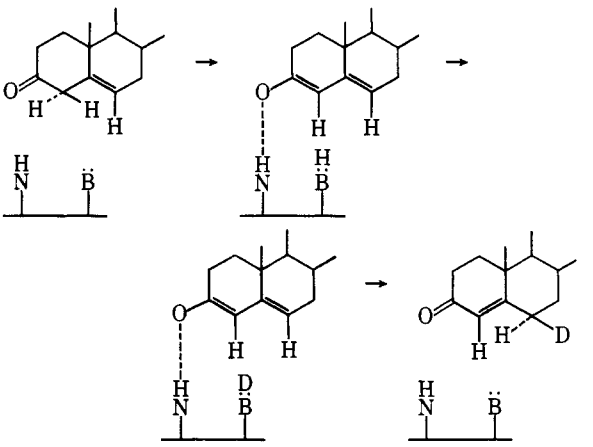


proceeds almost exclusively at the 6β position, while the conjugate enolate anion undergoes C-4 protonation. It therefore appears that the enolate anion is the intermediate formed during alkaline isomerization. Protonation and deprotonation at C-4 of the anion is probably faster than C-6 protonation, which generates the product.

Since deuterium is found in both the 4 and 6 positions in the enzymatic reaction, a similar intermediate appears to be formed by the rat adrenal isomerase. The following mechanism is postulated. The 4β proton (Werbin and Chaikoff, 1963, 1964) is abstracted by a basic group B: on the protein. The anion so formed



can be stabilized by hydrogen bonding of the carbonyl oxygen to an acidic group, *e.g.*, NH, on the protein. Protonation of the 6β position may be accomplished by the medium or, more probably, by B:H $\rightleftharpoons$ B:D.



To account for deuterium incorporation at C-4, the authors postulate that protonation of the enolate anion at C-4 is much faster than at C-6. The C-4 protonation can be accomplished either by the medium, or by a third, acidic, group on the protein, *e.g.*, NH_3^+, with inversion of configuration at C-4. The deuterium of B:could then arise by direct exchange of B:H with the medium as indicated above or by successive abstractions of 4β-H and loss either to the medium or, less frequently, to the 6β position. The deprotonation–reprotonation of C-4 might then be pictured as in Figure 2.

It might also be argued that the deuterium content of C-4 is the result of a reversible removal of the 4β-H by B: and exchange on B: to give B:D. It seems likely, however, that the single group on the enzyme would be stereospecific in removal and replacement of a proton and could not alone account for the occurrence of deuterium at another stereochemically distinct position. Deuterium at C-4 of the Δ^4-3-ketone cannot arise from exchange of B:H with the medium and replacement onto the 4β position.

The rather profound deuterium isotope effect suggests that the breaking of some CD bond is rate limiting. The proposed mechanism for incorporation of deuterium into C-4 involves an eventual cleavage of a C-4 deuterium bond by the enzyme. If reprotonation at C-4 is very rapid, then by the third abstraction of C-4 proton, the enzyme must remove a deuterium. The rate-limiting step would then be essentially the same as for the bacterial enzyme (Malhotra and Ringold, 1965). An additional possibility to be considered is that the enzyme is partially inactivated in D_2O.

Such a hypothesis could account for the variations in deuterium isotope effect for the enzymatic reaction in the presence and absence of pyridine nucleotide, *i.e.*, there may be less rapid D_2O inactivation of the enzyme in the presence of pyridine nucleotide.

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A Microanalytical Technique for the Quantitative Determination of Twenty-four Classes of Brain Lipids*

Michael A. Wells† and John C. Dittmer‡

ABSTRACT: Selective mild alkaline and acid hydrolyses are used to obtain water-soluble phosphate esters characteristic of the diacyl phosphoglycerides and plasmalogens of brain. These phosphate esters are separated by anion-exchange chromatography and quantitatively assayed. Phospholipids stable to hydrolysis are assayed after fractionation on silicic acid. The gangliosides, neutral lipids, and glycosphinogolipids are measured by specific spectrophotometric determination of characteristic components after an initial solvent fractionation and chromatography on Florisil. Complete analysis of 24 classes of lipids can be carried out on as little as 150 mg of brain tissue. Evidence for the occurrence in rat brain of plasmalogenic acid, choline plasmalogen, inositol plasmalogen, and phosphatidylglycerol is presented. Serine plasmalogen is absent.

No tissue has a more complex lipid compositon than brain and the degree of complexity has only become apparent over the years. It is not surprising then that methods devised in the past to analyze part or all of the different lipids have become obsolete. These methods have ranged from the pioneering attempts of Koch and Koch (1913) who assayed four major classes of lipids through the comprehensive analyses of Brante (1949) which distinguished 10 or 11 classes. More recently and of current value Dawson (1960) and Dawson *et al.* (1962) have developed a system of analysis based on selective hydrolysis procedures and paper chromatography and electrophoresis with which 11 phospholipids of ox and sheep brain have been determined. Also recently, Rouser *et al.* (1963, 1965) have described in general terms analytical procedures based on the fractionation of the intact lipids of brain. Data for 14 lipids of ox brain and 11 of human brain have been reported.

The success of Lester (1963) in quantitatively analyzing the phospholipids of yeast by selective hydrolysis and fractionation of the water-soluble phosphates on anion-exchange columns suggested the possibility of developing a similar technique which, coupled with other specific assays, could be used to analyze all the

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† Predoctoral Fellow, Institute of General Medical Sciences, U. S. Public Health Service. Present address: Department of Biochemistry, University of Washington, Seattle, Wash. 98105.

‡ Present address, Department of Biochemistry, Saint Louis University School of Medicine, Saint Louis, Mo. 63104.