ABSTRACT

THE RELATION BETWEEN BOAR ODOR INTENSITY
AND 5α-ANDROST-16-EN-3-ONE CONTENT
IN PORK FAT

By

Raymond H. Thompson, Jr.

The present investigation was undertaken to determine the relationship between boar odor scores for fat samples derived from cryptorchid (males with retained testicles) pigs and the levels of 5α-androst-16-en-3-one, which has been claimed to be responsible for this undesirable odor. In order to ascertain the levels of 5α-androst-16-en-3-one, a stable isotope dilution/carrier technique (Bieber, M.A. et al., 1972, Anal. Biochem. 47:264) was adapted for use on fat samples. After addition of the deuterium labeled 5α-androst-16-en-3-one to the fat sample, the mixture of deuterium and protium forms was purified. The resultant fraction was analyzed by a combination of gas chromatography and mass spectrometry. The ratio of the deuterium and protium forms was obtained by multiple ion detection using the accelerating voltage alternator accessory of an LKB 9000 gas chromatograph-mass spectrometer (Holland, et al., 1973, Anal. Chem. 45:308).

The addition of the labeled form of the steroid enabled small amounts of the compound to be partially purified and separated by gas chromatography without total loss through adsorption. Losses during isolation and analysis were automatically accounted for by the method since the ratio of the labeled and unlabeled species would remain constant. The amount of unlabeled 5α-androst-16-en-3-one was determined from the ratio of the labeled to the unlabeled species. This procedure was utilized in determining the level of 5α-androst-16-en-3-one in 21 fat samples from cryptorchid pigs.

Deuterium labeled forms of the five most common C_{19}-Δ^{16} steroids were synthesized in order to permit analysis by stable isotope dilution. The labeled C_{19}-Δ^{16} steroids synthesized were as follows: d_2-, d_3- and d_4-5α-androst-16-en-3-one; d_1- and d_4- 5α-androst-16-en-3α-ol; d_4-5α-androst-16-en-3β-ol; d_2-4,16-androstadien-3-one and d_2-5,16-androstadien-3β-ol.

Several fat samples from cryptorchid pigs were also rated for intensity of the undesirable (boar) odor by either a trained laboratory panel or by a meat industry panel and the values were correlated with the levels of 5α-androst-16-en-3-one determined by the isotope dilution procedure. The correlation coefficient for the meat industry panel and the level of 5α-androst-16-en-3-one was only 0.40, which was not statistically significant ($P < .05$). The corresponding correlation coefficient for the trained laboratory panel was 0.51

which was significant at $P<.05$. Although the trained laboratory panel apparently were able to detect the level of 5α-androst-16-en-3-one, the relationship only accounted for 25% of the variation. This indicated that 5α-androst-16-en-3-one was not solely responsible for the undesirable odor and that other related C_{19}-Δ^{16} steroids may account for the low relationship. Further support for this viewpoint is evident by the fact that several fat samples having high odor intensity scores were found on analysis to have only low levels of 5α-androst-16-en-3-one.

The correlation coefficient between the odor scores for the two panels was 0.52 ($P<.05$). Although the relationship was significant, the relationship accounted for only 27% of the variation in odor scores. Thus, the two panels did not closely agree on the odor. Some reasons for the possible differences are discussed.

Although the labeled C_{19}-Δ^{16} steroid standards were synthesized and are now available in our laboratory, time did not permit analysis. These labeled standard compounds will be useful in subsequent studies on the significance of C_{19}-Δ^{16} steroids in both pigs and human beings.

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INTRODUCTION

The undesirable odor frequently associated with the cooking of meat from uncastrated sexually mature male pigs (boars) has long been a problem to swine producers and the meat packing industry. This "perspiration-like" or "urine-like" odor is extremely offensive to many consumers. Approximately 65% of boar pigs have been reported to be effected, while only 1-5% of the females and castrated males have boar odor (Williams et al., 1963). Although only 0.0015% of all hogs are condemned for boar odor under federal meat inspection, it has been estimated that strict enforcement of the regulations could result in condemnation or restricted usage of 1,350,000 hogs or about 675,000,000 pounds of pork annually (National Provisioner, 1967).

Sink (1967) postulated that the odor was due to C_{19}-Δ^{16}-steroids and that they may function as sexual pheromones in pigs. Patterson (1968) isolated the C_{19}-Δ^{16}-steroid, 5α-androst-16-en-3-one, by vacuum distillation of heated boar fat and identified it as the major contributor to boar odor. Subsequent corraborative reports from two laboratories (Berry et al., 1971; Thompson et al., 1972) have confirmed the involvement of C_{19}-Δ^{16}-steroids in boar odor as suggested earlier by Sink (1967).

The aim of this investigation was to develop an accurate method for the assay of 5α-androst-16-en-3-one in pork fat, and to determine the extent to which the intensity of boar odor is related to the concentration of 5α-androst-16-en-3-one in the pig. A major portion of this study was devoted to the synthesis of deuterium labeled C_{19}-Δ^{16}-steroids to be used as internal standards and carriers in quantitative analysis by mass spectrometric reverse isotope dilution.

The technique of reverse stable-isotope dilution seemed uniquely well suited for assay of C_{19}-Δ^{16}-steroids in boar odor studies, and for studies of the metabolism of this class of compounds in the pig and in humans. The addition of a labeled form of a C_{19}-Δ^{16}-steroid would enable extremely small amounts of the compound to be partially purified and separated by gas chromatography without total loss by adsorption. Losses during the isolation would be corrected for since the ratio of the labeled and unlabeled species would not be expected to change. The amount of unlabeled steroid could be determined from the isotopic ratio resulting after the addition of a known amount of the labeled species.

REVIEW OF LITERATURE

Boar Odor in Pork

Meat from sexually mature boar (uncastrated male) pigs gives off a permeating undesirable odor upon heating, which is extremely offensive to many consumers. This odor has been characterized as "perspiration-like", "onion-like" or "urine-like" and occurs not only in boars, but also in cryptorchids and to a lesser extent in sows, barrows and gilts (Pearson et al., 1969). Lerche (1936) indicated that the meat of boars gives off an objectionable odor that becomes apparent as soon as the male pig reaches sexual maturity. Castration and holding for 57-68 days resulted in disappearance of the odor (Lerche, 1936).

Incidence of Boar Odor

Self (1957) reported that sex odor occurred as frequently in sows, gilts and barrows as in boars. Prior to this report other workers largely assumed that the problem was confined to sexually mature boars and cryptorchids (Pearson et al., 1969). Pearson et al. (1969) reviewed the incidence of sex odor and showed that there was a definite sex-dependency,

with 64% of all intact males exhibiting boar odor compared to only 1-5% for the females or the castrate males. A similar incidence of boar odor in pork carcasses was reported by the Meat Inspection Division of the United States Department of Agriculture (USDA, 1968). Their survey indicated that 57% of the boars and 33% of the cryptorchids examined gave off the objectionable odor, while approximately 20% more in each group were found to have a slight odor. Only 11% of the sows were found to give off a definite sexual odor, with 10% having a slight odor. The limited amount of data available on cryptorchids suggests that about 50% have sex odor, which would make the incidence somewhat lower than for boars (64%), but considerably higher than the 1-5% reported for gilts, sows and barrows by Pearson et al. (1969).

The discrepancies between the results of Self (1957) and those of other workers (Williams et al., 1963; USDA, 1968) may have been due to differences in the methods used for evaluating sex odor (Pearson et al., 1969). The testing procedure used by Self (1957) consisted of heating a portion of the diaphragm muscle to 200°F for evaluation by a panel selected without regard for their ability to recognize sex odor. The reliability of such a procedure is questionable on considering the variability observed in the ability of people to detect boar odor (Pearson et al., 1969). It is now known that a unique aspect of the sex odor problem

is the variable and sex-related ability of the human to detect the odor of the C_{19}-Δ^{16}-steroids, which are primarily responsible for the offensive odor (Griffiths and Patterson, 1970). Furthermore, the use of diaphragm muscle creates confusion because of possible contamination from the contents of the urinary and digestive tracts (Pearson et al., 1969). Finally, Self (1957) heated the sample to only 200°F, whereas, Craig et al. (1962) subsequently showed that a temperature of 212° F was required for optimum detection.

On occasion, terms such as boar odor and sex odor have been applied to more than one off-odor associated with pig fat (Self, 1957). Sex odor is volatilized on heating or cooking of pork or bacon containing the undesirable aroma, but can not be readily detected in the uncooked meat or cold precooked meat (Pearson et al., 1971). The term "sex odor" is meant to describe only this odor and not other odors associated with the live animal or its carcass.

Disposition of Carcasses with Sex Odor

Federal Meat Inspection Regulations require tha condemnation of carcasses that give off a "sexual" or "urine odor" and specify that disposal shall be determined by heating tests after chilling (USDA, 1973). The meat from all boars and cryptorchids not condemned may be used in comminuted, cooked meat food products or for rendering.

The seriousness of boar odor from the economic standpoint was emphasized in a recent article (National Provisioner, 1967). It was estimated that strict enforcement of the regulations on sex odor could result in condemnation of about one-half of the 300,000 boars and stags killed annually under federal meat inspection. In addition, 550,000 sows would be condemned and 500,000 more would be restricted for use in cooked sausage or for rendering. This would affect 1,350,000 hogs or about 675,000,000 pounds annually, without taking into account additional losses in fresh pork sausage or in Canadian style bacon (Pearson, 1972).

Indications are that only 0.0015% of all hogs are condemned for sex odor under federal inspection (National Provisioner, 1967). Efforts to enforce the regulations are frustrated by the varying levels of odor, the inability of many inspectors to consistently recognize the odor and the difficult task of heat testing every carcass (Pearson, 1972). Condemnation of all carcasses having boar odor would result in a serious economic loss, nevertheless, the pork industry cannot afford to market any meat or lard which gives off boar odor on heating.

Results of a study by Pearson et al. (1971) verified that boar meat can be used in some meat products eaten without further heating. Two large consumer taste panels were utilized to ascertain the acceptability of 22 different products differing only in that the pork utilized in them

did not contain boar pork. In general, the results indicated that products containing boar meat are not readily distinguishable from similar control products providing the kitchen and dining areas are kept distinctly separate, for it is during the cooking process that the objectionable odor becomes most apparent (Pearson et al., 1971).

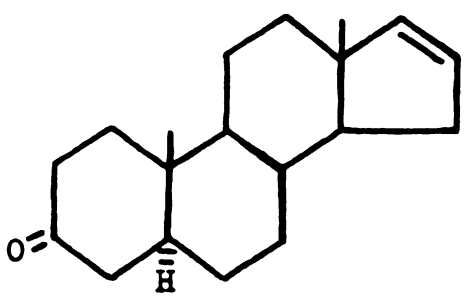
Chemical Identity of Boar Odor

One of the earliest investigations into the identity of the boar odor component(s) was a study by Craig et al. (1962), which established that the undesirable odor was localized in the fatty tissues of the carcass and was concentrated in the nonsaponifiable fraction of the fat. Subsequent efforts were made to identify the responsible components by fractionation of the nonsaponifiable material and gas chromatography, but these studies did not reveal the identity of the responsible components (Williams and Pearson, 1965).

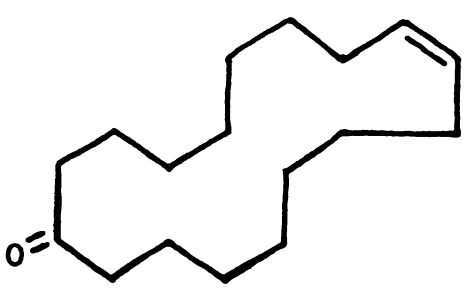
Sink (1967) published a theoretical paper in which he proposed that sex odor was caused by C_{19}-Δ^{16}-steroids and that these compounds were serving as sexual pheromones in the chemical communication between pigs. The theory was based in part on comments made much earlier by Prelog et al. (1944) about the musk-like odor of two C_{19}-Δ^{16}-steroids, 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol, both of which were isolated for the first time from swine

testicular tissue (Prelog and Ruzicka, 1944). Prelog et al. (1945) reported that the corresponding 5β-isomers were odorless and that the ketones, 4,16-androstadien-3-one and 5α-androst-16-en-3-one, possessed pronounced urine-like or perspiration-like odors. These workers also drew attention to the superficial structural similarities between 5α-androst-16-en-3-one and the structure of civetone and muscone. These two macrocyclic compounds were isolated by Ruzicka (1926a,b,c) and shown to have pheromonal significance in the sexual behavior of the civet cat and the musk deer, respectively.

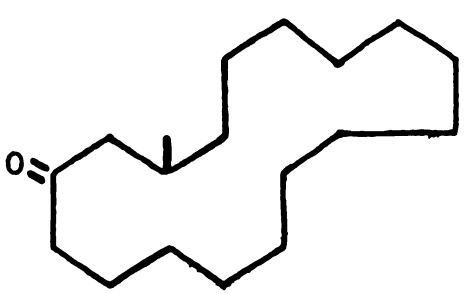
The similarity in the structures of civetone, muscone and 5α-androst-16-en-3-one as pointed out by Prelog and Ruzicka (1944) are shown below:



5α-Androst-16-en-3-one



Civetone



Muscone

Shortly after Sink (1967) published his theoretical paper on the possible relationship of boar odor to the C_{19}-Δ^{16}-steroids, Patterson (1968) reported the isolation and identification of 5α-androst-16-en-3-one in volatiles stripped from boar fat under high vacuum and at elevated temperatures (80° C). Gas chromatography of the complex mixture revealed many components, with the "taint" compound producing a very small response among the very last materials to be eluted. Patterson (1968) was able to identify the appropriate region of the chromatogram by smelling the column effluent after extinquishing the flame of the gas chromatographic detector. Collection of the effluent after repeated injections yielded enough material for mass spectrometric analysis. The mass spectrum suggested a molecular weight of 272, and an empirical formula of $C_{19}H_{28}O$.

Patterson (1968) then considered the ketosteroids of the androstane series, which have been described in the literature as possessing urine-like odors (Radt, 1959). The Steroid Reference Collection of the Medical Research Council of England was made available to Patterson (1968) so that he could perform olfactory tests of many steroids, most of which were not commercially available. Olfactory examination of a number of monoketoandrostanes and monoketoandrostenes revealed that several possessed a characteristic odor, which was identical in quality to the odor of the material isolated from boar fat. The strongest odors

were associated with steroids containing a keto group at position 3 on the steroid nucleus and a hydrogen atom on position 5, if present, in the α-orientation (Patterson, 1968). Chemical data and the "intense, urine-like odor" of 5α-androst-16-en-3-one as described by Radt (1959) prompted Patterson (1968) to further examine this compound. The compound was not available to him either commercially or from the Steroid Reference Collection and no published mass spectrum was available (Patterson, 1968). He made the steroid by oxidation of the corresponding alcohol, 5α-androst-16-en-3β-ol, which was available from the Medical Research Council Steroid Reference Collection. He found that the odor, gas chromatographic retention time and mass spectrum of 5α-androst-16-en-3-one agreed with that of the "taint" compound isolated from boar fat.

The findings of Patterson (1968) plus corroborative results by Beery and Sink (1971) and by Thompson *et al.* (1972) confirmed the involvement of C_{19}-Δ^{16}-steroids in boar odor as suggested by Sink (1967). Isolation of 5α-androst-16-en-3-one from the nonsaponifiable material of boar fat (Beery and Sink, 1971; Thompson *et al.*, 1972) supported the earlier studies by Craig and Pearson (1959), Craig *et al.* (1962) and Williams and Pearson (1965) showing that sex odor was associated with the fatty tissues, and more specifically with the nonsaponifiable material.

Odor Characteristics of C_{19}-Δ^{16}-Steroids

5α-androst-16-en-3-one has a pronounced perspirative, urine-like odor, which is characteristic of a number of androstane steroids of similar structure (Patterson, 1968). The olfactory properties of the C_{19}-Δ^{16}-steroids have been referred to in a number of papers (Kloek, 1961; Comfort, 1971a; 1971b; Beets, 1971) and have been reviewed recently by Katkov (1971).

The characteristic odors of this group of compounds have been useful in detecting the presence of very small quantities in tissues, plasma or urine extracts (Brooksbank and Haslewood, 1950; 1952). For example, 5α-androst-16-en-3-one was detected in boar fat extracts as it emerged from a gas chromatographic column (Patterson, 1968). Brooksbank and Haslewood (1950; 1952) were able to smell the musk-like odor of a compound (later identified as 5α-androst-16-en-3α-ol) in hydrolyzed urine extracts. Gower *et al.* (1970) described how the odor of 5α-androst-16-en-3-one could be detected on the hot syringe needle after injecting the plasma extract from the spermatic vein onto a gas chromatographic column.

The characteristic musk odor is not restricted to C_{19}-Δ^{16}-steroids. The 3-hydroxy-5α-androstanes also possess a musk-like odor (Prelog *et al.*, 1945), although the 5β-epimers are odorless. Of thirty-three steroids investigated by Beets (1962), the musk odor was detected in thirteen

derivatives of androstane.

Many ketosteroids of the androstane series are described in the literature as possessing urine-like odors (Radt, 1959). These compounds include 5α-androstan-3-one, 5α-androst-2-en-17-one, 5α-androst-3-en-17-one, 5α-androst-16-en-3-one and 5β-androst-16-en-3-one. The last compound was reported as having a distinct, but weaker urine-like odor than that of the 5α-epimer, which Radt (1959) has described as being intense.

Patterson (1968) reported that the odor of 5α-androstan-3-one was strong while that of the 5β-epimer was weak. He also stated that 4-androsten-3-one and 4,16-androstadien-3-one possessed strong odors, while 3,5-androstadien-17-one possessed a weak odor. He observed that some monoketoandrostanes possessed no odor. These included 5α-androstan-17-one, 5α-androst-2-en-17-one and 5α-androstan-11-one.

A unique aspect of sex odor is the variation in and possible sex-related ability to perceive this odor (Comfort, 1971a; Sink, 1973). To some consumers the sex odor is acceptable or even pleasant, while to others the slightest hint of this odor is enough to cause serious objection to or even complete rejection of food products containing the undesirable aroma (Sink, 1973). Several researchers (Patterson, 1968; Pearson et al., 1969; Sink, 1973) have noted that some people are consistently able to perceive small amounts of the odor, while others cannot detect it.

Griffiths and Patterson (1970) noted a sex-related difference in the ability to detect 5α-androst-16-en-3-one. Analysis of olfactory responses of fifty men and fifty women to a pure sample of 5α-androst-16-en-3-one showed that 92% of the women, but only 46% of the men could smell this steroid. For the men and women able to detect the odor, there was no significant difference between sexes in the individual threshold values. However, women found the odor significantly more unpleasant than did men, with most of the women subjects rating the odor as extremely unpleasant. On a scale of 1 to 9 (extremely pleasant to extremely unpleasant) the average score for women was 7.26 compared with 6.22 for men. This has important practical implications since women, rather than men, are most often involved in the preparation and cooking of pork or bacon and make decisions as to whether or not a product is acceptable.

A study by Klock (1961) of 100 men and 100 women revealed that 29% of the men could not smell 3α-hydroxy-5α-androst-16-ene, 38% described the odor as faint and 33% as strong. The corresponding percentages for women were 22% (no odor), 36% (faint odor) and 42% (strong odor).

Griffiths and Patterson (1970) recorded threshold values for 5α-androst-16-en-3-one in 30 individuals selected for their ability to smell the compound. Threshold values were determined for the dry residue of the pure compound that had been applied in ether solution to a 5 cm^2 area on a watch-glass. The values extended over a 2000-fold range of concentration

from 0.049 to 100.0 ng. These threshold values refer to the concentration on the watch-glass and not the concentration in the air, which obviously is very much lower. Griffiths and Patterson (1970) found for both sexes that the most sensitive subjects reported the odor to be more objectionable. On smelling the same concentrations of 5α-androst-16-en-3-one, women found the odor more unpleasant than did men having identical odor thresholds.

Occurrence of C_{19}-Δ^{16}-Steroids

The existence of 16-dehydro-C_{19} steroids in the pig (Prelog and Ruzicka, 1944) and in man (Brooksbank and Haslewood, 1950) has been recognized for many years, but very little is known of their biological significance (Brooksbank et al., 1972). The occurrence of C_{19}-Δ^{16}-steroids has been reviewed by Gower (1972) and more current information can be found in papers by Brophy and Gower (1972), Loke and Gower (1972), Saat et al. (1972), Brooksbank et al. (1972) and Gustafsson (1973). Comparative aspects of steroid metabolism in human and boar testes tissue was the subject of recent papers by Ruokonen (1973) and Ruokonen and Vihko (1974a,b).

C_{19}-Δ^{16}-Steroids in the Pig

The first report of C_{19}-Δ^{16}-steroids in the pig was by Prelog and Ruzicka (1944), who isolated relatively large amounts of 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol from boar testes. These same workers and subsequent investigators (Gower, 1972) noted that large quantities of the C_{19}-Δ^{16}-steroids occurred in pig testes, while the amount of androgenic steroids was comparatively much lower. Recently, Booth (1972), Claus et al. (1971) and Ruokonen and Vihko (1974a) have shown conclusively that the C_{19}-Δ^{16}-steroids are quantitatively more important than androgens in the pig. In fact, Ahmad and Gower (1968) demonstrated that the metabolism of pregnenolone and progesterone is biased in favor of the formation of C_{19}-Δ^{16}-steroids rather than androgens.

Claus et al. (1971) have shown that 5α-androst-16-en-3-one and testosterone increase with age, both in testis tissue and in peripheral blood plasma of boars. In testes, the concentration of 5α-androst-16-en-3-one was ten times higher than that of testosterone. The level of 5α-androst-16-en-3-one varied from 100 to 310 ng/g in comparison to values for testosterone of 6 to 37 ng/g testes (Claus et al., 1971). In peripheral blood plasma, the concentration of 5α-androst-16-en-3-one and testosterone was nearly the same, and increased directly with age in the boar from 6 to 22 ng/ml plasma (Claus et al., 1971). Plasma values in females (0.8 to 2.7 ng/ml) and in castrated males (1.3 to 2.7 ng/ml) were 9.5-

and 8-fold lower, respectively, than corresponding values of 6.0 to 22.3 ng/ml for boars (Claus et al., 1971).

Claus et al. (1971) found relatively high concentrations of 5α-androst-16-en-3-one in boar fatty tissue (1.03 to 7.49 μg/g) and in boar parotid salivary gland (0.17 to 11.43 μg/g). 5α-androst-16-en-3-one was not found in the fatty tissue or the parotid gland of females or castrate males, in contrast to the situation in peripheral blood where appreciable amounts of this compound were detected (Claus et al., 1971).

Ruokonen and Vihko (1974a,b) have also observed that C_{19}-Δ^{16}-steroids occupy a quantitatively important position in boar testis tissue. The principle compounds in the unconjugated fraction were 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol (Ruokonen and Vihko, 1974a). In the monosulfate fraction of boar testicular tissue, Ruokonen and Vihko (1974a) found 5,16-androstadien-3β-ol was also one of the most abundant steroids along with 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol. No disulfate conjugates were detected despite a careful search with a level of detection of 5 ng/g testis tissue. Ruokonen and Vihko (1974a) did not detect 5α-androst-16-en-3-one or testosterone in the testicular tissue of the boar with a level of detection of 5 ng/g. In contrast to this, Claus et al. (1971) reported that the concentration of 5α-androst-16-en-3-one in boar testis ranged from 100 to 310 ng/g, while the concentration of testosterone was 6 to 37 ng/g.

Analysis of boar urine (Gower and Katkov, 1969; Gower et al., 1970) has revealed the presence of 5α-androst-16-en-3β-ol, conjugated as glucosiduronates, at a level of approximately 250 μg/l. This contrasts with the situation in human urine where the predominant C_{19}-Δ^{16}-steroid is the corresponding 3α-ol isomer present as glucosiduronates (Brooksbank and Haslewood, 1950; 1952). The 3α-ol isomer possesses an intense odor, which is characteristic of human urine (Kloek, 1961) and explains the choice of the term "urine-like" frequently used to describe boar odor in pork. The term "perspiration-like" also appears to be an appropriate description for boar odor since it is now known that human sweat also contains 5α-androst-16-en-3-one, the steroid principally responsible for boar odor in pigs (Gower, 1972).

C_{19}-Δ^{16}-Steroids in the Human

Brooksbank (1962) found that relatively large quantities of the odorous compound 5α-androst-16-en-3α-ol are excreted in human urine, with excretion values of approximately 1 mg/day for men and 0.4 mg/day for women. Urinary 5α-androst-16-en-3α-ol excretion is very small in infants and children but increases at puberty to a maximum in the young adult. Thereafter, excretion decreases to lower values in old age (Cleveland and Savard, 1964; Brooksbank, 1962).

Brooksbank et al. (1969) isolated 4,16-androstadien-3-one from normal human blood plasma, and subsequently showed that its concentration was higher in the male than in the female (Brooksbank et al., 1972). The only other paper located which deals with C_{19}-Δ^{16}-steroids in human plasma was by Gower and Stern (1969), who reported the presence of 5α-androst-16-en-3α-ol in the peripheral plasma of a woman with a virilizing adrenocortical carcinoma. After adrenalectomy, the 5α-androst-16-en-3α-ol was no longer detectable. Although clearly related to the endocrine function of the testis, no specific function has yet been attributed to C_{19}-Δ^{16}-steroids in man (Brooksbank et al., 1972).

There is some evidence that the Δ^{16}-androstenes are precursors for 3,16,17-trihydroxy-androstanes. In this connection, Brooksbank et al. (1972) found four different isomers of 3,16,17-trihydroxy-androstanes in human urine after administration of radioactive 4,16-androstadien-3-one to a normal male and a normal female subject. Gustafsson (1973) showed that 4,16-androstadien-3-one, 5α-androst-16-en-3α-ol, 5α-androst-16-en-3β-ol and 5,16-androstadien-3β-ol were metabolized to 3,16β,17α-trihydroxylated steroids in liver microsomal preparations.

Biosynthesis of C_{19}-Δ^{16}-Steroids in the Boar

Boar testis tissue has been shown to convert pregnenolone and progesterone into C_{19}-Δ^{16}-steroids *in vitro* (Gower and Ahmad, 1967; Ahmad and Gower, 1968; Katkov and Gower, 1968; 1970). The conversion of pregnenolone and progesterone to androgen is small by comparison (Ahmad and Gower, 1968).

Katkov and Gower (1970) have shown that 5,16-androstadien-3β-ol is produced from pregnenolone by boar testis *in vitro*, and is then converted into 4,16-androstadien-3-one. 4,16-androstadien-3-one can be converted to the A-ring saturated alcohols (5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol) by way of 5α-androst-16-en-3-one as shown by Gower (1972) in Figure 1. 4,16-androstadien-3-one can also be formed directly from progesterone *in vitro*, furnishing an alternative pathway for formation of C_{19}-Δ^{16}-steroids as shown in Figure 1. The pathway by way of 5,16-androstadien-3β-ol has been reported by Saat *et al.* (1972) to be the predominant one in boar testes. Bicknell and Gower (1971) have reported similar results from experiments with human testes.

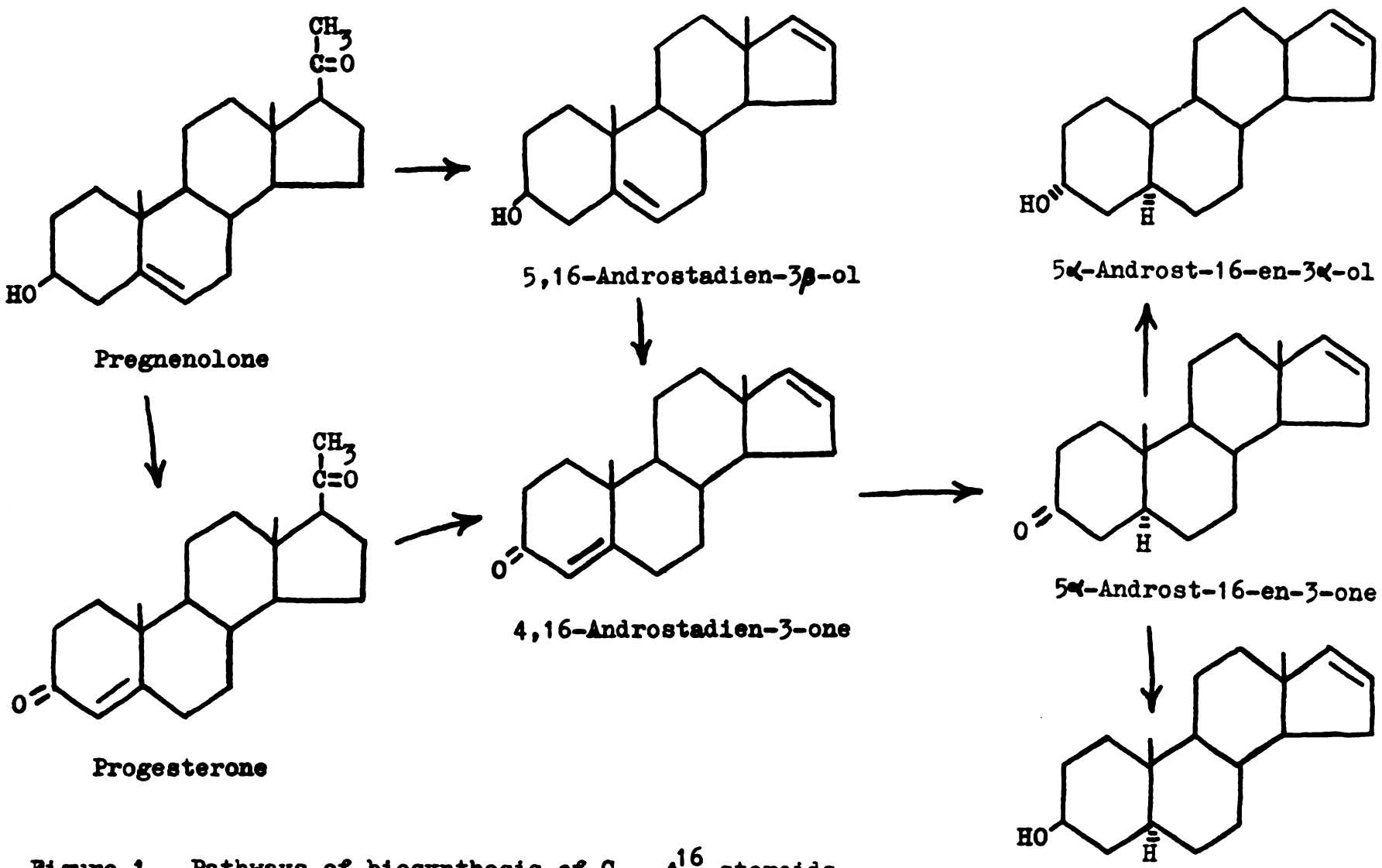


Figure 1. Pathways of biosynthesis of C_{19}-Δ^{16}-steroids in boar testis *in vitro*. Taken from Gower (1972).

A number of steroids have been tested as intermediates in the conversion of pregnenolone and progesterone to C_{19}-Δ^{16}-steroids, but none of them have been shown to be effective (Gower, 1972). The compounds tested included the following: testosterone, 3β-hydroxy-5-androstene-17-one, 4,16-pregnadiene-3,20-dione and 17α-hydroxy-4-pregnene-3,20-dione by Ahmad and Gower (1968); epitestosterone and 3β,17α-dihydroxy-5-pregnene-20-one by Gower and Ahmad (1967); 16α-hydroxy-4-pregnene-3,20-dione by Matsui and Fukushima (1970); and 5-androstene-3β-ol by Katkov and Gower (1970).

The 17α-hydroxyl-derivatives of pregnenolone and progesterone are generally recognized as intermediates in the formation of androgens in the testes (Eik-Nes, 1970). However, 17α-hydroxy-pregnenolone and 17α-hydroxy-progesterone are not converted to C_{19}-Δ^{16}-steroids in boar testes, suggesting that the C_{19}-Δ^{16}-steroids are produced from pregnenolone and progesterone by a unique pathway not requiring 17α-hydroxylation before side-chain cleavage (Gower, 1972)

Loke and Gower (1972) considered the possibility that other dihydroxylated C_{21}-steroids might be intermediates in C_{19}-Δ^{16}-steroid biosynthesis. They reported that radioactive 5-pregnene-3β,20β-diol was formed during the biosynthesis of 5,16-androstadiene-3β-ol in homogenates of boar testes. However, they could not exclude the possibility of the conversion of 5-pregnene-3β,20β-diol to pregnenolone before the formation of 5,16-androstadiene-3β-ol. Although Ruokonen and Vihko (1974a) did not detect 5-pregnene-3β,20β-diol in

boar testes, they did detect its monosulfated metabolite, 5α-pregnane-3β,20β-diol.

The enzyme system involved in the conversion of pregnenolone to 5,16-androstadiene-3β-ol in boar testes has been named "andien-β synthetase" by Loke and Gower (1971). Katkov and Gower (1970) have shown that the enzyme system requires NADPH and O_2 for activity. Gower and Loke (1971) studied the enzyme system in homogenates and subcellular fractions of boar testes and showed the activity to be mostly in the microsomal fraction.

Katkov et al. (1972) have shown that 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol are formed by reduction of 5α-androst-16-en-3-one in boar submaxillary glands. However, they found no evidence for the conversion of C_{21}-steroids into C_{19}-Δ^{16}-steroids in the submaxillary glands. Therefore, it seems likely that the C_{19}-Δ^{16}-steroids are formed in the testes and transported to the salivary glands where reduction of the ketone occurs. This could explain the occurrence of 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol in boar saliva (Gower, 1972).

The adrenal cortex in the pig has a limited capacity for C_{19}-Δ^{16}-steroid biosynthesis (Gower, 1972), which may explain the occurrence of boar odor in sows, barrows and gilts. It has been shown that 5α-androst-16-en-3α-ol, 5α-androst-16-en-3β-ol and 4,16-androstadiene-3-one are formed from pregnenolone and from progesterone in the adrenal cortex in vitro, but only in very small quantities compared with the testes (Gower and Ahmad, 1967; Ahmad and Gower, 1968).

Analysis of 5α-Androst-16-en-3-one in Adipose Tissue

Two techniques for determination of 5α-androst-16-en-3-one in adipose tissue have been published. The first technique, which was developed by Patterson (1969) and later used by Fuchs (1972) and Stinson (1972), utilizes vacuum distillation followed by saponification, and separation by thin layer chromatography and gas chromatography. The second technique, which was developed by Claus et al. (1971), involves the addition of a radioactive internal standard (5α-^{3}H-androst-16-en-3-one) to the sample followed by two separate column chromatographic steps, followed by thin layer and gas chromatography.

Distillation Procedure

Patterson (1969) used kidney fat instead of subcutaneous fat, since kidney fat contains less connective tissue and can be obtained without damaging the carcass. In this connection, Patterson (1969) reported that 5α-androst-16-en-3-one is present in similar concentrations in both kidney fat and subcutaneous fat, but gave no values to substantiate this claim.

Patterson (1969) used samples of kidney fat (150 g), which were chilled, cut into small pieces, minced and then

melted at 55°C. The liquified fat was filtered through butter muslin using a warm Büchner filter funnel connected to an aspirator. The fat was then dried with anhydrous sodium sulfate and filtered through Whatman No. 1 filter paper under vacuum to give a clear oil.

For vacuum distillation, Patterson (1969) poured a 44 g sample of the filtered fat into the lower section of a "pot-still". The upper half of the "pot-still" consisted of a cold finger containing liquid nitrogen. When the two sections were joined together with an O-ring they formed an efficient, short-path, molecular still in which the distance from the fat surface to the condensing surface was only a few centimeters. The fat was distilled with stirring for 6 hours at 1 mm vacuum, while the base of the still was immersed in water at 80°C.

Patterson (1969) removed the distillate from the surface of the cold finger by rinsing with small volumes of ethyl ether. These solutions were then combined and concentrated in vacuo. The residue was saponified for 15 minutes with 0.1 N aqueous NaOH at room temperature. Then the solution was poured into 90 ml of distilled water and the nonsaponifiable material was extracted with two-30 ml volumes of diethyl ether-hexane (3:17). The combined extracts were dried with anhydrous sodium sulfate and concentrated to 250 ul. A 50 ul aliquot was purified by thin layer chromatography and the appropriate zone was recovered for quantitative

analysis of 5α-androst-16-en-3-one by gas chromatography. Patterson (1969) performed gas chromatographic analysis by comparing peak heights obtained with 10 ul injections of the fraction containing 5α-androst-16-en-3-one with standards for this compound. Internal standards were not used during either the isolation procedure or the gas chromatographic analysis. The efficiency of the overall procedure was determined by checking recoveries of known quantities of 5α-androst-16-en-3-one added to fat, which had been previously distilled to remove this compound.

Chromatographic Technique

Claus et al. (1971) used a chromatographic method in which fatty tissue (5 g) of an unspecified type was cut into small pieces. After addition of 0.148 μC of tritiated 5α-androst-16-en-3-one as an internal standard, the sample was homogenized in 24 ml 0.1 N NaOH. The homogenate was increased to a total volume of 50 ml by the addition of another 25 ml portion of 0.1 N NaOH. It was then extracted with 250 ml of dichloromethane-ethyl acetate (1:1) in a 500 ml separatory funnel. The extract was washed with 50 ml of distilled water, dried over anhydrous sodium sulfate and evaporated to dryness in vacuo.

Claus et al. (1971) dissolved the residue in 2 ml cyclohexane and applied it to the first silica gel column (1.5 cm x 30 cm), after which it was eluted with cyclohexane-

ethyl acetate (98:2). The fractions containing radioactivity were pooled, dried and applied to the second silica gel column, which differed from the first column only by being smaller in size (1 cm X 28 cm). The radioactive fractions were pooled, dried and further purified by chromatography on silica gel thin layer plates developed in benzene-ethyl acetate (99:1). The thin layer zone, which contained the radioactivity, was removed and eluted with 5 ml of ethyl acetate. An aliquot was withdrawn for calculation of the recovery by counting its radioactivity. The remainder of the sample was used for analysis by gas chromatography using 4-androstene-3,17-dione as a chromatographic internal standard. Recovery of the radioactive 5α-androst-16-en-3-one was reported to be 50-60% when measured just prior to the gas chromatographic analysis.

Boar Odor Intensity and 5α-Androst-16-en-3-one Content

Fuchs (1972) and Newell *et al.* (1973) reported positive correlations between the concentration of 5α-androst-16-en-3-one in pork fat and the odor scores determined by a panel of experts, selected for their ability to smell 5α-androst-16-en-3-one and trained to distinguish its odor from other odors associated with heated pork fat. Both Fuchs (1972) and Newell *et al.* (1973) used the vacuum distillation-gas

chromatographic technique of Patterson (1969) to determine the concentration of 5α-androst-16-en-3-one. Newell et al. (1973) reported that the correlation coefficient between odor intensity and the quantity of 5α-androst-16-en-3-one was 0.53. Fuchs (1972) evaluated 19 samples and reported a correlation coefficient of 0.75.

Fuchs (1972) excluded the three samples with the highest concentration of 5α-androst-16-en-3-one (5.3, 5.8 and 8.0 μg/g fat) from his regression calculations. He reported that the odor intensity reached a maximum value at about 2-4 μg/g fat, after which there was no incremental increase in odor with increasing concentrations of 5α-androst-16-en-3-one. The concentration of 5α-androst-16-en-3-one ranged from 0.3 to 8.0 μg/g fat.

Newell et al. (1973) showed that odor scores from an untrained panel did not correlate significantly with the quantity of 5α-androst-16-en-3-one in the fat. Members of the untrained panel were able to detect the odor of 5α-androst-16-en-3-one, but they were not trained to specifically distinguish this odor from other odors that are apparent when pork fat is heated.

EXPERIMENTAL

Materials

Chemicals

The common reagents were reagent grade. Special reagents are listed below:

Aluminum isopropoxide, electronic grade	
Chloroiridic acid ($H_2IrCl_6 \cdot 6H_2O$)	Ventron Corporation Alfa Products P.O.Box 159 Beverly, Mass.
Lithium wire, packed under mineral oil	
Methyllithium, 2M in ether	
Lithium aluminum hydride	Chemicals Procurement Laboratories, Inc. 18-17 130th Street College Point, N.Y.
p-toluenesulfonylhydrazine	
Ammonia-d_3	Merck and Company, Inc. Isotopes 111 Central Avenue Teterboro, N.J.
Deuterium oxide, D_2O	
Lithium aluminum deuteride	
Methyl alcohol-d	
Trimethylphosphite, 97%	Aldrich Chemical Co. 940 West St. Paul Ave. Milwaukee, Wisconsin
3β-Hydroxy-5-androsten-17-one	

3α-Hydroxy-5α-androstan-17-one	Sigma Chemical Co. P.O.Box 14508 St. Louis, Mo.
3β-Hydroxy-5α-androstan-17-one	

5α-Androst-16-en-3-one	104 mg	Gifts from The Upjohn Co. Kalamazoo, Michigan
5α-Androst-16-en-3α-ol	22 mg	
5α-Androst-16-en-3β-ol	50 mg	
4,16-Androstadien-3-one	100 mg	
5,16-Androstadien-3β-ol	50 mg	

Solvents

General solvents	All solvents were redistilled before use
Benzene, acetone, cyclohexane, ethyl acetate, ethyl ether, hexane methanol and ethanol - "Distilled in Glass"	Burdick and Jackson Laboratories, Inc. Muskegon, Michigan

Chromatography Supplies

Silica Gel H	Brinkmann Instruments, Inc. EM Reagents Division Westbury, N.Y.
Thin layer plates, Type PLQF "pre-absorption silica gel, 1 mm thickness, 20 × 20 cm	Quantum Industries 341 Kaplan Drive Fairfield, N.J.
Activated Alumina, F-20	Alcoa Chemicals
Gas chromatography packing materials 3% and 5% OV-1 on Supelcoport, 100/120 mesh	Supelco Incorporated Bellefonte, Pa.
5% SP-2401 on Supelcoport, 100/120 mesh	

Fat Samples from Cryptorchid Pigs

The objective of this project was to determine the concentration of 5α-androst-16-en-3-one in back fat from cryptorchid pigs that had previously been evaluated for boar odor by employees of two meat packing companies. Each company was to supply 40 back fat samples (5 lbs each-free of skin) that had been evaluated for boar odor by procedures used under normal operating conditions. The fat was removed from the carcasses of cryptorchid pigs in the cooler and evaluated in the quality control laboratory by smelling the odors produced upon touching the fat with a hot iron. The samples were frozen, packaged in dry ice and shipped to East Lansing by air freight. Upon arrival in East Lansing the samples were coded with numbers taken from a table of five-digit random numbers and stored in a freezer at -20°C.

The samples were supposed to include a representive number in each of the following categories: no boar odor, slight boar odor, strong boar odor and pronounced boar odor. Although packer A provided a total of 40 samples, only 23 were evaluated according to the above system. The 23 samples were classified as: none-3, slight-8 and pronounced-12. The other 17 samples were rated by other descriptive terms such as very slight-3, slightly less than pronounced-1, slightly pronounced-7, moderate-4, moderately intense-1 and

and very pronounced-1. Samples from packer B arrived nine months late and included only samples described as having no odor-17 and slight odor-25. No strong or pronounced samples were sent because they reported that none were found.

Purification and Quantitative Determination of 5α-Androst-16-en-3-one in Fat Samples

The level of 5α-androst-16-en-3-one in samples of fat was determined by a reverse isotope dilution/carrier technique (Sweeley et al., 1966; Samuelsson et al., 1970; Bieber et al., 1972; Gordon and Frigerio, 1972). A known amount of stable isotope labeled steroid (usually 21 μg of 6,6-d_2-5α-androst-16-en-3-one) was added to the sample to serve as a carrier and as an internal standard. The mixture of protium and deuterium form of 5α-androst-16-en-3-one was isolated and the ratio of protium form and deuterium form was found by combined gas chromatography-mass spectrometry. The amount of 5α-androst-16-en-3-one in the original mixture was calculated from the measured ratio and the known amount of deuteriated material added.

Preparation of Fat Samples

Fat samples were prepared as described by Patterson (1968,1969). A 150 g portion of fat was homogenized in a

Waring blender and the liquified fat was then warmed to 55°C. The warm fat was filtered through four layers of cheese cloth using a warm Büchner filter funnel connected to an aspirator. The liquified fat was dried by mixing with anhydrous sodium sulfate for 30 minutes and then filtering through Whatman #1 filter paper on a warm Büchner filter funnel connected to an aspirator. The drying procedure was repeated for each sample until a clear oil was obtained.

Saponification of Fat Samples

Fat was saponified according to a procedure recommended for assay of vitamin E (Bunnel, 1967). A 2 g fat sample was transferred to a 100 ml Erlenmeyer flask containing 15 ml ethanol, 100 mg pyrogallol and a few boiling chips. The flask was connected to a reflux condenser and the solvent refluxed for 5 minutes to displace air from the flask. Approximately 1 g of potassium hydroxide pellets was added through the reflux condensor and the refluxing continued for 30 minutes.

The flask was cooled and the contents transferred to a separatory funnel which contained 15 ml of hexane and 15 ml of water. The saponification mixture was shaken for 1 minute. The extraction was repeated twice with 8 ml of hexane. The combined extracts were washed 5 times with water followed by drying over anhydrous sodium sulfate. The hexane extract was evaporated to dryness and the residue was redissolved in 250 ul of ethyl acetate.

Treatment of Fat with Lithium Aluminum Hydride

De Luca (1974) recommended treatment with lithium aluminum hydride ($LiAlH_4$) as an alternative to saponification for fractionation of pork fat samples. The sample (usually 2 g, prepared as described above) was dissolved in 40 ml of tetrahydrofuran in a 50 ml Erlenmeyer flask fitted with a ground glass stopper. The mixture was dried over anhydrous sodium sulfate for 4 hours, and then the sodium sulfate was removed by filtration. The solution was transferred to a 50 ml conical shaped centrifuge tube. Approximately 0.32 g of $LiAlH_4$ was added slowly, and the reaction mixture was held at room temperature for 72 hours with occasional stirring.

The excess $LiAlH_4$ was removed by centrifugation and decanting. The supernatant was transferred to a 250 ml separatory funnel and the excess $LiAlH_4$ was destroyed by dropwise addition of ethanol. The solution was washed 3 times with 60 ml of 0.1N HCl and 3 times with 60 ml of water. After drying over sodium sulfate, the solution was evaporated to dryness and the residue was weighed.

Urea Inclusion Compounds

The residue (usually 1.7 g) obtained after $LiAlH_4$ treatment of fat samples was dissolved in methanol (30 ml/g) previously saturated with urea at room temperature (Mareno and Roncero, 1964). After 2 hours the crystals were separated

from the solution by filtration. The filtrate was transferred to a 250 ml separatory funnel containing 100 ml of water and the solution was extracted 3 times with 60 ml of ethyl ether. The ether extracts were combined and washed 3 times with 60 ml of water. After sodium sulfate drying, the solution was evaporated to dryness and the residue was weighed.

The included compounds were freed by treatment with water. The crystals were dissolved in 150 ml of water and the solution was extracted 3 times with 60 ml of ether. The combined extracts were washed 3 times with 60 ml of water, dried over sodium sulfate and evaporated to dryness.

Vacuum Distillation

The analysis of 5α-androst-16-en-3-one involving vacuum distillation was performed essentially as described by Patterson (1969). For vacuum distillation, a 55 g sample of filtered and dried fat was poured into the bottom of a high capacity vacuum sublimator (K-855700, Kontes Glass Company). A Teflon-coated stirring bar was added to the container. The flask and condenser were sealed without grease using an "O"-ring, and the assembled unit was connected to a vacuum manifold by a ball and socket joint. The base of the sublimator was submerged in a glycerol bath that was resting on a magnetic stirrer. The temperature of the glycerol bath was controlled within narrow limits by a circulating heater (HAAKE, Model FJ).

It was determined that a bath temperature of 87°C gave the desired 80°C temperature in the fat during vacuum distillation.

Liquid nitrogen was added to the condenser section of the sublimator and also to another trap interposed between the manifold and the mechanical vacuum pump (Cenco, HYVAC 14). The stopcock on the sublimator was opened and the sublimator-manifold unit was evacuated very slowly by gradually opening a small precision vacuum valve (K-82505, Kontes Glass Company). The speed of stirring was carefully controlled to avoid splattering of the fat during the initial phase of evacuation, after which the speed was adjusted so that the stirring bar revolved at approximately one revolution per second. A large bore vacuum valve was opened as soon as it was apparent that no splattering would occur and the system was thoroughly degassed.

The pumping system included an oil diffusion pump (Cenco, #93423), which was connected to the system as soon as the pressure dropped to 1 mm Hg. The mechanical pump was used to produce the required vacuum at the outlet of the oil diffusion pump. The distillation was continued for 6 hours after the pressure first dropped to 10^{-3} mm Hg. The glycerin bath was maintained at 87°C. The liquid nitrogen traps were resupplied with refrigerant throughout the 6 hour distillation period.

At the conclusion of the distillation period the stopcock on the sublimator was closed, and the sublimator, while still under high vacuum, was removed from the manifold and glycerin bath. The sublimator remained at room temperature and high vacuum until opened for analysis of the distillate. Then air was slowly readmitted and the sublimator was disassembled. The distillate on the condenser was removed by rinsing the glass surface with four 5 ml volumes of ethanol and four 5 ml volumes ethyl ether. The combined solvents were removed on a rotary vacuum evaporator. The residue was dissolved in 10 ml of 0.1N KOH and saponified for 15 minutes at room temperature. The solution was poured into 90 ml of distilled water and the nonsaponifiable material was extracted with three 30 ml volumes of ether-hexane (3:17). The combined extracts were dried over sodium sulfate, transferred to a 5 ml Pierce Reactivial (Pierce Chemical Company) and concentrated to 0.5 ml under a stream of nitrogen gas.

Thin Layer Chromatography

Preparative thin layer chromatography was performed on "pre-absorption" silica gel thin layer plates (Type PLQF, 1 mm thickness, 20 × 20 cm, Quantum Industries). The sample was applied to the center of the plate. A solution of authentic C_{19}-Δ^{16}-steroids was applied to the edges of the plate to serve as a marker. The plate was developed in benzene-ethyl acetate (95:5) in a tank under saturated conditions.

Following development, the center of the plate was covered with glass to protect the sample. Then the sides of the plate were sprayed with a sulfuric-acetic acid mixture (1:1) to visualize the C_{19}-Δ^{16}-steroid standard. The appropriate region of the plate was removed and placed in a small fritted glass filter funnel (#36060, 2ml, medium porosity, Corning Glass Works). The sample was recovered from the silica gel by a 1 hour continuous elution with four 1 ml volumes of chloroform-methanol (2:1) as recommended by VandenHeuvel (1967). The solution was collected in a 5 ml Pierce Reactivial (Pierce Chemical Company) and evaporated to dryness with a stream of nitrogen gas. The residue was redissolved in 50 ul of ethyl acetate and stored in a desiccator at -10°C.

Liquid Column Chromatography

Newly synthesized C_{19}-Δ^{16}-steroids were purified by chromatography on Silica Gel H. The glass column, 110 cm X 2 cm (i.d.), had a solvent reservoir at the top and a sintered glass disk and Teflon stopcock at the bottom. Two kinds of column material were used: (1) a 20 g column of Silica Gel H for separations of steroids differing in the number and position of hydroxyl and carbonyl groups; and (2) a 30 g column of $AgNO_3$-impregnated Silica Gel H for separation of steroids differing in the number of carbon-carbon double bonds. Silica Gel H was used without further preparation as received from the supplier. The $AgNO_3$-impregnated Silica Gel H was

prepared by the method of Katkov and Gower (1970) and stored in the dark in a desiccator over phosphorous pentoxide.

Silica Gel H Column. This procedure was adapted from that of Claus et al. (1971). The column was filled to a height of 14 cm with 20 g of Silica Gel H suspended in hexane. The column was prewashed with 100 ml of benzene. The sample was dissolved in a minimum amount of benzene (usually 2 ml) and applied to the column. Elution was carried out with benzene-ethyl acetate (9:1,v/v) and the eluate was collected in 10 ml fractions. Nitrogen pressure was applied to achieve a flow rate of 1 ml/minute. Each fraction was examined by gas chromatography using a 5% SP-2401 column at 220°C. 5α-Androst-16-en-3α-ol was eluted in fractions 9 and 10 and 5α-androst-16-en-3β-ol was eluted in fractions 12,13,14 and 15.

$AgNO_3$-Impregnated Silica Gel Column. The procedure of Katkov and Gower (1970) was used with only minor modifications. The column was prepared as follows: the column was filled with hexane and 4 g of alumina was poured into it followed by 30 g of the impregnated gel. The alumina layer served as an adsorbent for any $AgNO_3$ displaced from the upper layer (Katkov and Gower, 1970). The column was prewashed with 100 ml of benzene. The sample was applied in a benzene solution (2 ml) and eluted with benzene-ethyl acetate (3:1,v/v). Nitrogen pressure was applied to achieve a flow rate of 1 ml/minute. Gas chromatographic analysis of each 10 ml

fraction was performed as described above. Fractions 12 through 19 contained 5α-androst-16-en-3-one and fractions 23 through 33 contained 4,16-androstadien-3-one.

Gas Chromatography

Gas chromatographic analysis was done with a Beckman GC-4 instrument with a hydrogen flame detector using a 6 ft × 2 mm (i.d.) glass column packed with 5% SP-2401, 3% OV-1 or 5% OV-1 on 100/120 mesh Supelcoport. The column had been previously cleaned, silanized and packed with suction by the procedure recommended by Horning et al. (1967). Helium carrier gas flow was adjusted to 22 ml/minute. Hydrogen and oxygen flows were set at 60 ml/minute and 300 ml/minute, respectively. Additional helium (carrier-makeup gas) was introduced into the hydrogen ahead of the detector at 45 ml/minute to improve the signal to noise ratio of the detector. The samples, dissolved in ethyl acetate, were injected directly onto the column. Authentic C_{19}-Δ^{16}-steroids were used as standards in the analysis.

Gas Chromatography-Mass Spectrometry (GC-MS)

Combined GC-MS was carried out on an LKB-9000, interfaced to a dedicated minicomputer (PDP-8/I, Digital Equipment Company) for data acquisition and reduction (Sweeley et al., 1970). Data was displayed on a Tektronic Model 4002A storage scope with keyboard terminal and a Tektronic Model 4601 hard copy unit.

The coiled glass GC column was 6 ft in length by 2 mm (i.d.) and was packed with 5% SP-2401 or 3% OV-1 on 100/120 mesh Supelcoport. Mass spectral measurements were recorded at 70 eV ionizing energy with full accelerating voltage of 3.5 kV and 60 uA trap current.

The level of steroid in GC effluents was determined by mass spectrometric selected ion monitoring with the aid of computer-control of fine focus, data acquisition, reduction and display (Holland et al., 1973). The ratio of protium and deuterium forms of 5α-androst-16-en-3-one was determined by continuous recording of the intensities of the molecular weight ions. The lower mass of the pair was first focused by manual adjustment of the magnetic field strength. The higher mass was then focused by lowering the accelerating voltage. The areas and heights of each ion peak was determined by the computer and the ratio of protium and deuterium forms was determined. A blank ratio of these ions for the pure reference deuterium form was obtained and this was subtracted from the isotopic abundance ratios obtained for the samples.

Synthesis of C_{19}-Δ^{16}-Steroids

6,6-d_2-5α-Androst-16-en-3-one

The activated hydrogens in 4,16-androstadiene-3-one were exchanged for deuteriums by equilibration in alkaline deuteriomethanol/deuterium oxide. The exchange procedure was adapted from a procedure used by Djerassi and Tökés (1966) for deuteriation of 5α-pregn-9-en-12-one. A solution of 25 mg of 4,16-androstadiene-3-one in 5 ml of deuteriomethanol was saturated with 20% sodium deuterioxide in deuterium oxide and heated under reflux for 3 days. A few drops of deuteriomethanol were added whenever the solution became turbid due to supersaturation. The refluxing solution was protected from air and moisture by maintaining the system under an atmosphere of nitrogen. After cooling, the solution was diluted with 10 ml of dry ethyl ether and washed with two 5 ml volumes of deuterium oxide in a dry 60 ml separatory funnel. The ether solution was dried over anhydrous sodium sulfate. The solvent was evaporated on a rotary vacuum evaporator and the dry residue was stored in a dessicator over phosphorous pentoxide.

The product, 2,2,4,6,6-d_5-4,16-androstadiene-3-one, was converted to 2,2,4,6,6-d_5-5α-androst-16-en-3-one by lithium-ammonia reduction (Dryden, 1972). Approximately 3 ml of ammonia was transferred into a 10 ml flask fitted with a

dewar condenser (9253, Ace Glass Incorp.) and a side arm pressure equalizing addition funnel (9494T, Ace Glass Incorp.). The condenser was refrigerated with dry ice/isopropanol.

Approximately 2 mg of clean lithium wire was added to the liquid ammonia and an intense blue color appeared in the solution as the lithium dissolved. Color persistence for at least 5 minutes indicated that the ammonia was suitable for use. Rapid disappearance of the color indicated that the ammonia was contaminated with colloidal iron and should not be used (Campbell, 1972).

Approximately 25 mg of the deuteriated steroid in 2 ml of tetrahydrofuran (freshly distilled from $LiAlH_4$) was added to the lithium/ammonia solution in a slow stream. The mixture was refluxed with stirring for 1/2 hour and then the reaction was stopped by the addition of 37 mg of anhydrous ammonium chloride. Approximately 6 ml of ethyl ether was added to the reaction flask and the ammonia was allowed to evaporate. The product was extracted from the reaction vessel with three 5 ml volumes of ethyl ether. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated on a rotary vacuum evaporator.

The product, 2,2,4,6,6-d_5-5α-androst-16-en-3-one, was dissolved in 10 ml of methanol-water (9:1) and refluxed for 12 hours to remove deuteriums in the α-position to the ketone. The solution was diluted with 15 ml of water and extracted with three 15 ml volumes of ethyl ether. The combined extracts

were dried over anhydrous sodium sulfate and evaporated to dryness on a rotary vacuum evaporator. The residue was redissolved in methanol-water and the back-exchange was repeated to insure removal of all the exchangeable deuteriums. The steroid was reextracted and dried in the same way. The purity of the compound was established by thin layer and gas chromatography and mass spectrometry. Attempts to crystallize the product were not successful. The yield of 6,6-d_2-5α-androst-16-en-3-one was estimated by gas chromatography to be 10 mg, resulting in an overall yield of 40%.

5,6,6-d_3-5α-Androst-16-en-3-one

This material was prepared in the same way as 6,6-d_2-5α-androst-16-en-3-one except that deuterioammonia was used in the lithium-ammonia reduction.

The deuterium exchange of 4,16-androstadiene-3-one was achieved by refluxing 87 mg of the steroid in 15 ml of deuteriomethanol saturated with 20% sodium deuterioxide in deuterium oxide. The refluxing solution was protected from air and moisture by maintaining the system under an atmosphere of nitrogen. Refluxing was begun before the addition of sodium deuterioxide in deuterium oxide because it was found that serious degradation occurred in the previous exchange when the alkali was added prior to thorough "degassing" of the steroid solution. After 1 hour of refluxing, the 20% sodium deuterioxide in deuterium oxide was added dropwise until turbidity indicated that the solution was saturated.

The reflux was continued for 3 days with periodical additions of deuteriomethanol (9 ml total) as needed to maintain the volume of solution.

After cooling, the solution was diluted with 15 ml of deuterium oxide and the steroid was extracted with two 15 ml volumes of ethyl ether. The combined ether extracts were dried over sodium sulfate and the solvent was removed under vacuum. The residue of 2,2,4,6,6-d_5-4,16-androstadiene-3-one was stored in a desiccator over phosphorous pentoxide.

The deuteriated Δ^4-3-keto steroid was converted to the corresponding 3-keto steroid by reduction in a solution of lithium in deuterioammonia. The reaction was carried out in a 25 ml triple-neck flask fitted with a pressure equalizing addition funnel (9494T, Ace Glass Incorp.) and a small dewar condenser (9253, Ace Glass Incorp.) joined to a drying tube. All of the glassware was previously dried for 24 hours at 110°C and 30 inches of vacuum. The condenser was filled with dry ice/isopropyl alcohol, and a small Teflon coated magnetic stirring bar was added to the flask. The bottom of the flask was immersed in a second dry ice/isopropanol bath to provide more condensing surfaces during the addition of the deuterioammonia to the reaction apparatus.

Approximately 4 ml of deuterioammonia and 7 mg of clean lithium wire were added to the flask. An intense blue color developed in the solution when the lithium dissolved but the color did not persist for more than a few

minutes. The stubborn disappearance of the color indicated that the lithium was being consumed by impurities in the deuterioammonia (Campbell, 1972). This situation necessitated the addition of several 7 mg portions of lithium wire before the blue color could be maintained in the deuterioammonia solution.

A solution of approximately 87 mg of the 2,2,4,6,6-d_5-4,16-androstadiene-3-one in 6 ml of tetrahydrofuran (freshly distilled from $LiAlH_4$) was slowly added with stirring to the lithium/deuterioammonia solution using the addition funnel.

After a 30 minute refluxing period the reaction was stopped by the careful addition of 130 mg of ammonium chloride (previously dried at 110°C and 30 inches of vacuum for 2 hours). The flask was removed from the refrigerant bath and 10 ml of ethyl ether was added to the flask. The deuterioammonia was allowed to evaporate at room temperature.

The flask was rinsed several times with ethyl ether and the combined ether extracts were washed with water in a 60 ml separatory funnel. The ether solution was dried over sodium sulfate and the solvent was removed in vacuum.

The product, 2,2,4,5,6,6-d_6-5α-androst-16-en-3-one, was back exchanged to remove α-deuteriums by refluxing for 2 hours in 25 ml of methanol and 10 ml of water containing 0.2 mg of sodium hydroxide. The steroid material was recovered by extraction with ethyl ether. The combined extracts were washed with water to remove the alkali, dried over sodium sulfate and evaporated to dryness under vacuum.

The product was a mixture of 36 mg of 5,6,6-d_3-5α-androst-16-en-3-one and 36 mg of 2,2,4,5,6,6-d_6-5α-androst-16-en-3β-ol. These two deuteriated steroids were purified by preparative thin layer chromatography. The purity of the 5,6,6-d_3-5α-androst-16-en-3-one was established by gas chromatography and mass spectrometry.

5,16-Androstadiene-3β-ol

5,16-Androstadien-3β-ol was prepared according to a procedure published by Matthews (1968) and Matthews and Hassner (1972). The procedure involved methyllithium reduction of the toluenesulfonylhydrazone derivative of the corresponding 17-keto steroid.

3β-hydroxy-5-androstene-17-one (12.2 g) and p-toluenesulfonylhydrazine (10.2 g) were dissolved in 212 ml of hot methanol and refluxed for 16 hours. The reaction mixture was concentrated on a steam bath to a volume of approximately 170 ml and cooled to room temperature. The crystallized steroid hydrazone was recovered by filtration, washed with 40 ml of methanol and dried in a vacuum oven at 65°C for 12 hours. The reaction yielded 16.7 g of 3β-hydroxy-5-androstene-17-one tosylhydrazone.

The hydrazone (16.7 g) was dissolved in 628 ml of 1,2-dimethoxyethane (freshly redistilled from $LiAlH_4$) in a 1 l flask fitted with a 250 ml addition funnel, a drying tube and a magnetic stirring bar. A 2.05 M ether solution of

methyllithium (30 ml) was transferred to the addition funnel using appropriate precautions to avoid an explosion or fire that can occur when methyllithium is exposed to a moist atmosphere. The methyllithium/ether solution was transferred to the addition funnel using a 30 cc syringe fitted with a needle sufficiently long to withdraw the liquid from the reagent bottle through a small hole in the cap. The solution was withdrawn from a cold bottle which contained a precipitate in order to avoid the mineral oil impurity present in the reagent (Matthews, 1968).

The methyllithium solution was added to the hydrazone solution over a 60 minute period. The highly colored reaction mixture was stirred for 7 hours before it was stirred into 1250 ml of ice water. The precipitated material was digested for 12 hours on a warm steam bath to aid its removal by filtration. The filter cake was washed with water and dried under vacuum at 50°C for several hours.

The product was recrystallized from ethanol-water to give a first crop of 4.5 g and a second crop of 2.8 g of 5,16-androstadiene-3β-ol, a total yield of 61%. The purity of the product was established by thin layer and gas chromatography and mass spectrometry.

17-d_1-5α-Androst-16-en-3α-ol

The procedure was adapted from one described by Matthews (1968) and Matthews and Hassner (1972). 3α-hydroxy-5α-androstane-

17-one (14.4 g) and p-toluenesulfonylhydrazine (12.2 g) were refluxed together in 250 ml of methanol for 12 hours. The solution was concentrated on a steam bath to a volume of approximately 180 ml and cooled to room temperature to promote crystallization of the hydrazone. The hydrazone was recovered by filtration, washed with 50 ml of methanol and dried in a vacuum oven at 65°C for several days. The yield of tosylhydrazone was 18.9 g.

The tosylhydrazone derivative was reduced with methyllithium as previously described with the exception that deuterium oxide (100 ml) was added slowly to the reaction mixture just before it was stirred into the ice water. The resulting material was digested on a warm steam bath for 24 hours before the agglomerated precipitate was filtered, washed with water and dried in a vacuum oven for 3 hours at 45°C. Recrystallization from ethanol-water gave 5.3 g of 17-d_1-5α-androst-16-en-3α-ol. The purity of the product was established by thin layer and gas chromatography and mass spectrometry.

16,17-d_2-5,16-Androstadien-3β-ol

The hydrogen atoms at C-16 of 3β-hydroxy-5-androsten-17-one (12.2 g) were exchanged for deuterium atoms by refluxing for 16 days in 212 ml of deuteriomethanol. p-Toluenesulfonylhydrazine (10.2 g) was added to the solution and the tosylhydrazone derivative of the steroid was formed by refluxing

for an additional 16 hours in the deuteriomethanol solution. The volume of solution was reduced by approximately 20% by directing a stream of nitrogen into the warm flask. Crystallization of the tosylhydrazone occurred as the solution cooled. The crystals were removed by filtration, washed with 40 ml of methanol and dried under vacuum at 65°C for 12 hours. The reaction yielded 16.6 g of steroid hydrazone.

The hydrazone was dissolved in 730 ml of 1,2-dimethoxyethane and treated with methyllithium as previously described. The highly colored reaction mixture was stirred for 7 hours. Then deuterium oxide (150 ml) was added slowly using an addition funnel and the mixture was stirred for an additional 6 hours. The reaction mixture was stirred into 900 ml of water and the resulting mixture was digested on a warm steam bath for 12 hours to aid the recovery of the product by filtration. The filter cake was washed with water and then dried in a vacuum oven at 50°C for 5 hours. Recrystallization from ethanol-water yielded 7.2 g of 16,17-d_2-5,16-androstadien-3β-ol. The purity of the product was established by thin layer and gas chromatography and mass spectrometry.

16,17-d_2-4,16-Androstadien-3-one

This compound was prepared by Oppenauer oxidation of 16,17-d_2-5,16-androstadien-3β-ol using a procedure described by Matthews (1968). A solution of 6 g of 16,17-d_2-5,16-androstadien-3β-ol in 300 ml of toluene and 47 ml

of cyclohexanone was distilled for 30 minutes to eliminate traces of moisture. The distillate (approximately 10 ml) was removed from the apparatus using a Stark-Dean moisture receiver.

A solution of 3.1 g of aluminum isopropoxide in 30 ml of toluene was added to the mixture and the combined solution was refluxed for 2 hours. Water (80 ml) was added and the volatile components were removed by 4 hours of steam distillation. The residue was extracted with 500 ml of chloroform and then with 300 ml of dichloromethane. The combined extracts were washed with water, dried over anhydrous sodium sulfate and concentrated to 25 ml under vacuum. Hexane (100 ml) was added to the concentrate and the solution was further concentrated until crystallization was imminent. The crystals were recovered by filtration and rinsed with a minimum amount of hexane before being dried for 6 hours in a vacuum oven at 35°C. The yield of the reaction was 2.9 g of 16,17-d_2-4,16-androstadiene-3-one, the purity of which was established by thin layer and gas chromatography and mass spectrometry.

6,6,16,17-d_4-5α-Androst-16-en-3-one

16,17-d_2-4,16-Androstadiene-3-one (previously dried for 24 hours at 50° C over P_2O_5) was converted to 2,2,4,6,6,16,17-d_7-4,16-androstadiene-3-one by refluxing 2 g

of the steroid for 4 days in 110 ml of deuteriomethanol containing 10 drops of 20% sodium deuterioxide in deuterium oxide. At the conclusion of this equilibration period the perdeuterio-steroid was recovered from the deuteriomethanol solution by crystallization brought about by the addition of deuterium oxide. The crystallized steroid was recovered by filtration and washed with a small amount of deuterium oxide. This product was dried for 3 hours in a vacuum oven at 40°C and then for 8 hours in a desiccator over P_2O_5 at 50°C. The exchange procedure yielded 1.70 gm of 2,2,4,6,6,16,17-d_7-4,16-androstadiene-3-one.

The 2,2,4,6,6,16,17-d_7-4,16-androstadiene-3-one was converted to the corresponding 5α-H-3-one steroid by reduction of the Δ^4-double bond in lithium/ammonia solution. Lithium wire (0.25 g) was dissolved in 250 ml of ammonia in a 500 ml flask fitted with an addition funnel and a dewar condenser. The ammonia was freshly distilled from sodium and passed through a refrigerated trap (-21°C) to remove any water contaminating the ammonia. A solution of 1.7 g of 2,2,4,6,6,16,17-d_7-4,16-androstadiene-3-one in 25 ml of tetrahydrofuran (freshly redistilled from $LiAlH_4$) was added in a slow stream to the lithium/ammonia solution. The reaction mixture was stirred at reflux for 1 hour by a Teflon coated stirring bar. The reaction was stopped by the addition of a saturated ammonium chloride in tetrahydrofuran until the blue color disappeared. The ammonia was allowed to evaporate and the product was extracted with ethyl ether. The ether

extract was washed 2 times with 1 volume of 0.1N HCl, 2 times with 1 volume of 0.1N $NaHCO_3$ and 3 times with 1 volume of water. The solution was dried over sodium sulfate and the solvent was removed in vacuum. The residue was purified by chromatography on a column containing $AgNO_3$-impregnated silica gel (prepared as described earlier), and then recrystallized from acetone-hexane. The yield was 67 mg of 2,2,4,6,6,16,17-d_7-5α-androst-16-en-3-one.

The α-deuteriums were removed by equilibration in methanol-water and the final product, 6,6,16,17-d_4-5α-androst-16-en-3-one, was recrystallized from the same solvents. Thin layer chromatography, gas chromatography and mass spectrometry were used to establish the purity of the product.

6,6,16,17-d_4-5α-Androst-16-en-3α-ol

The procedure was adapted from one published by Wheeler and Wheeler (1972). A solution of 6,6,16,17-d_4-5α-androst-16-en-3-one (0.63 g), chloroiridic acid (0.32 g), trimethyl phosphite (3.3 ml) and water (6.5 ml) in 2-propanol (49 ml) was boiled under reflux for 5 days. After cooling, the reaction mixture was transferred to a 250 ml separatory funnel containing 50 ml of ether and 50 ml of water. The product was extracted 3 times with 50 ml of ether. The combined ether extracts were dried over anhydrous sodium sulfate and then evaporated to dryness.

The residue was purified by chromatography on a column packed with silica gel impregnated with $AgNO_3$ as described earlier. The product was further purified by chromatography on a second silica gel column without $AgNO_3$. The appropriate fractions were combined and evaporated to dryness. The product was recrystallized from acetone-hexane to yield 0.11 g of 6,6, 16,17-d_4-5α-androst-16-en-3α-ol. The product purity was established by thin layer and gas chromatography and mass spectrometry.

6,6,16,17-d_4-5α-Androst-16-en-3β-ol

The procedure was adapted from one published by Tökés and Throop (1972). A mixture of 6,6,16,17-d_4-5α-androst-16-en-3-one (145 mg) and lithium aluminum hydride (66 mg) in dry ether (15 ml, freshly distilled from lithium aluminum hydride) was heated under reflux for 3 hours. The excess hydride was then decomposed by careful dropwise addition of water. The reaction mixture was washed with 0.1N HCl to remove the residue from the hydride. Then the solution was washed with water, dried with sodium sulfate and evaporated to dryness.

Gas chromatographic analysis of the product revealed that it consisted of 96% of the 3β-hydroxy compound and 4% of the corresponding 3α-hydroxy compound. The 3β-hydroxy steroid was purified by chromatography on a silica gel column as outlined earlier and crystallized from acetone-hexane. The reaction yielded 73 mg of 6,6,16,17-d_4-5α-androst-16-en-3β-ol. The purity of the product was established by gas chromatography and mass spectrometry.

RESULTS AND DISCUSSION

Comparisons of Levels of 5α-Androst-16-en-3-one and Boar Odor Scores

Boar odor is not usually a serious problem in meat packing plants processing only female and castrate male hogs. Occasionally, however, cryptorchid pigs enter the plant and go undetected until the discovery of testicles in the abdominal cavity upon evisceration. The carcasses must be tested for boar odor in the cooler so that any possessing boar odor can be restricted for use in comminuted cooked products, for rendering or condemned for use as human food. The carcasses are tested by heating the back fat with a hot iron and smelling the heated fat to detect the odors produced. The test is simple in theory, but in practice it is difficult because of the presence of other odors in the plant and also because of variations in the ability of different inspectors to detect the undesirable odor.

The USDA proposed an amendment to the federal meat inspection regulations that would exclude all cryptorchid pork from the fresh pork market (National Provisioner, 1967). Otherwise, they argued that it was impossible to insure cryptorchid pork containing boar odor was excluded from the

fresh meat market because of the difficulties associated with the testing procedure. The American Meat Institute, a special interest group representing meat packers, argued that the ruling was unnecessary since packing plant personnel could test cryptorchid pork for boar odor. However, USDA officials questioned whether or not the packers would be any more effective than the USDA inspectors in insuring that cryptorchid pork with the undesirable odor did not reach the market place.

The objective of this study was to determine whether packing plant personnel could estimate the levels of 5α-androst-16-en-3-one in backfat of cryptorchid pigs by the hot iron test for boar odor. This involved comparing the results of the olfactory tests with actual levels of 5α-androst-16-en-3-one as determined by chemical analysis. These results are shown in Table 1 where 21 cryptorchid samples, previously tested for odor by personnel of a meat packing firm, are listed in order of increasing concentration of 5α-androst-16-en-3-one.

In general, the odor rankings of the plant personnel do not accurately reflect the changing concentration of 5α-androst-16-en-3-one as can be seen in Table 1. This is shown graphically in Figure 2 where the odor scores are plotted against the concentration of 5α-androst-16-en-3-one. For the purposes of this comparison the odor scores were assigned numerical values as follows: no boar odor, 0; very slight, 1; slight, 2; moderate, 3; slightly pronounced, 4;

pronounced, 5; and very pronounced, 6.

The odor scores did not correlate significantly ($P<.05$) with the concentration of 5α-androst-16-en-3-one ($r=0.40$), which is in contrast to reports by Fuchs (1972) and Newell et al. (1973) who found correlation coefficients of 0.75 and 0.53, respectively, between odor intensity and the level of 5α-androst-16-en-3-one. The discrepancy between the results reported herein and those of Fuchs (1972) and Newell et al. (1973) is due, in part, to differences in the make-up of the odor panels, and to differences in the instructions given to them. The significant correlations reported by Fuchs (1972) and Newell et al. (1973) were obtained using panels composed of individuals selected for their ability to smell 5α-androst-16-en-3-one and trained to distinguish its odor from other odors associated with heated pork fat. Therefore, it is not surprising that they were able to obtain positive correlations between 5α-androst-16-en-3-one concentration and odor assignments. In contrast, the plant personnel used in this study were asked to rank the samples according to the degree of undesirable odor, generally referred to as boar odor. Their task was to score the samples according to the intensity of the undesirable odor which was assumed to be unacceptable to consumers of meat products.

The panel composed of packing plant personnel is more like the untrained panel used by Newell et al. (1973). Members of this panel could detect the odor of 5α-androst-16-en-3-one, but they were not trained to specifically distinguish this

TABLE 1. Comparison of the Concentration of 5α-Androst-16-en-3-one and Boar Odor Intensity Scores by Employees of a Meat Processing Plant

Sample Number	Concentration of 5α-Androst-16-en-3-one μg/g fat	Boar Odor Intensity[a]
10174	<0.10	0
14736	<0.10	0
13025	<0.10	2
74761	<0.10	2
31751	<0.10	1
15486	0.19	5
62342	0.22	4
63267	0.42	5
88651	0.42	4
75122	0.61	2
39528	0.67	2
17820	0.82	3
08899	1.12	3
00556	1.23	3
43253	1.24	5
94015	1.45	6
14924	1.56	4
70312	1.88	2
70707	1.88	5
02985	5.70	4
00911	6.97	5

a. Intensity: 0 = none, 1 = very slight, 2 = slight, 3 = moderate, 4 = slightly pronounced, 5 = pronounced, 6 = very pronounced.

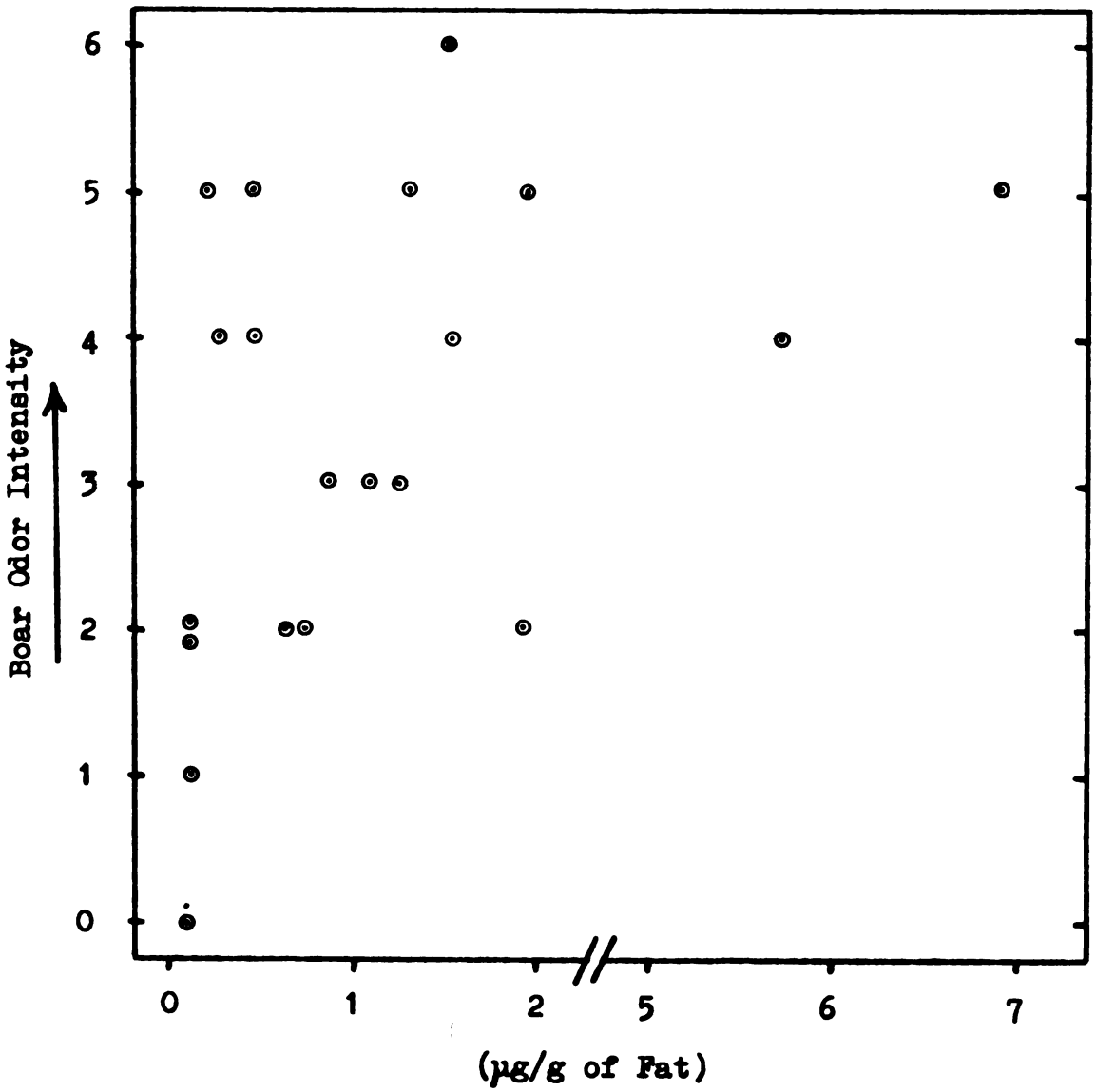


Figure 2. Graph of 5α-Androst-16-en-3-one vs. Boar Odor Intensity Scores

odor from other odors that are apparent when pork fat is heated. Their odor scores did not correlate significantly with the level of 5α-androst-16-en-3-one in fat (Newell et al., 1973), which is in agreement with the results of the packing house personnel as reported herein.

The same fat samples were odor tested in the laboratory by a three member panel composed of individuals with demonstrated ability to smell low levels of 5α-androst-16-en-3-one, 5α-androst-16-en-3α-ol, 4,16-androstadien-3-one and 5,16-androstadien-3β-ol. The boar odor intensity, rated on a scale of 0 (no boar odor) to 9 (pronounced boar odor), are shown in Table 2 where the fat samples are listed in order of increasing concentration of 5α-androst-16-en-3-one.

The average scores of this panel were positively correlated ($r=0.51$, $P<.05$) with the level of 5α-androst-16-en-3-one. Even though the correlation coefficient for the level of 5α-androst-16-en-3-one and the odor scores was significant, it accounted for only 25% of the variation. This compares closely with the correlation of 0.53 obtained in a similar study by Newell et al. (1973) with a trained panel. The correlation of 0.73 obtained by Fuchs (1972) is not directly comparable with the correlation of 0.51 obtained in this study because Fuchs (1972) decided to exclude from his calculations those samples having the highest concentrations of 5α-androst-16-en-3-one (5 to 8 μg/g fat).

The "urine-like" and "perspiration-like" nature of boar odor appears to result from a complex interplay of several

Table 2. Comparison of the Concentration of 5α-Androst-16-en-3-one and Boar Odor Intensity Scores by Laboratory Panel

Sample Number	Concentration of 5α-Androst-16-en-3-one	Boar Odor Intensity [a]
	μg/g of fat	
10174	0.10	0.0
14736	0.10	1.3
13025	0.10	1.8
74761	0.10	0.0
31751	0.10	0.3
15486	0.19	1.3
62342	0.22	3.3
63267	0.42	0.8
88651	0.42	3.3
75122	0.61	1.8
39528	0.67	1.5
17820	0.82	5.5
08899	1.12	0.5
00556	1.23	2.0
43253	1.24	1.3
94015	1.45	8.5
14924	1.56	4.8
70312	1.88	1.8
70707	1.88	3.0
02985	5.70	6.0
00911	6.97	4.8

a. Intensity: 0 (no boar odor) to 9 (pronounced boar odor).

undesirable odors, with 5α-androst-16-en-3-one being an important contributor. As can be seen in Tables 1 and 2, a high concentration of this steroid is a good indication that the undesirable odor will be evident when the fat sample is heated. However, a low level of 5α-androst-16-en-3-one does not guarantee freedom from undesirable odors. Therefore, it is unlikely that a chemical assay for 5α-androst-16-en-3-one alone would be helpful as an objective test for boar odor, but an assay that included other C_{19}-Δ^{16}-steroids, especially the highly odoriferous 5α-androst-16-en-3α-ol, might give a better indication of the extent of odor to be expected in the sample.

During the evaluation of samples in this laboratory the distinctive musky odor of 5α-androst-16-en-3α-ol was noticeable in several of the samples, especially sample #88651 and #14924. This observation prompted efforts to prepare a deuterium labeled form of this compound to be used as an internal standard in an assay using mass spectrometric reverse isotopic dilution. The labeled compound was prepared as outlined earlier but unfortunately there was not enough time to run the assay.

The failure of the meat plant personnel to discriminate between samples with various levels of 5α-androst-16-en-3-one does not discredit the hot iron technique as a way of selecting cryptorchid animals with no boar odor. All animals with levels of 5α-androst-16-en-3-one greater than 0.1 μg/g fat were rated as having at least slight boar odor, which would disqualify

them for use in the fresh pork market. However, the selection of carcasses eligible for use in cooked, comminuted products (less than pronounced odor), as against carcasses to be condemned or for rendering (pronounced odor), may be a more difficult task based on the observations presented here.

Procedure for the Assay of 5α-Androst-16-en-3-one

The original plan called for the assay of 5α-androst-16-en-3-one by the vacuum distillation method of Patterson (1969). However, alternative procedures were considered very early for several reasons. First, the distillation step required constant supervision, and even with careful attention to details it was difficult to reproduce the distillation conditions. Another serious objection was the absence of a suitable way to correct for manipulative losses during the procedure. In addition, the saponification step was found to result in some destruction of 5α-androst-16-en-3-one. Coupled with these chemical and manipulative losses was the uncertainty associated with gas chromatographic analysis performed without an internal standard.

The technique of mass spectrometric stable isotope dilution seemed uniquely well suited for this analysis. Losses during the entire procedure could be automatically corrected

for through the use of a deuterium labeled form of 5α-androst-16-en-3-one as an internal standard. Secondly, the sample size could be substantially reduced because the deuterium form of 5α-androst-16-en-3-one could serve as carrier for the smaller amount of the protium form being measured.

The original intention was to check the accuracy of the distillation technique by comparisons with the results from a few samples analyzed by reverse isotope dilution. However, this became impossible when the distillation apparatus was destroyed by a fire in the laboratory. It was the loss of this equipment that prompted the use of the reverse isotope dilution/carrier procedure for analysis of all of the samples. This was made possible by the generous cooperation of Drs. C.C. Sweeley and J.F. Holland of the Mass Spectrometry Laboratory in the Department of Biochemistry at Michigan State University.

The stable isotope dilution/carrier technique was used to measure the levels of 5α-androst-16-en-3-one which are given for cryptorchid fat samples in Tables 1 and 2. Following the addition of the deuterium labeled internal standard (usually 21 μg of 6,6-d_2-5α-androst-16-en-3-one), the mixture of deuterium and protium forms was purified by saponification and TLC. The resultant fraction was analyzed by GC to check its purity before analysis by combined GC-MS. The GC trace shown in Figure 3 is typical of the results obtained for all fat samples. It shows that the purification procedure involving saponification and TLC resulted in a relatively pure sample

with 5α-androst-16-en-3-one eluting at about 7 minutes. The peak at 7 minutes contained both the protium form of 5α-androst-16-en-3-one, which was originally present in the sample, and the d_2-form added as an internal standard and carrier.

Figure 4 shows a GC trace obtained for one sample to which no deuterium labeled standard had been added. No measureable amount of 5α-androst-16-en-3-one can be seen at 7 minutes. It is evident from the GC trace in Figure 4 that ordinary GC techniques without carrier could not be used to assay this sample. This sample was easily assayed by combined GC-MS when an excess of deuterium labeled 5α-androst-16-en-3-one was added as a carrier and internal standard. The sample was found to contain 5α-androst-16-en-3-one at a level of 0.42 μg/g fat (sample #88651, Table 3).

The mass spectrometer was used to determine the relative amounts of protium and deuterium form present in the 5α-androst-16-en-3-one peak for each of the samples. Acting as a very specific and sensitive GC detector, the mass spectrometer registered the intensity of the molecular weight ions of the two forms as they emerged from the column. The molecular weight ions of the protium form (m/e 272) and for the d_2-form (m/e 274) can be seen in the abbreviated mass spectra shown in Figure 5. Figure 6 shows the result obtained by monitoring the intensity of m/e 272 and 274 during the elution of a typical sample. Peak heights were used in the isotopic ratio determinations.

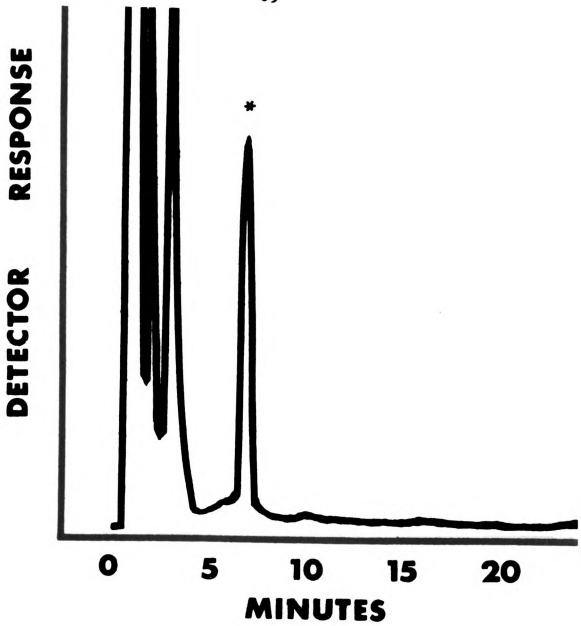


Figure 3. Gas Chromatogram of 5α-Androst-16-en-3-one Isolated with Deuterium Labeled Carrier.

This material was isolated from 2 g of backfat to which had been added 21 μg of 6,6-d_2-5α-androst-16-en-3-one. The asterisk identifies the peak containing the protium and deuterium forms of 5α-androst-16-en-3-one. Conditions: 6 ft x 2 mm column packed with 3% SP-2401, operated at 183°C.

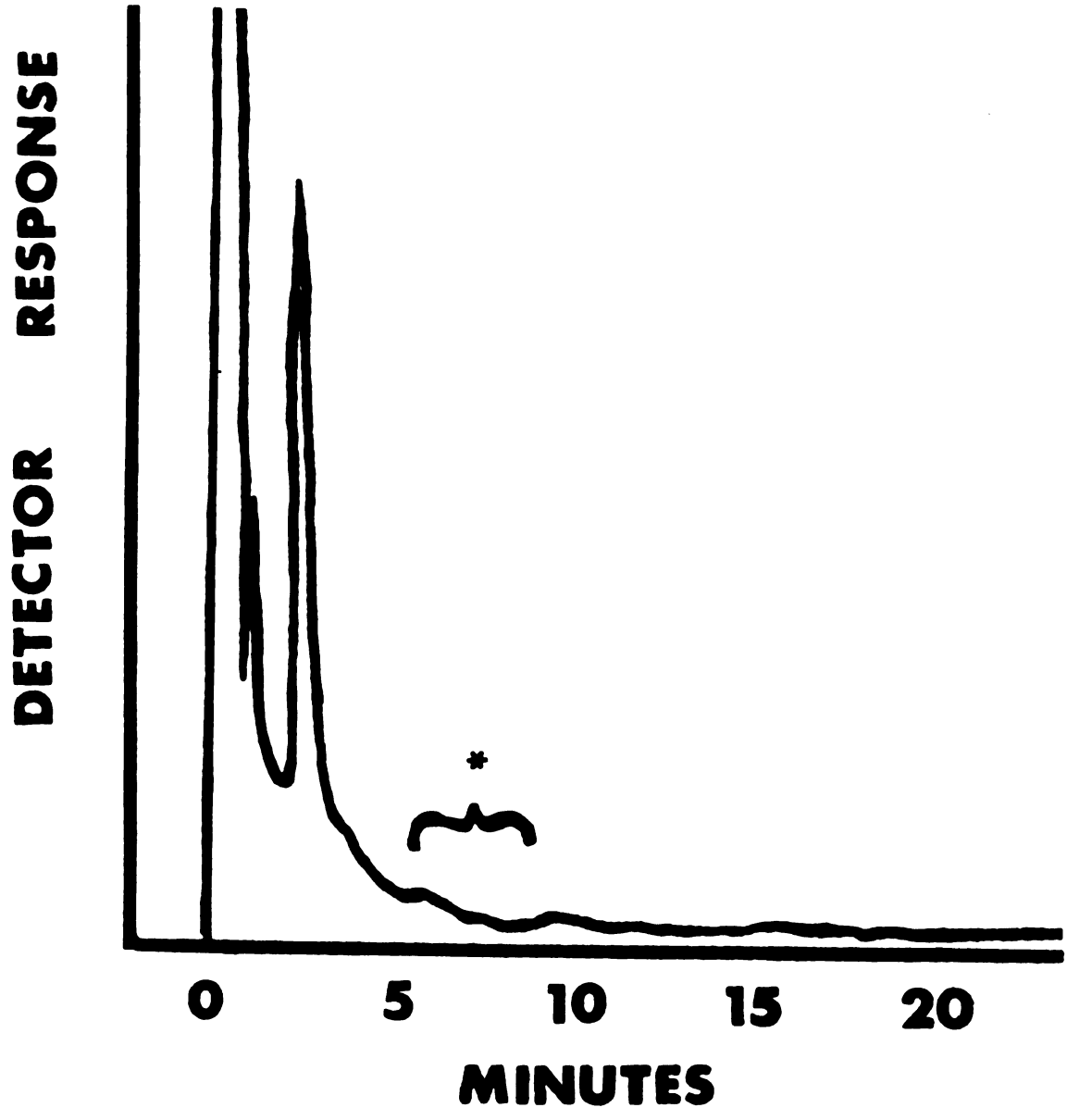


Figure 4. Gas Chromatogram of 5α-Androst-16-en-3-one Isolated without Carrier.

This material was isolated from 2 g of backfat. The asterisk denoted the region of the chromatogram corresponding to the retention time of 5α-androst-16-en-3-one. Operating conditions are the same as those described in the legend to Figure 3.

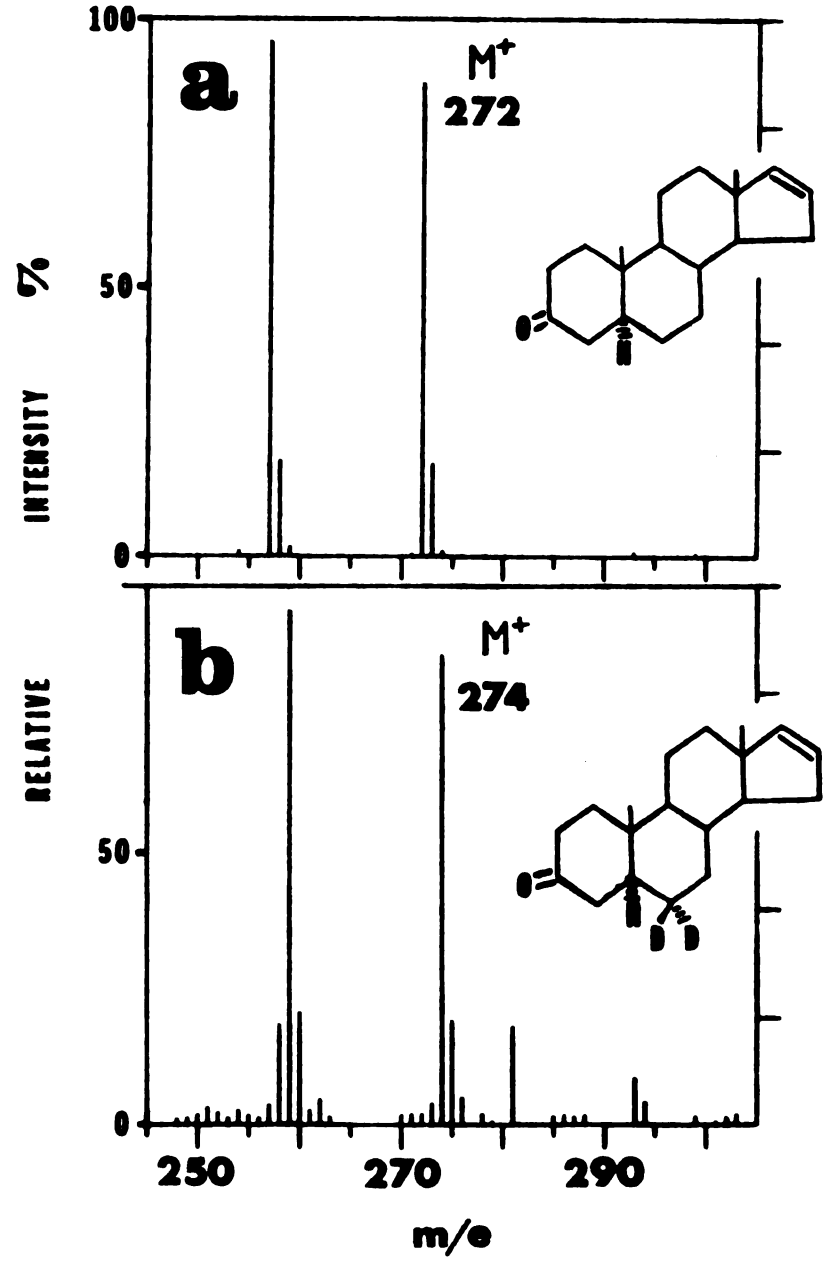


Figure 5. Abbreviated Mass Spectra of Protium and Deuterium Forms of 5α-Androst-16-en-3-one.

a) 5α-Androst-16-en-3-one; b) 6,6-d_2-5α-Androst-16-en-3-one Spectra recorded at 70.0 eV with a GC inlet consisting of a 6 ft × 2 mm glass column packed with 3% SP-2401. The complete mass spectra are shown in Figure 7.

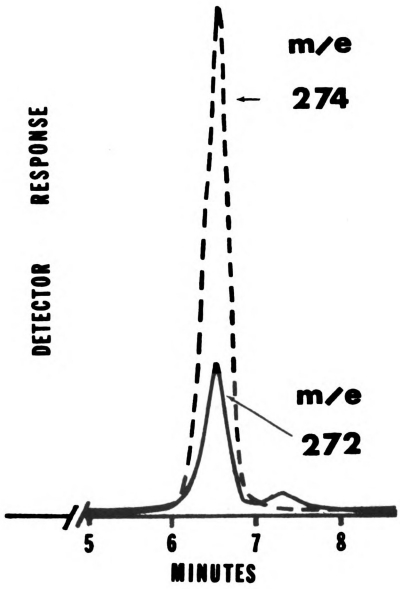


Figure 6. Molecular Ion Intensity Recordings for Protium and Deuterium Forms of 5α-Androst-16-en-3-one.

Selected ion monitoring of the M^+ ions for the p-form (m/e 272) and d_2-form (m/e 274) as they elute from the GC column. The sample was the same one shown in Figure 3. Operating conditions are given in the legend to Figure 5.

The isotopic abundance ratios obtained for the experiment are shown in Table 3 where the samples are listed in the order of their analysis. The concentration of 5α-androst-16-en-3-one (column D) was obtained by multiplication of the difference in the protium/deuterium ratio (column B) by the amount of deuterium carrier added to the original sample. The difference in protium/deuterium ratio was obtained by subtraction of the ratio at ion pair m/e 272/274 in the pure deuteriated carrier from the ratio obtained for each sample (column A). This difference was taken to be proportional to the amount of protium form present in the sample.

In the analysis of some samples (#14736, 13025, 10175 and 74761) the protium/deuterium ratios (column A in Table 3) were smaller than the protium/deuterium ratio for the pure deuteriated carrier. There are several possible explanations for this incongruity.

The samples may have contained an impurity that co-chromatographed with 5α-androst-16-en-3-one and produced an ion at m/e 274, thereby reducing the ratio of m/e 272/274 below that of the added deuteriated carrier. The SP-2401 liquid phase of the GC column does not produce a measurable ion at m/e 274. Therefore, column bleed was ruled out as a possible source of an inflated intensity value for m/e 274.

If the deuteriated carrier was not sufficiently pure to begin with, it could have become more purified during its isolation from the fat sample. A lowering of the

Table 3. Assay of 5α-Androst-16-en-3-one

sample	protium/deuterium ratio		μg carrier added to 2 g fat sample	μg 5α-Androst-16-en-3-one/g of fat
	Ratio A	Difference B (A-.024)	C	D (B•C/2)
carrier	.024	--	--	--
carrier	.024	--	--	--
31751	.032	.008	21	<0.10 [b]
14924	.173	.149	21	1.56
75122	.082	.058	21	0.61
14736	.020	(-) [a]	21	<0.10 [a]
62342	.045	.021	21	0.22
63267	.064	.040	21	0.42
13025	.016	(-) [a]	21	<0.10 [a]
88651	.064	.040	21	0.42
43253	.142	.118	21	1.24
02985	.566	.542	21	5.70
70312	.203	.179	21	1.88
08899	.131	.107	21	1.12
10174	.018	(-) [a]	21	<0.10 [a]
17820	.102	.078	21	0.82
74761	.016	(-) [a]	21	<0.10 [a]
39528	.088	.064	21	0.67
94015	.162	.138	21	1.45
15486	.042	.018	21	0.19
00556	.141	.117	21	1.23
00911	.688	.664	21	6.97
70707	.203	.179	21	1.88

a. Ratio (A) was smaller than ratio for carrier. Therefore, values of <0.10 were assigned. b. The ratio (A) was only slightly larger than ratio for carrier. Since the difference (B) may not be significant, an arbitrary value of <0.10 was assigned.

ratio of m/e 272/274 could then occur if the impurities which were removed had been partly responsible for the ion intensity at m/e 272 in the pure deuteriated carrier. Errors of this type could have been minimized if the background value (ratio of m/e 272/274 for the carrier) had been determined on carrier material that had been treated in the same way as the samples.

For the reasons discussed above, the concentration of 5α-androst-16-en-3-one in these four samples were assigned values of <0.1 μg/g of fat. Sample #31751, with a calculated level of 0.08 μg/g, was also reported as having <0.1 μg/g of fat because it was considered doubtful that a ratio difference (column B of Table 3) as small as 0.008 could be significant in view of the possible errors in the assay, which are discussed below.

Possible Errors in the Assay Procedure

The data presented in Tables 1, 2 and 3 is considered to be sufficiently accurate to serve the purposes of this study, and perhaps more reliable than other published data for similar samples assayed without any corrections for losses (Patterson, 1969; Fuchs, 1972; Stinson *et al.*, 1972; Newell *et al.*, 1973). Nevertheless, the levels of 5α-androst-16-en-3-one reported herein are considered to be approximations because of the possibility of errors caused

by "memory" effects in the GC-MS instrument, and the reliance on single measurements for each sample.

Errors in isotopic abundance measurement could have resulted from background due to a carry-over effect from previous samples examined ("memory effect"). According to Beynon (1960) the only solution in such cases is to remove all memory of the previous sample which can be accomplished by repeatedly flushing with the new sample until a constant isotopic abundance ratio is achieved. Ratio measurements made in this study were based on only single injections of each sample so it is quite likely that some errors may have resulted, especially in cases where a low level sample was preceded by a much more concentrated one.

The first step in reverse isotope dilution analysis should be the preparation of a working curve showing a linear relationship between observed isotopic abundance ratios and actual weight ratios upon injection of a dilution series comprised of mixtures containing protium and deuterium forms of the compound being measured. The use of such a curve would eliminate errors arising from differences in ion intensities between the protium and deuterium forms which may result from fragmentation isotope effects and mass discriminations in the mass spectrometer.

No correction was made for the cross-contribution of the M^++2 natural satellite isotope peak from the protium form (M^+, m/e 272) and the ion intensity measured for the

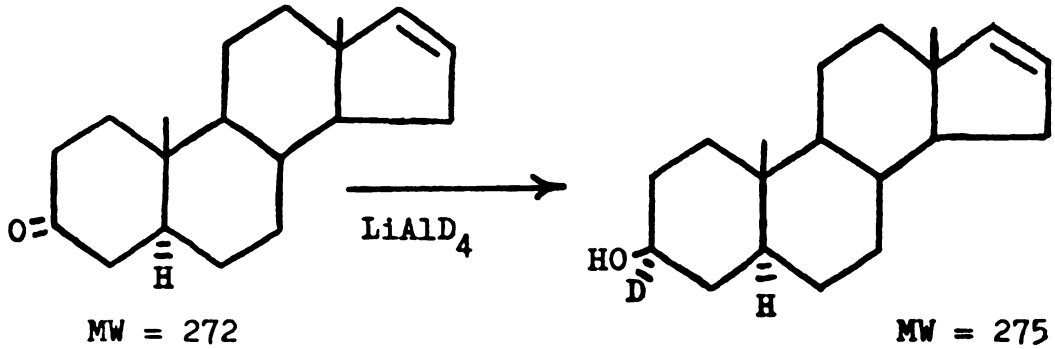
d_2-form which also appears at m/e 274. Since the intensity of the M^++2 satellite ion will be only approximately 0.12% of the intensity of the M^+ ion, this correction can be ignored for isotopic abundance measurements on mixtures containing small amounts of the protium form.

Even though the reverse isotope dilution method did not give absolute values for 5α-androst-16-en-3-one, exact concentrations are not required to relate to odor scores. The levels of 5α-androst-16-en-3-one reported herein are in agreement with the findings of other workers (Claus et al., 1971; Rhodes and Patterson, 1971; Fuchs, 1972; Stinson et al., 1972; Newell et al., 1973) who reported concentrations of 0.1 to 7 μg/g of fat in studies using fat samples from uncastrated male hogs.

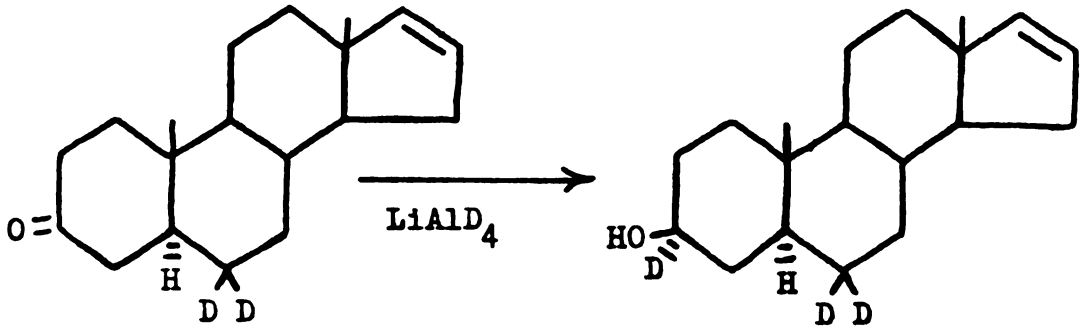
Purification by $LiAlH_4$ Reduction and Urea Clathrate Formation

Consideration was given to a purification scheme involving $LiAlH_4$ reduction and urea clathrate formation instead of saponification for the removal of glyceride esters during the purification of 5α-androst-16-en-3-one from fat samples. This was prompted by the desire to reduce the time and labor requirements of the assay and to reduce destruction of 5α-androst-16-en-3-one. Results of preliminary experiments showed that these combined steps yielded 0.6 g of material from a 2 g fat sample. However, the samples prepared in this way were not sufficiently pure for GC-MS analysis without additional preparative chromatography steps.

Reduction of the glyceride esters with $LiAlH_4$ would be accompanied by reduction of the 3-ketone group of 5α-androst-16-en-3-one. The resulting 3β-alcohol would be indistinguishable from 5α-androst-16-en-3β-ol originally present in the sample. However, by the use of $LiAlD_4$ instead of $LiAlH_4$ it would be possible to distinguish between the two since any alcohol formed during the reduction would have deuterium incorporated at the 3α-position. Therefore, any 5α-androst-16-en-3-one (MW 272) present in the original sample would be converted to 3α-d_1-5α-androst-16-en-3β-ol (MW 275), and a carrier/internal standard such as 6,6-d_2-5α-androst-16-en-3-one (MW 274) would be converted to 3α,6,6-d_3-5α-androst-16-en-3β-ol (MW 277) as shown below.



MW = 272 MW = 275



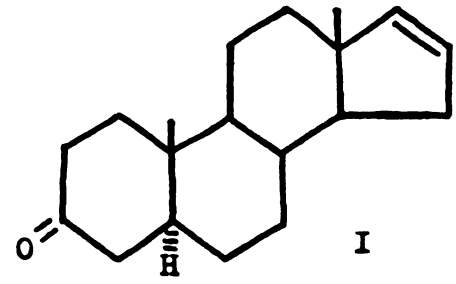
MW = 274 MW =277

The ratio of the two isotopic forms could be determined by measuring the intensities of their molecular weight ions (d_1-form, m/e 275; d_3-form, m/e 277) in GC-MS analysis. Then the concentration of 5α-androst-16-en-3-one originally present in the sample could be calculated by multiplication of the isotopic abundance ratio by the weight of carrier added to the sample.

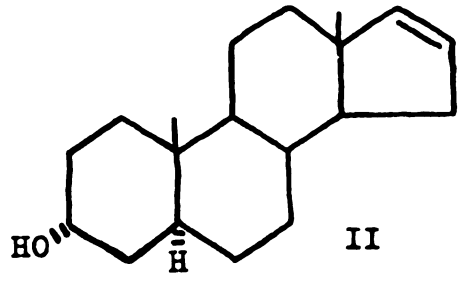
Although time did not permit an evaluation of the entire procedure described above, the proposed method appears to be superior in some respects to the methods described earlier which involved saponification.

Deuterium Labeled C_{19}-Δ^{16}-Steroids

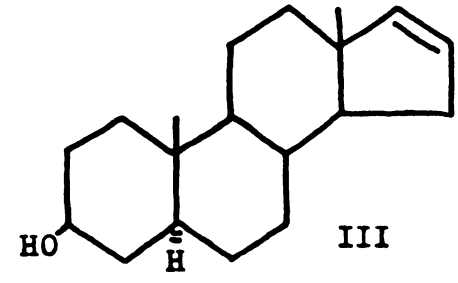
Deuterium labeled steroids were prepared to be used as internal standards and carriers in the assay of C_{19}-Δ^{16}-steroids by mass spectrometric reverse isotope dilution. The general features of the synthetic steps are discussed in this section, while the details of the reactions are given under Experimental. Deuterium labeled forms of the following five steroids were prepared: 5α-androst-16-en-3-one (I), 5α-androst-16-en-3α-ol (II), 5α-androst-16-en-3β-ol (III), 5,16-androstadien-3β-ol (IV), and 4,16-androstadien-3-one (V). The structural formulas for each steroid are shown below:



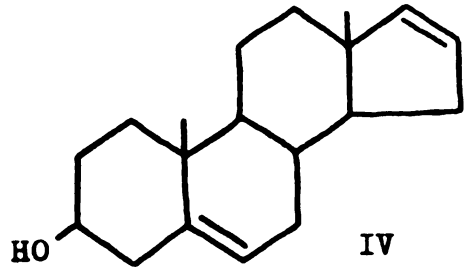
I



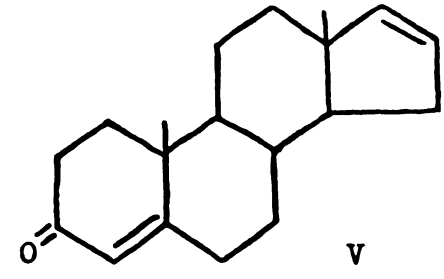
II



III



IV

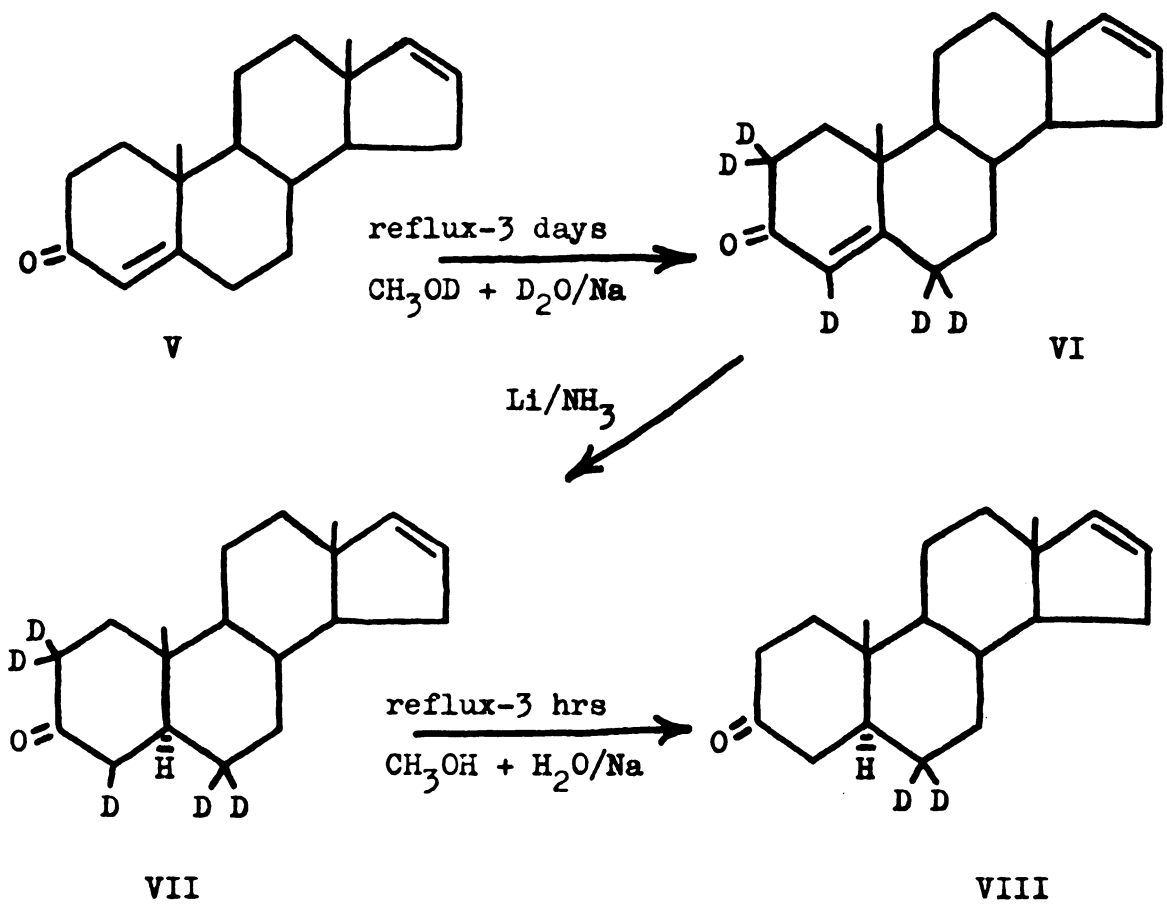


V

6,6-d_2-5α-Androst-16-en-3-one

One convenient method for preparing a site-specifically deuteriated form of 5α-androst-16-en-3-one would be to exchange the four activated hydrogens in the α-position of the carbonyl group. However, the resultant material would not have been suitable as an internal standard in isotope dilution analysis because the label would back exchange with protons in the medium.

An alternative approach was selected which led to the introduction of two deuteriums at carbon-6, three carbon atoms away from the carbonyl group (Shapiro et al., 1964). The synthetic steps are shown below.



V VI VII VIII

In the first step, the activated hydrogens in 4,16-androstadien-3-one (V) were exchanged with deuteriums by equilibration in methanol-OD/D_2O. The product (VI) was reacted with a solution of lithium metal in liquid ammonia (Li/NH_3). The Li/NH_3 reduced only the Δ^4-double bond and yielded 2,2,4,6,6-d_5-5α-androst-16-en-3-one (VII). The exchangeable deuteriums at C-2 and C-4 were removed by back exchange in methanol-OH/H_2O. The two deuteriums attached to C-6 are no longer exchangeable since the enone structure that existed in the original material has been lost by saturation of the double bond between C-4 and C-5. These reaction steps resulted in a 40% yield (10 mg) of 6,6-d_2-5α-androst-16-en-3-one (VIII).

The mass spectrum of 6,6-d_2-5α-androst-16-en-3-one (VIII) is shown in Figure 7a. By comparison with the mass spectrum of the unlabeled form, shown in Figure 7b, it can be seen that the molecular weight ion (M^+) at m/e 272 has been shifted to m/e 274 and the ion at m/e 257 (M^+-CH_3) has been shifted to m/e 259 in the labeled steroid. Either of these ions could be monitored for the measurement of 5α-androst-16-en-3-one (I) in gas chromatographic effluents by selected ion monitoring mass spectrometry.

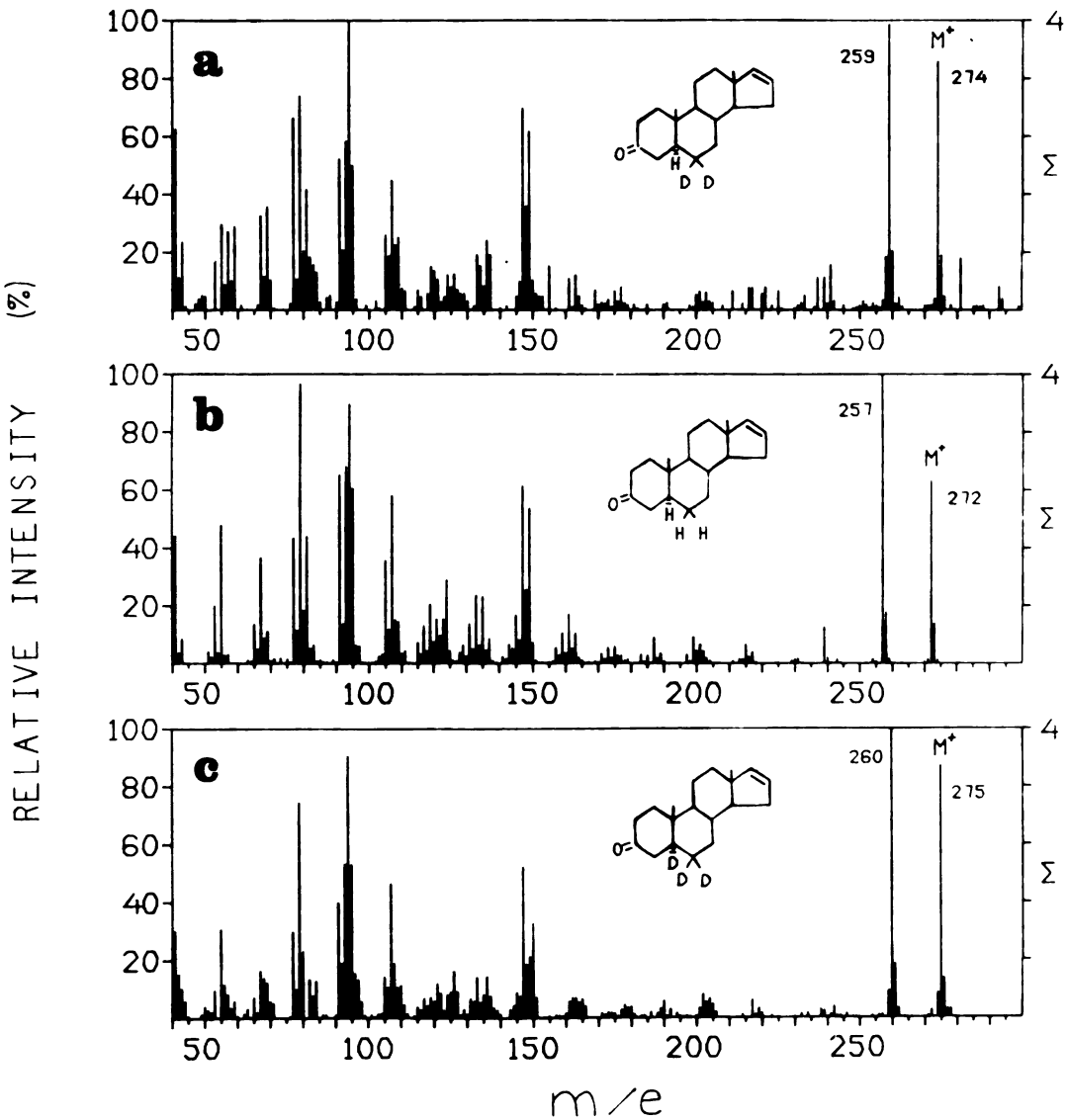
The 6,6-d_2-5α-androst-16-en-3-one (VIII) prepared in this experiment was used as an internal standard and carrier in the assay of 5α-androst-16-en-3-one (I) in fat samples evaluated earlier for boar odor intensity. The results were discussed earlier herein.

Figure 7. Mass Spectra of Protium and Deuterium Forms of 5α-Androst-16-en-3-one.

a) 6,6-d_2-5α-Androst-16-en-3-one; b) 5α-Androst-16-en-3-one;

c) 5,6,6-d_3-5α-Androst-16-en-3-one

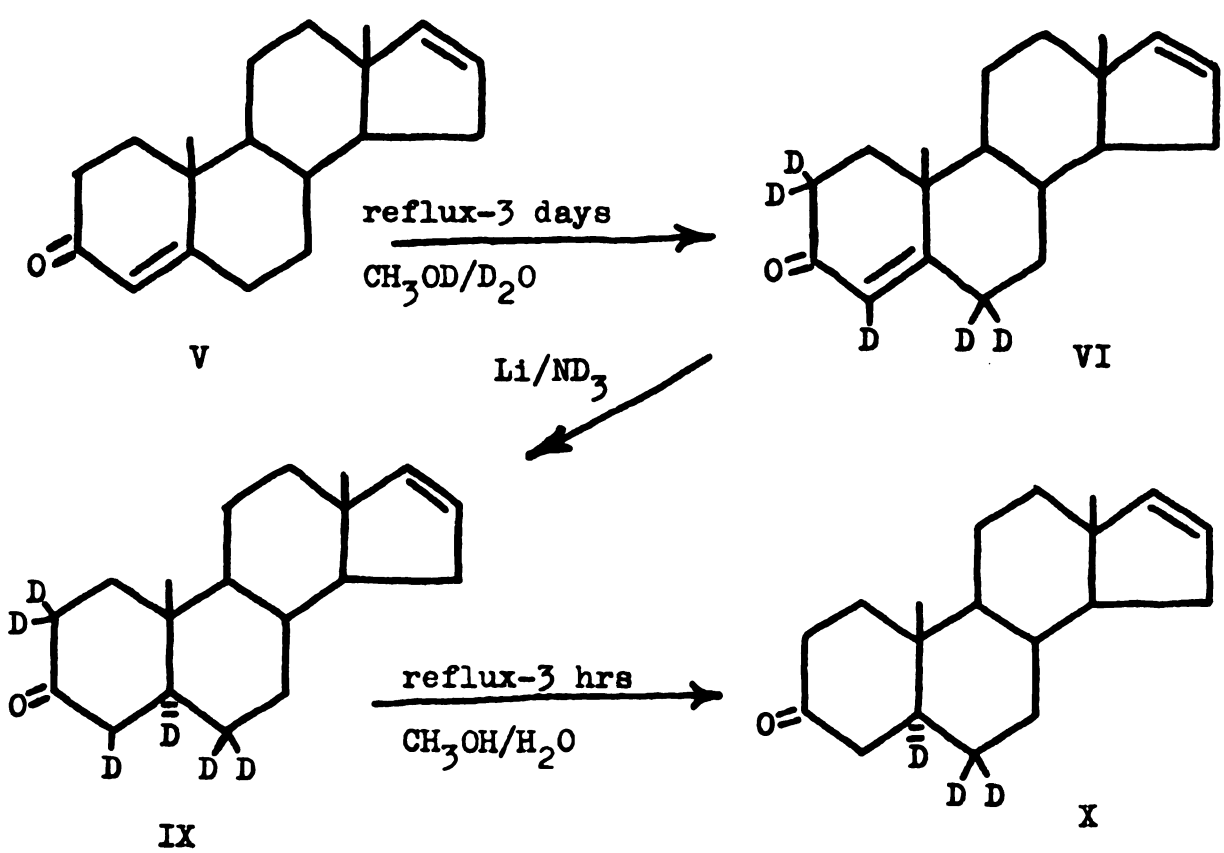
Mass spectra were recorded at an ionizing potential of 70 eV. The GC inlet consisted of a glass column (6 ft x 2 mm) packed with 3% SP-2401 on Supelcoport (100/120 mesh) operated isothermally at 190°.


a
b
c
259
M+
274
257
M+
272
260
M+
275
D D
H H
D D
Σ
RELATIVE INTENSITY (%)
m/e
100
80
60
40
20
50
100
150
200
250
4

5,6,6-d_3-5α-Androst-16-en-3-one

This synthesis was undertaken because it was thought that a d_3-form of 5α-androst-16-en-3-one would be a better internal standard than the d_2-form due to lower values for background. In this case, the term "background" refers to the relative amount of m/e 272 (M^+ for the unlabeled form) in the mass spectrum of the labeled form. Also, the use of a d_3-labeled standard would eliminate the cross contribution that would exist between the natural isotope peak at $M^+ + 2$ in the unlabeled form and the M^+ peak in a d_2-form.

d_3-5α-androst-16-en-3-one was prepared as previously described for the d_2-form except that deuterioammonia was used in the lithium/ammonia reduction instead of ordinary ammonia as shown below.



The starting material, 4,16-androstadien-3-one (V), was subjected to hydrogen-deuterium exchange prior to reduction with Li/ND_3, and then the deuteriums in the α-position of (IX) were removed by back exchange. The products of the above reactions consisted of 34 mg (41% yield) of 5,6,6-d_3-5α-androst-16-en-3-one (X) and an equal amount of the corresponding 3β-alcohol. The production of substantial amounts of alcohol was predictable since over-reduction to an alcohol is a common side reaction due to the presence of D_2O in commercially available ND_3 (Tökés and Throop, 1972).

The mass spectrum of 5,6,6-d_3-5α-androst-16-en-3-one (X), shown in Figure 7c, shows that the M^+ and M^+-CH_3 ions now appear at m/e 275 and 260 as expected. Unfortunately, the spectrum also shows a background ion at m/e 272 which was not expected. The intensity of m/e 272 for this d_3-form was expected to be much smaller than m/e 272 in the d_2-form shown in Figure 7b. It was thought that the additional deuterium introduced at C-5 in the formation of the d_3-form would reduce the background at m/e 272 by an amount proportional to the percentage of deuterium added at C-5.

The intensity of m/e 272 taken together with the intensity of m/e 287 suggested that the d_3-form was chemically impure, and that m/e 272 was a chemical impurity and not an isotopic impurity. Thin layer chromatography purification removed both the ions at m/e 272 and 287, and resulted in a d_3-product with a background (intensity of m/e 272 / m/e 275) of 8 parts per thousand.

Perdeuterio 5α-Androst-16-en-3β-ol and 5α-Androst-16-en-3α-ol

The mass spectra of 5α-androst-16-en-3α-ol (II) and 5α-androst-16-en-3β-ol (III), shown in Figure 8a and 9a, are different only in the intensity of the ions produced. Both compounds yield relatively intense M^+ ions (m/e 274) and M^+-CH_3 ions (m/e 259) that would be good candidates for mass spectrometric selected ion monitoring of these compounds in gas chromatographic effluents.

5α-Androst-16-en-3β-ol and 5α-androst-16-en-3α-ol both undergo the loss of two hydrogens from their M^+ ions and M^+-CH_3 ions upon electron ionization. Therefore, it would be desirable if the deuterium labeled internal standards contained three or more deuteriums. Otherwise, the measurement of the intensity of m/e 274 and 259 (M^+ and M^+-CH_3 for the unlabeled steroids) would be complicated because of contributions to those masses from the d_2-labeled internal standard.

A convenient way to prepare a d_3-version of 5α-androst-16-en-3β-ol would have been to reduce the previously prepared 6,6-d_2-5α-androst-16-en-3-one (VIII) with $LiAlD_4$ as shown below:

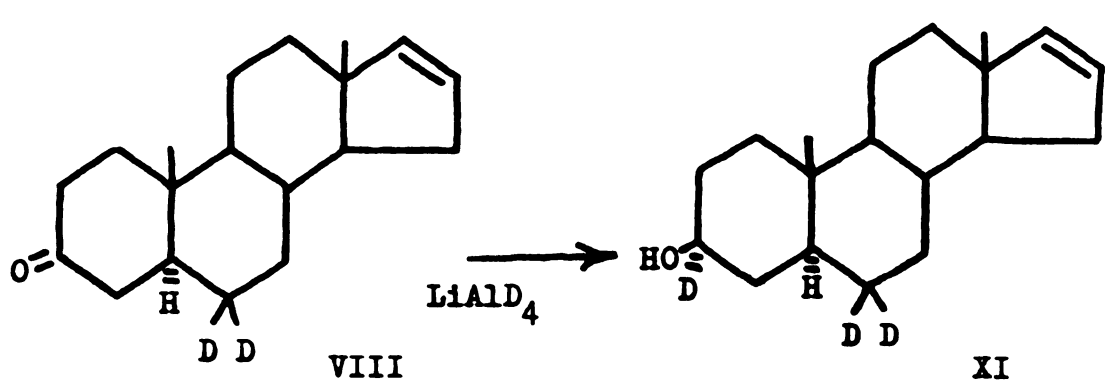


Figure 8. Mass Spectra of Protium and Deuterium Forms of 5α-Androst-16-en-3α-ol.

a) 5α-Androst-16-en-3α-ol; b) 6,6,16,17-d_4-5α-Androst-16-en-3α-ol

Operating conditions are given in the legend to Figure 7.

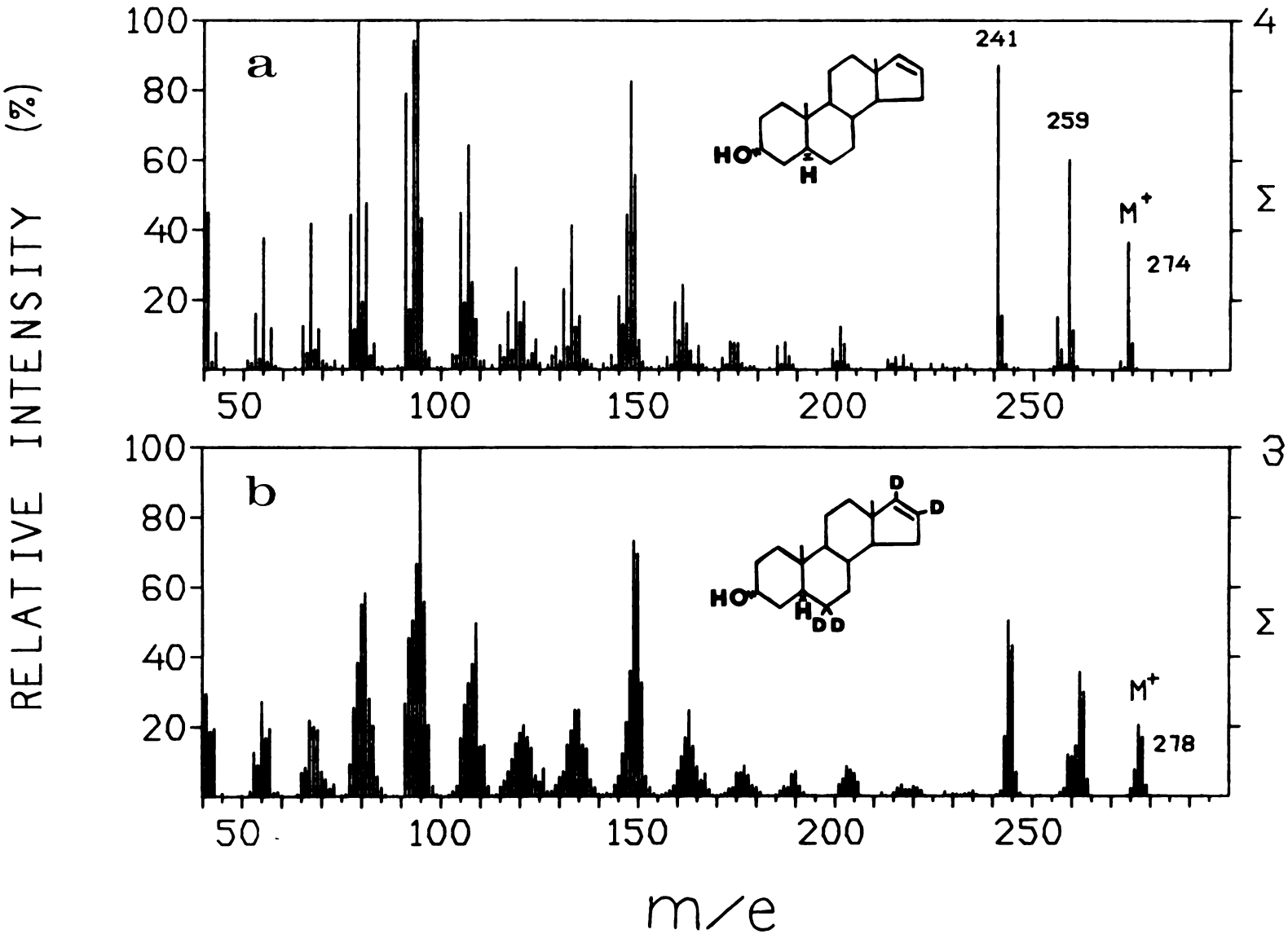
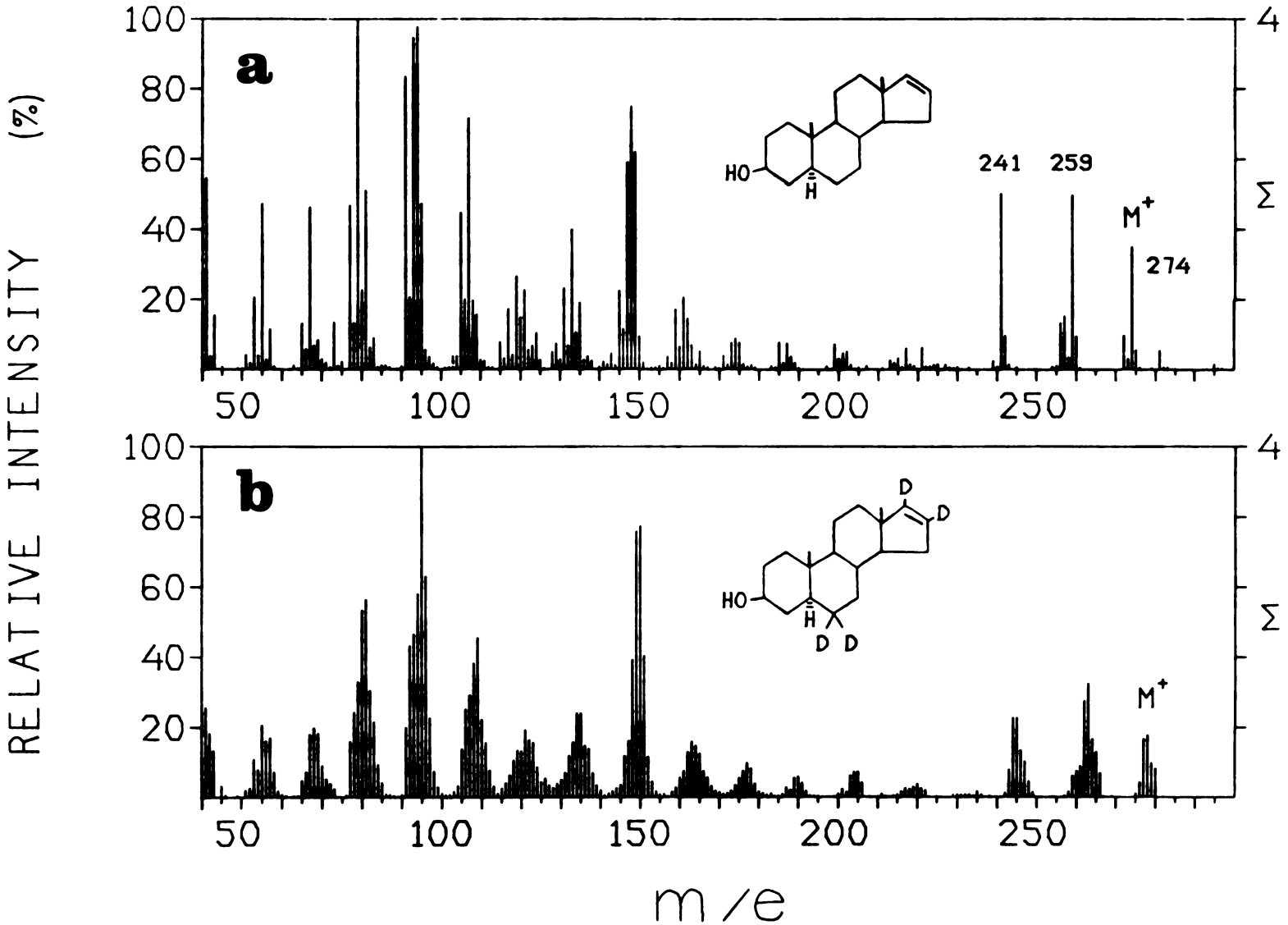

a
b
100
80
60
40
20
50
100
150
200
250
241
259
M+
274
278
HO
H
D
D
DD
4
3
Σ
RELATIVE INTENSITY (%)
m/e

Figure 9. Mass Spectra of Protium and Deuterium Forms of 5α-Androst-16-en-3β-ol.

a) 5α-Androst-16-en-3β-ol; b) 6,6,16,17-d_4-5α-Androst-16-en-3β-ol

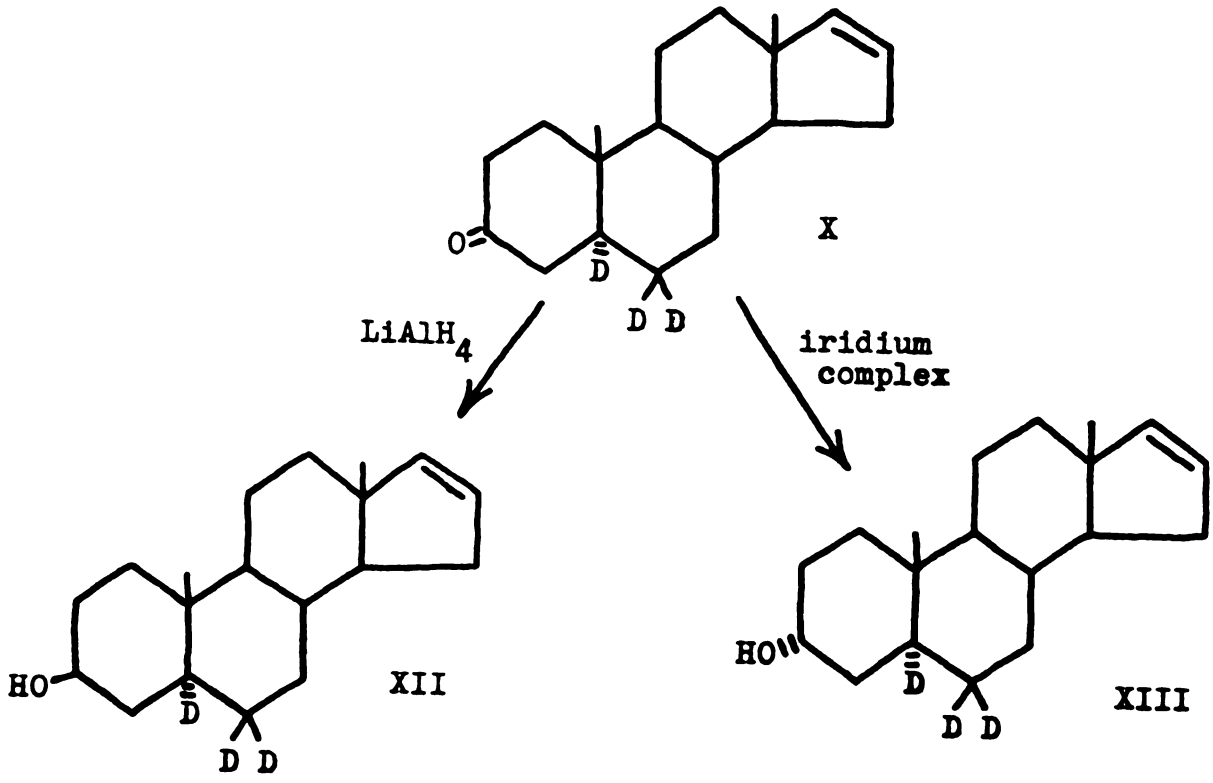
Operating conditions are given in the legend to Figure 7.


a
b
100
80
60
40
20
50
100
150
200
250
4
Σ
HO
H
241
259
M+
274
D
D D
RELATIVE INTENSITY (%)
m/e

The product obtained from the above reaction, 3α,6,6-d_3-5α-androst-16-en-3β-ol (XI), would be expected to be of high isotopic purity since Tökés and Throop (1972) reported that the isotopic purity of products from $LiAlD_4$ reduction are usually equivalent to that of the $LiAlD_4$ (99 atom % D).

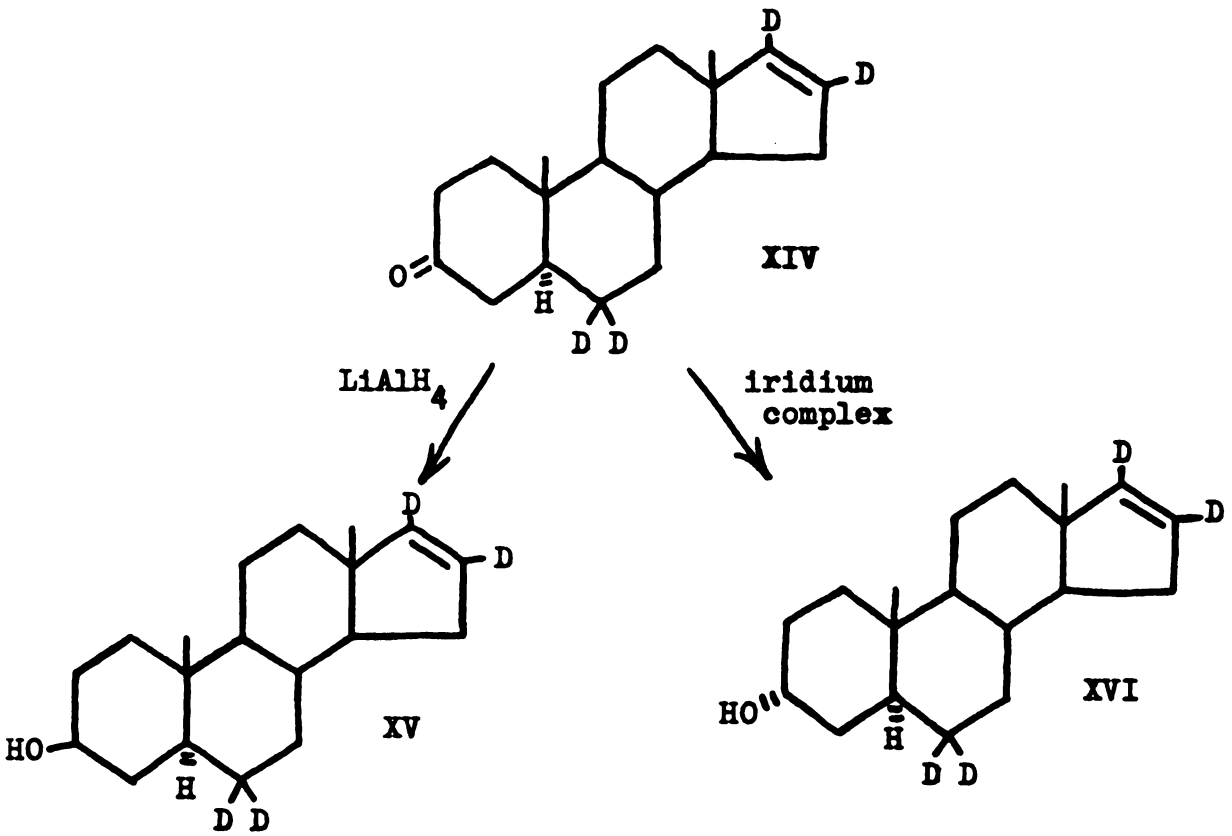
While the above procedure would have been convenient for preparing the 3β-alcohol, the 3α-epimer could not be made by this procedure since little or none of the 3α-alcohol can be obtained by reducing 3-keto-5α-steroids with $LiAlD_4$ (Wheeler and Wheeler, 1972). However, as much as 92% of the 3α-alcohol can be obtained by reduction of 3-keto-5α-steroids with iridium complex catalysts (Haddad et al., 1964). Although there are no reports describing the introduction of deuterium during this reaction, it seems possible that this could be accomplished if the reaction were performed in isopropanol-OD and D_2O instead of protio-solvents. However, this was not attempted.

An alternative approach was considered which involved the use of 5,6,6-d_3-5α-androst-16-en-3-one (X) as starting material instead of the d_2-form (VIII). The use of d_3 starting material would eliminate the need to incorporate deuterium during the reduction with iridium complex catalyst. For example, the 5,6,6-d_3-5α-androst-16-en-3-one (X) prepared earlier could be reduced with $LiAlH_4$ to obtain 5,6,6-d_3-5α-androst-16-en-3β-ol (XII), while reduction with the iridium complex could be used to obtain 5,6,6-d_3-5α-androst-16-en-3α-ol (XIII) as shown on the following page.



Although the procedure just described seemed at first to be a promising approach, the method was abandoned because of the difficulties in making, handling and storing the large quantities of ND_3 required for the synthesis of 5,6,6-d_3-5α-androst-16-en-3-one (X). Tökés and Throop (1972) described the preparation of ND_3 by reaction of magnesium nitride with D_2O, but this technique was not well suited for the production of large amounts of ND_3. Large scale preparation of ND_3 by a counter current exchange procedure has been described by Chevallier and Jillian (1968), but this was not attempted. Fétizon and Gore (1966) used deuteriated propylamine instead of ND_3 and their results suggested that deuteriated propylamine should be used in subsequent synthetic work of this type.

The technique finally selected for preparation of d-labeled 5α-androst-16-en-3β-ol and 5α-androst-16-en-3α-ol involved the reduction of d_4-labeled 5α-androst-16-en-3-one (XIV). The 3β-alcohol (XV) was prepared from the corresponding 3-ketone (XIV) by reduction with $LiAlH_4$, while the 3α-alcohol (XVI) was prepared from similar material by reduction with iridium complex catalyst as shown below. The preparation of the d_4-labeled starting material (XIV) will be discussed later.



The mass spectra of the perdeuterio 5α-androst-16-en-3β-ol (XV) and 3α-ol (XVI) are shown in Figure 9b and 8b, respectively. An important feature in the mass spectrum of both compounds is

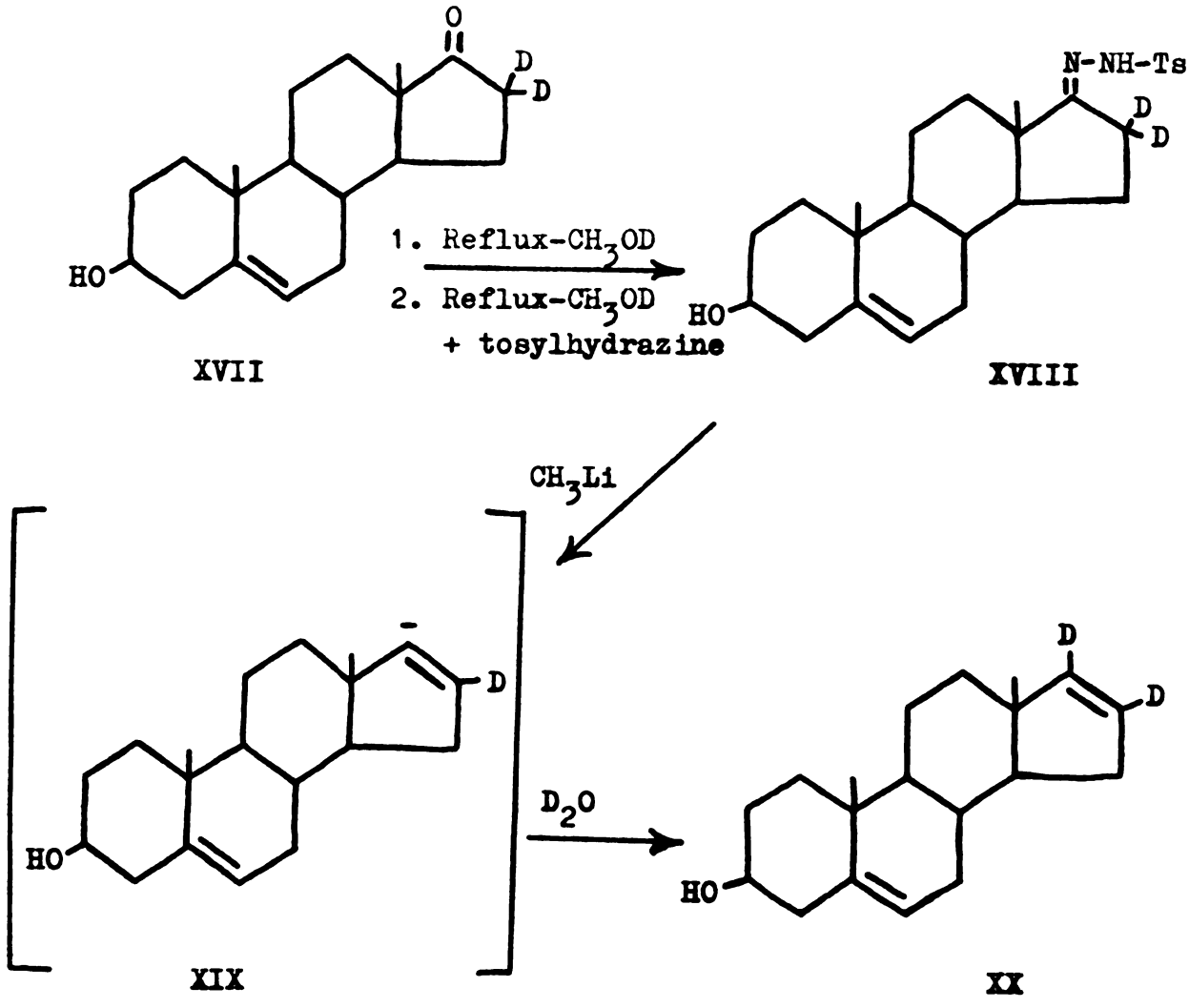
the absence of a background ion at m/e 274. The very low intensity of this ion suggests that both compounds would be good internal standards for mass spectrometric isotopic dilution studies of these two important C_{19}-Δ^{16}-steroids.

While both d_4-labeled standards contain isotopic impurities (d_1, m/e 275; d_2, m/e 276; d_3, m/e 277), these should not interfere with reverse isotopic dilution analyses. The d_3-impurity probably originated from low deuterium incorporation at C-17, which is discussed later in connection with the synthesis of 16,17-d_2-5,16-androstadien-3β-ol. The appearance of an ion at m/e 266 in the mass spectrum of the d_4-labeled 3β-alcohol (Figure 9b) is curious because it represents the unlikely loss of 14 amu's from the M^+ ion, which suggests that it originated from a chemical impurity and that the steroid should be purified further. The ion at m/e 280 was also unexpected, and may originate from a chemical impurity.

16,17-d_2-5,16-Androstadien-3β-ol

An effort was made to prepare this compound to be used as an internal standard and carrier in the assay of 5,16-androstadien-3β-ol, an important intermediate in boar testes (Saat *et al.*, 1972) and in human testes (Bicknell and Gower, 1971), and one of the most abundant steroids in boar testes (Ruokonen and Vihko, 1974a). The procedure was analogous to the one used by Matthews (1968) to make 5,16-androstadien-3β-ol except that a d_2-labeled 17-keto steroid (XVII) was used as starting material

and D_2O was added in the final step instead of H_2O. The sequence of reactions are shown below:



XVII XVIII XIX XX

The d_2-starting material (XVII) was prepared by enolic exchange in methanol-OD. This exchange was purposely done without the aid of a base catalyst so that the hydrazone derivative (XVIII) could be formed simply by addition of tosylhydrazine to the methanol-OD solution of 17-keto steroid.

Decomposition of the hydrazone (XVIII) with CH_3Li yielded the stabilized anion complex (XIX), and addition of D_2O resulted in the introduction of one deuterium at C-17 (Shapiro and Heath, 1967).

A 53% yield of dideuterio-5,16-androstadien-3β-ol(XX) was obtained, but the isotopic purity of this material was disappointingly low as can be seen by comparison of its mass spectrum (Figure 10a) with that of the protium form (Figure 10b). The deuterium labeled product (XX) contained as much as 45% of a d_1-impurity (m/e 275) and 4% of a d_0-impurity (m/e 274).

The low isotopic purity was not caused by incomplete deuterium exchange at C-16 of the 17-keto starting material (XVII). This was ruled out after comparison of its mass spectrum (Figure 11a) and the spectrum of the protium form (Figure 11b). For example, the spectrum of the labeled form shows that two deuteriums were added because the M^+, M^+-CH_3, M^+-H_2O and $M^+-(CH_3+H_2O)$ ions appear 2 amu's higher in the deuterium labeled form. The possibility that only one deuterium was attached to C-16 and that the other one was on the hydroxyl oxygen was excluded because the M^+-H_2O ion appears at m/e 272 (M^+-18) instead of m/e 271 (M^+-19). Also, the $M^+-(CH_3 + H_2O)$ ion is found at m/e 257 (M^+-33) instead of m/e 256 (M^+-34). Furthermore, comparison of other ions in the spectra of the deuterium and protium forms reveals mass differences of either 2 amu's or no mass differences, a result consistent with both deuteriums being located on

Figure 10. Mass Spectra of Deuterium and Protium Forms of 5,16-Androstadien-3β-ol.

a) 16,17-d_2-5,16-Androstadien-3β-ol; b) 5,16-Androstadien-3β-ol

Operating conditions are given in the legend to Figure 7.

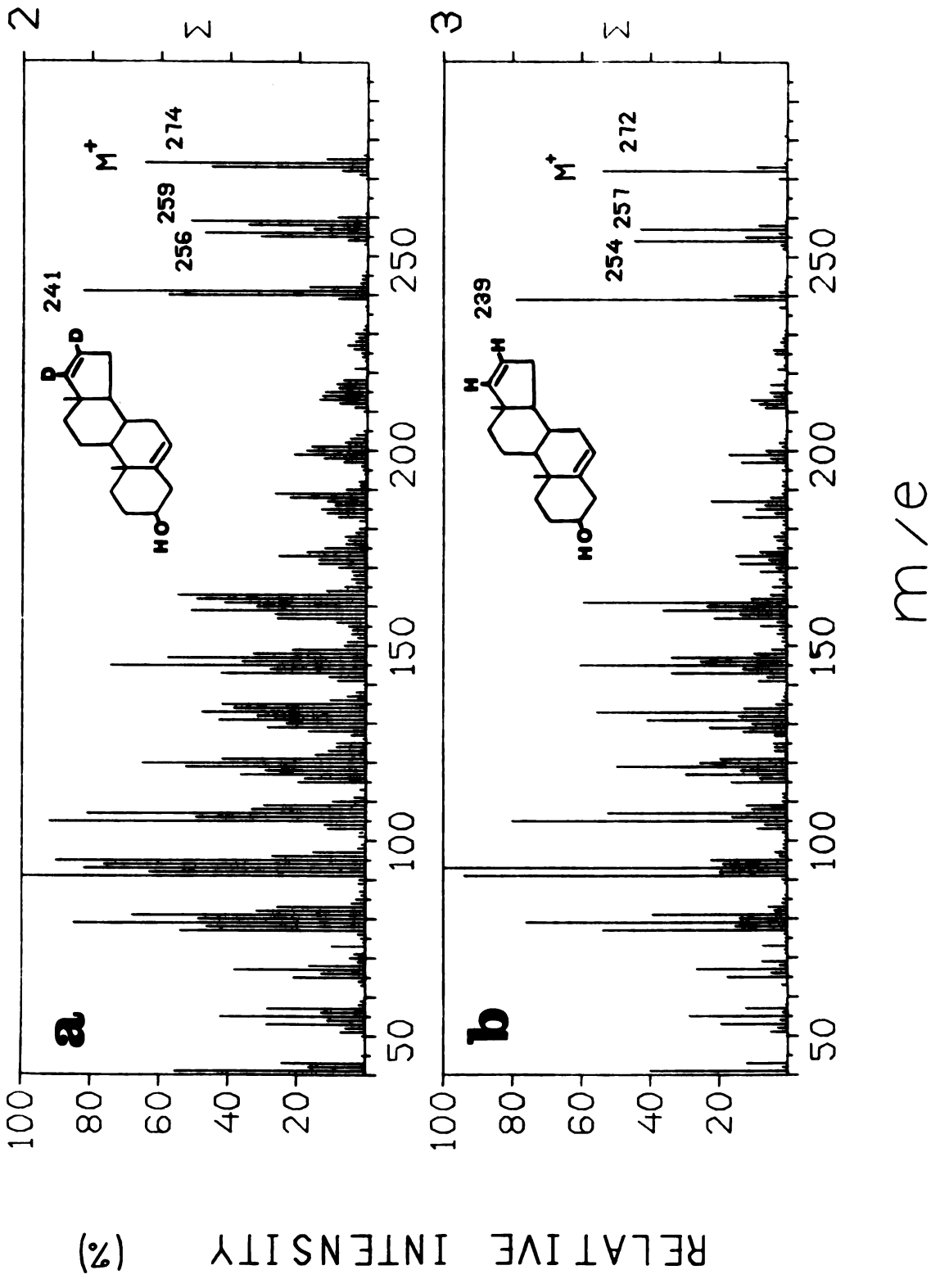
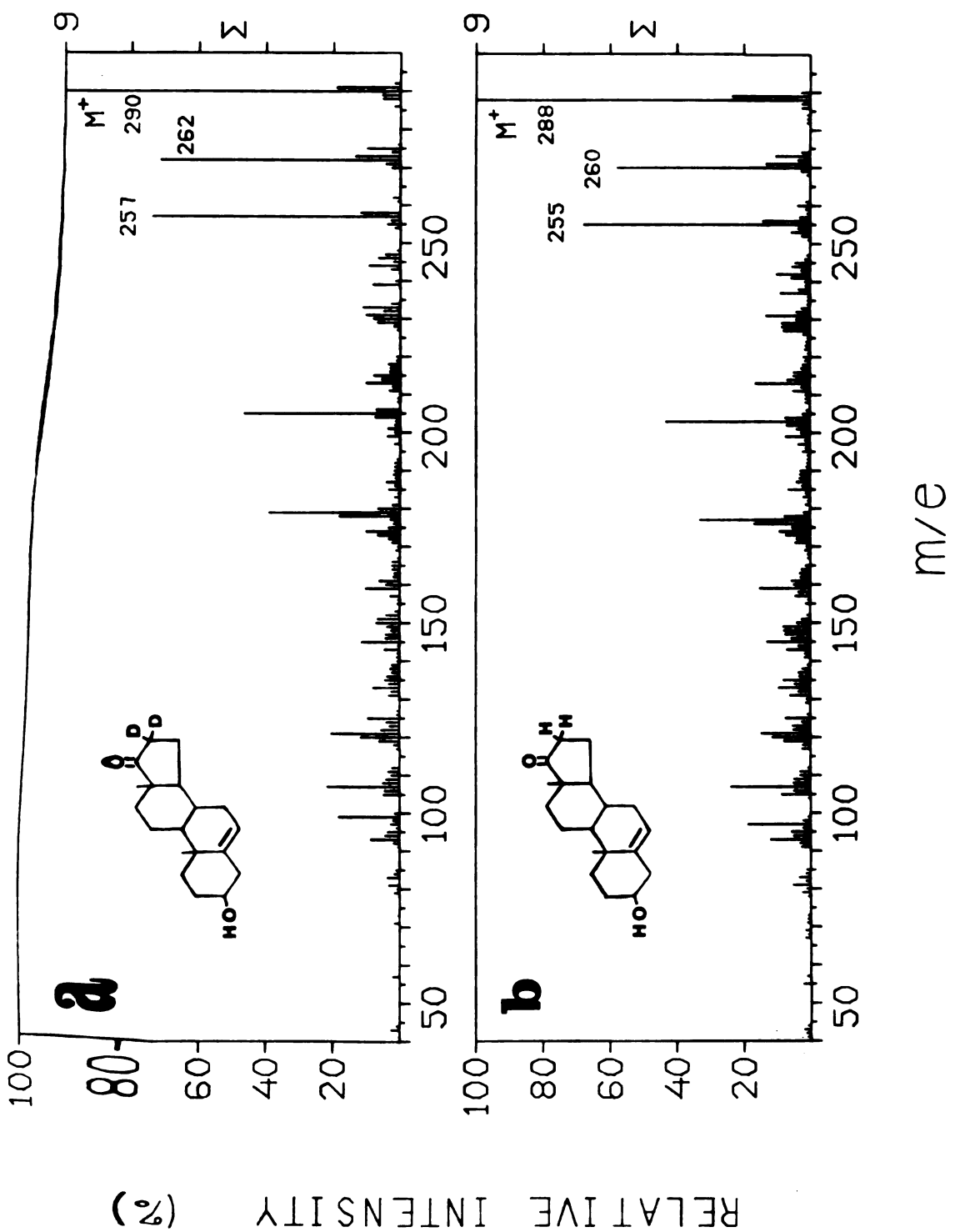

RELATIVE INTENSITY (%)
m/e
a
b
M+
241
256
259
274
239
254
257
272
HO
D
H

Figure 11. Mass Spectra of Deuterium and Protium Forms of 3ß-hydroxy-5-androsten-17-one.

a) 3ß-hydroxy-5-[16,16-d_2]androsten-17-one

b) 3ß-hydroxy-5-androsten-17-one

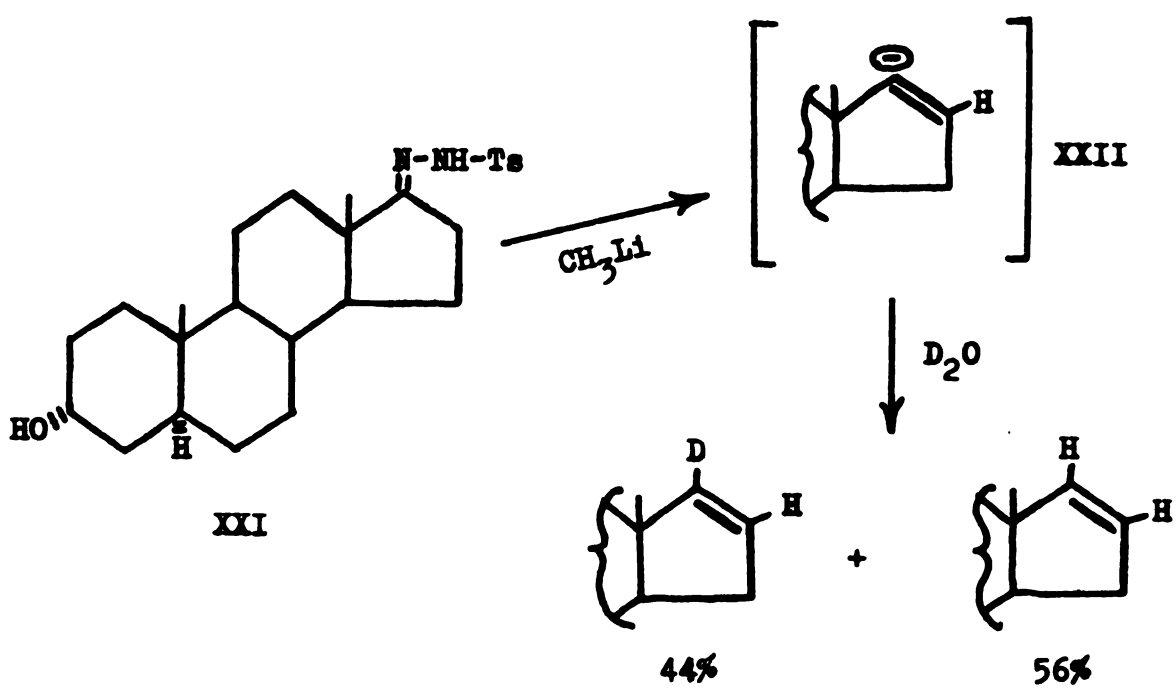
Mass spectra were obtained via the direct probe inlet and recorded at an ionizing potential of 22.5 eV.


a
b
M+
290
262
257
288
260
255
HO
D
H
100
80
60
40
20
50
100
150
200
250
m/e
RELATIVE INTENSITY (%)

one end of the molecule at C-16.

The view that both deuteriums were on C-16 is supported by the results of other workers who prepared 16,16-d_2-17-keto-steroids in high isotopic purity (89%-d_2; 11%-d_1) after only 4 hours exchange in methanol-OD/deuterioxide Tokes et al., 1967). The absence of base catalyst in the procedure described herein is counterbalanced by the longer exchange period which was used (7 days).

The results of a separate experiment indicate that the low isotopic purity was due to competition between the ether solvent and D_2O for protionation (deuteriation) of the anion intermediate (XIX). In this experiment the tosylhydrazone derivative of 3α-androst-5α-androstan-17-one (XXI) was treated with methyllithium and then D_2O was added as shown below:



The isotopic purity of the product was low (Figure 12a; 44%-d_1 and 56%-d_0) which supported the suspicion that the low isotopic purity observed earlier for 16,17-d_2-5,16-androstadien-3β-ol (XX) was the result of competition between D_2O and ether, and not the result of incomplete enolic exchange at C-16. Shapiro and Heath (1967) speculated about this competition in explaining the results of their experiments in which they obtained 17-d_1-16-androstene with an isotopic purity of 60%-d_1 and 40%-d_0 using diethyl ether as solvent. By contrast, Fischer et al. (1965) and Tökés and Djerassi (1969) obtained 89%-d_1 and 93%-d_1, respectively, when a similar reaction was performed with dioxane as solvent. These observations suggest that subsequent labeling experiments of this type should be performed in dioxane solvent instead of dimethoxyethane or diethyl ether in order to achieve the highest possible isotopic purity.

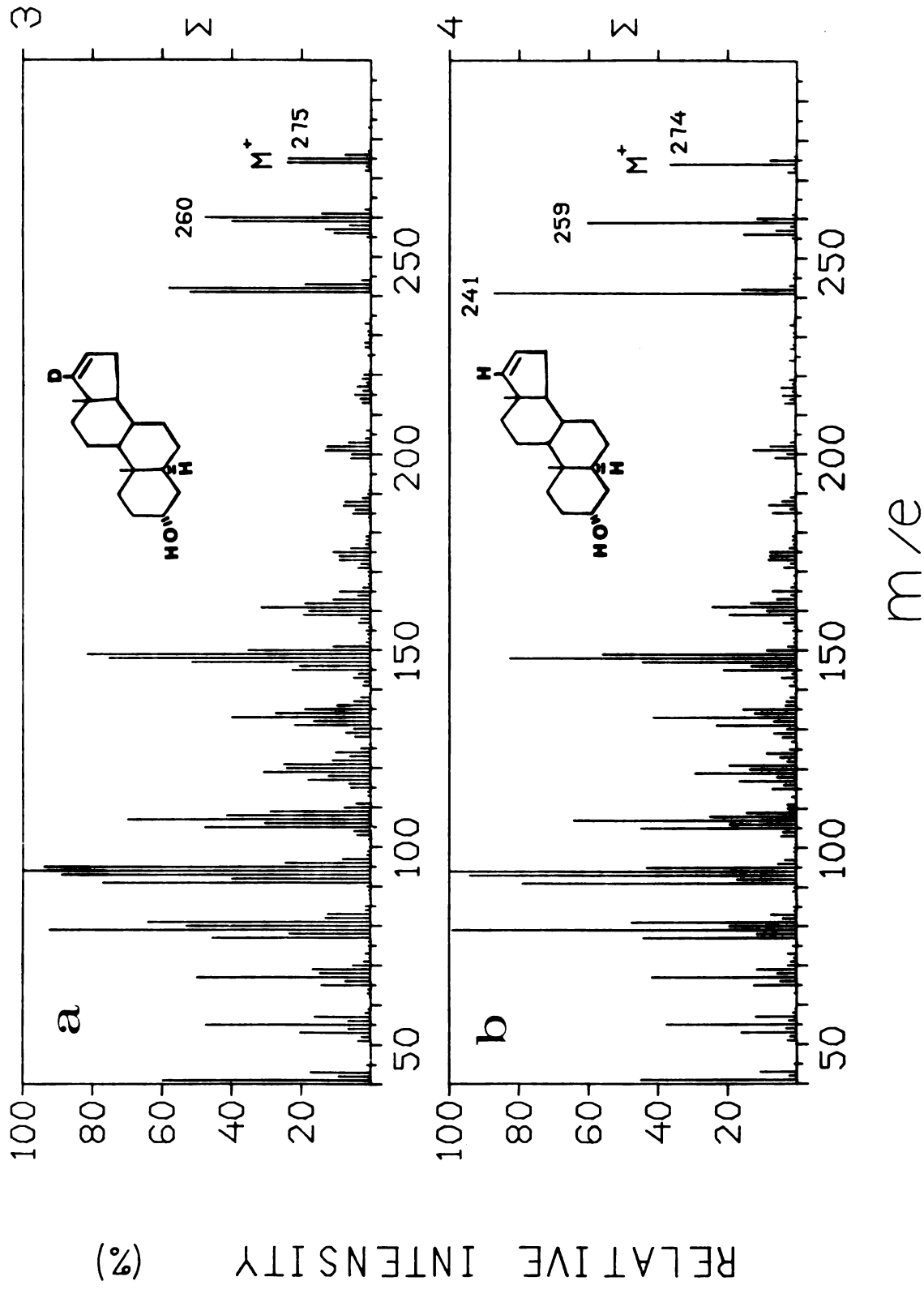
Even a d_2-form of 5,16-androstadien-3β-ol of high isotopic purity would not be a convenient internal standard because of its tendency to give M^+-2 ions upon mass spectral analysis. The M^+-2 ion can best be seen in the spectrum of the pure unlabeled form shown in Figure 10b. The M^+-2 ion arising from the d_2-labeled internal standard would contribute to the variable background which must be substracted from measurements made of the M^+ ion of the protium form.

The 16,17-d_2-5,16-androstadien-3β-ol (XX) could be transformed into a d_3, d_4, d_5 or d_7-form by procedures used by

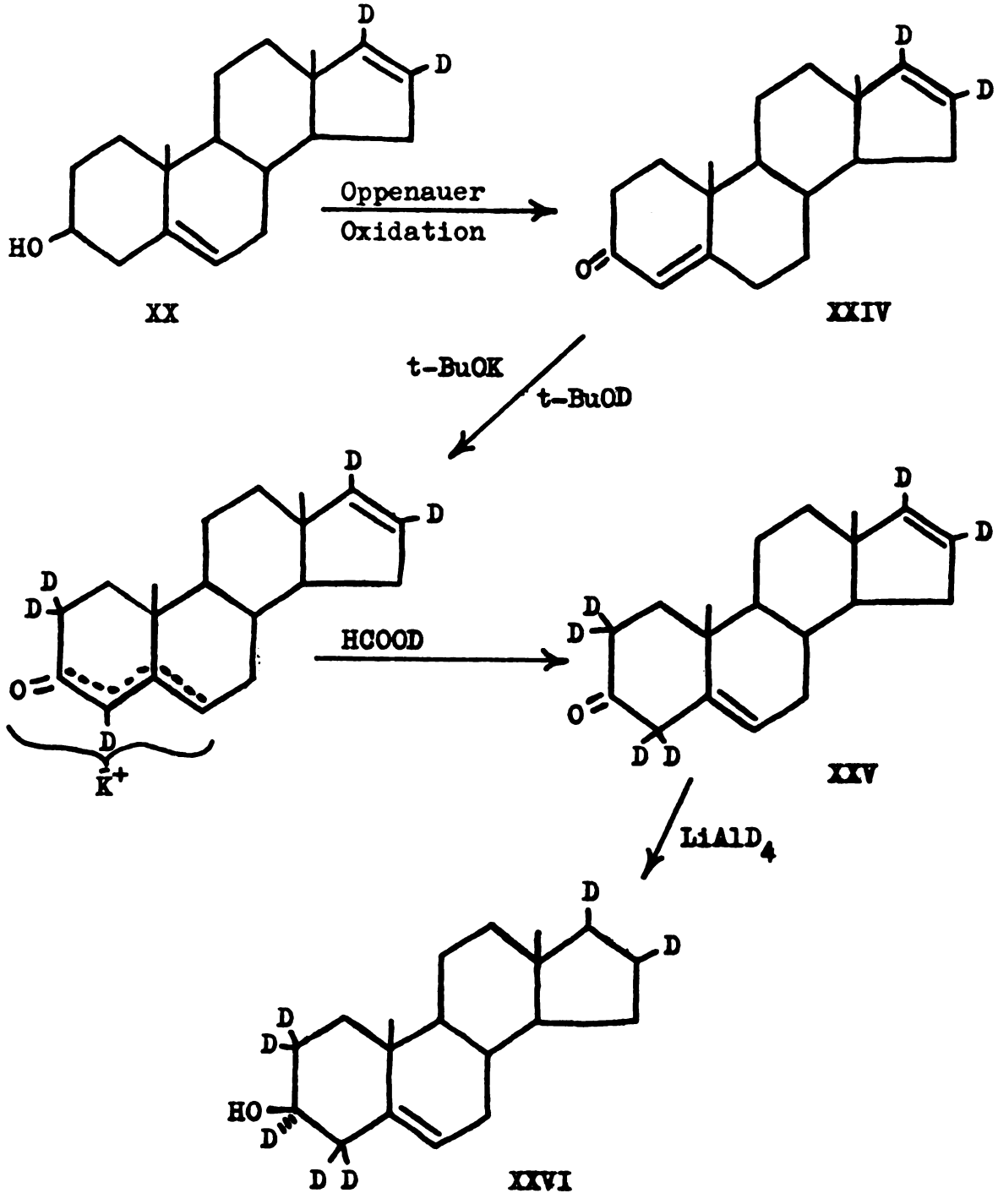
Figure 12. Mass Spectra of Monodeuterated and Protium Forms of 5α-Androst-16-en-3α-ol.

a) 17-d_1-5α-Androst-16-en-3α-ol; b) 5α-Androst-16-en-3α-ol

Operating conditions are given in the legend to Figure 7.


a
b
M+
275
260
274
259
241
HO
D
H
m/e
RELATIVE INTENSITY (%)

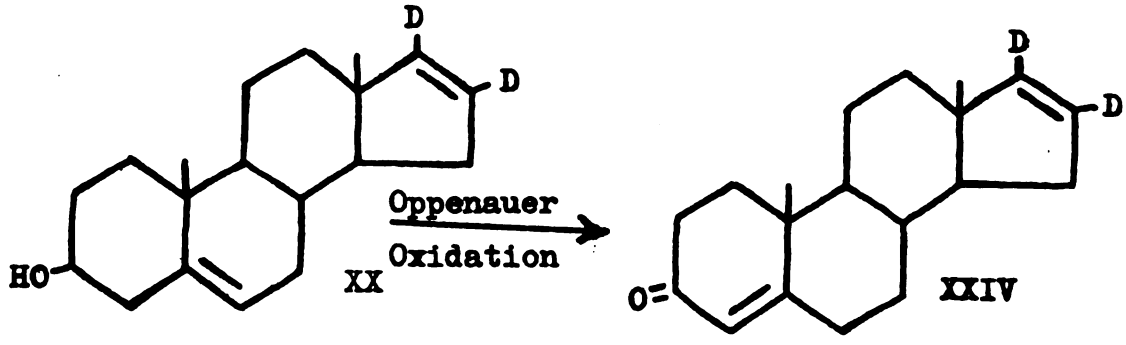
Bjorkhem *et al.* (1973,1974) for preparation of other Δ^5-3β-ol steroids with deuterium label on C-2, C-3 and C-4. The proposed scheme of reactions is shown below:



The first step involves the conversion of the Δ^5-3β-ol steroid (XX) to the corresponding Δ^4-3-one (XXIV) by Oppenauer oxidation, described later herein. Deconjugation of the Δ^4-3-one by equilibration in t-butyl alcohol-OD and potassium t-butoxide, followed by acetic acid-COOD protonation (deuteriation) of the anion would yield the perdeuterio Δ^5-3-one (Ringold and Malhotra, 1962). This material (XXV) could be treated immediately with $LiAlD_4$ which would result in the Δ^5-3β-ol (XXVI) with as many as 7 deuteriums. Alternatively, a d_3-form (3α,16,17-d_3), a d_4-form (4,4,16,17-d_4) or a d_5-form (3α,4,4,16,17-d_5) could be prepared by variations of this procedure as shown by Björkhein et al. (1974) in work with 3β-hydroxy-5-androsten-17-one. Perdeuterio-5,16-androstadien-3β-ol made in this way would be a useful internal standard and carrier in the assay of 5,16-androstadien-3β-ol in the tissues and fluids of pigs and humans.

16,17-d_2-4,16-Androstadien-3-one

The d_2-form of 4,16-androstadien-3-one (XXIV) was prepared from 16,17-d_2-5,16-androstadien-3β-ol (XX) by Oppenauer oxidation performed according to the procedure described by Matthews (1968).



The reaction gave an 88% yield (5.3 g) of d-labeled 4,16-androstadien-3-one (XXIV), but no plans were made to use this material as an internal standard/carrier after the extent of its isotopic impurity was revealed by the results of mass spectral analysis shown in Figure 13a and b.

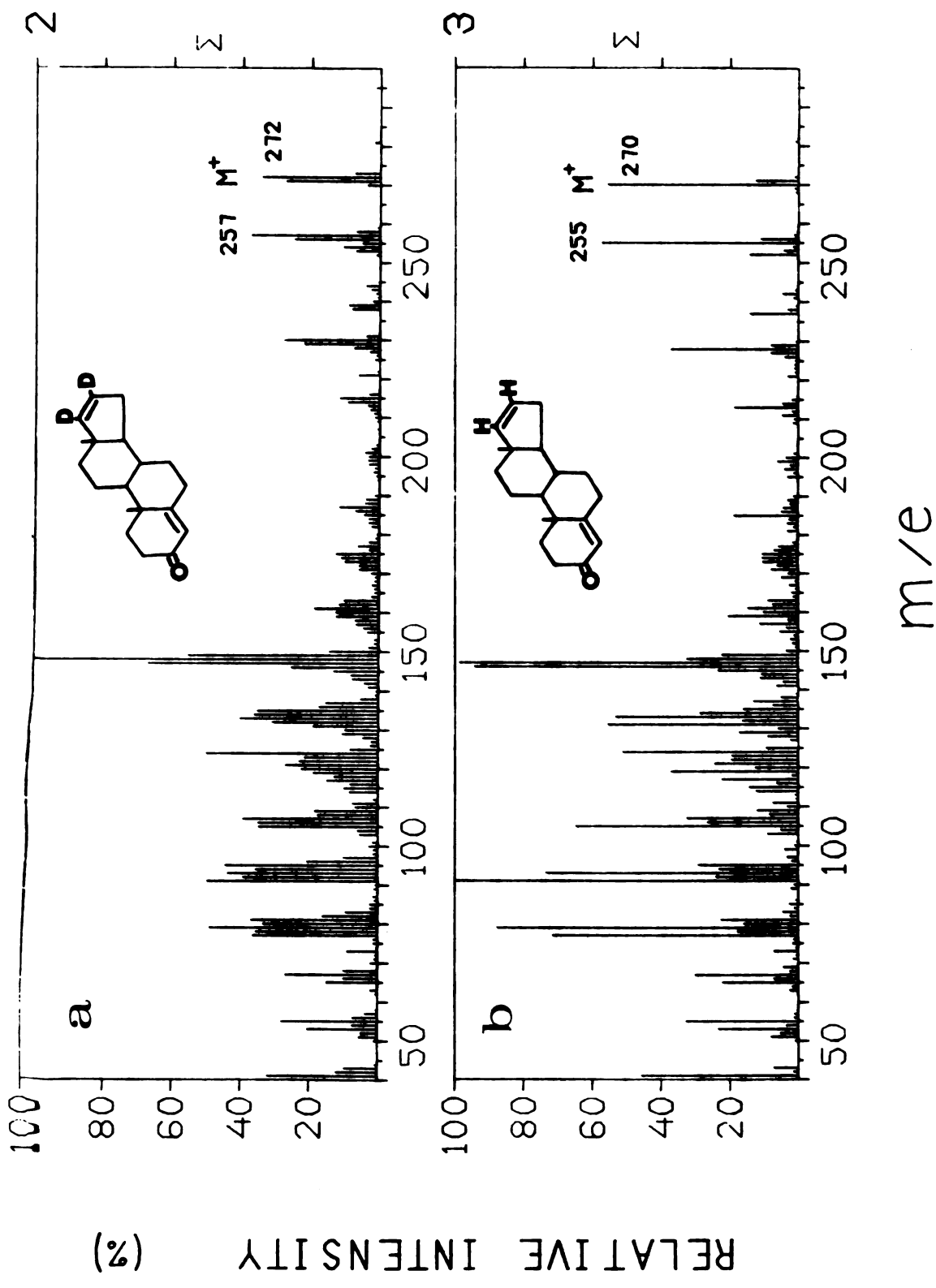
Unfortunately, this synthetic experiment was performed before checking the isotopic purity of the starting material (XX), and as a result, no useable material was obtained for use as a carrier in the assay of 4,16-androstadien-3-one. However, the product was valuable as starting material for the preparation of other deuterium labeled standards, including 5α-androst-16-en-3-one (XIV), 5α-androst-16-en-3β-ol (XV) and 5α-androst-16-en-3α-ol (XVI).

The d_0 and d_1 impurities seen in the mass spectrum of 4,16-androstadien-3-one (Figure 13a) were a direct result of the low isotopic purity observable in the mass spectrum of the starting material (XX) shown in Figure 10a. It is now known that a more pure d-labeled 5,16-androstadien-3β-ol (XX) can be prepared with only minor modifications of the methods, as discussed earlier herein.

Figure 13. Mass Spectra of Deuterium and Protium Forms of 4,16-Androstadien-3-one.

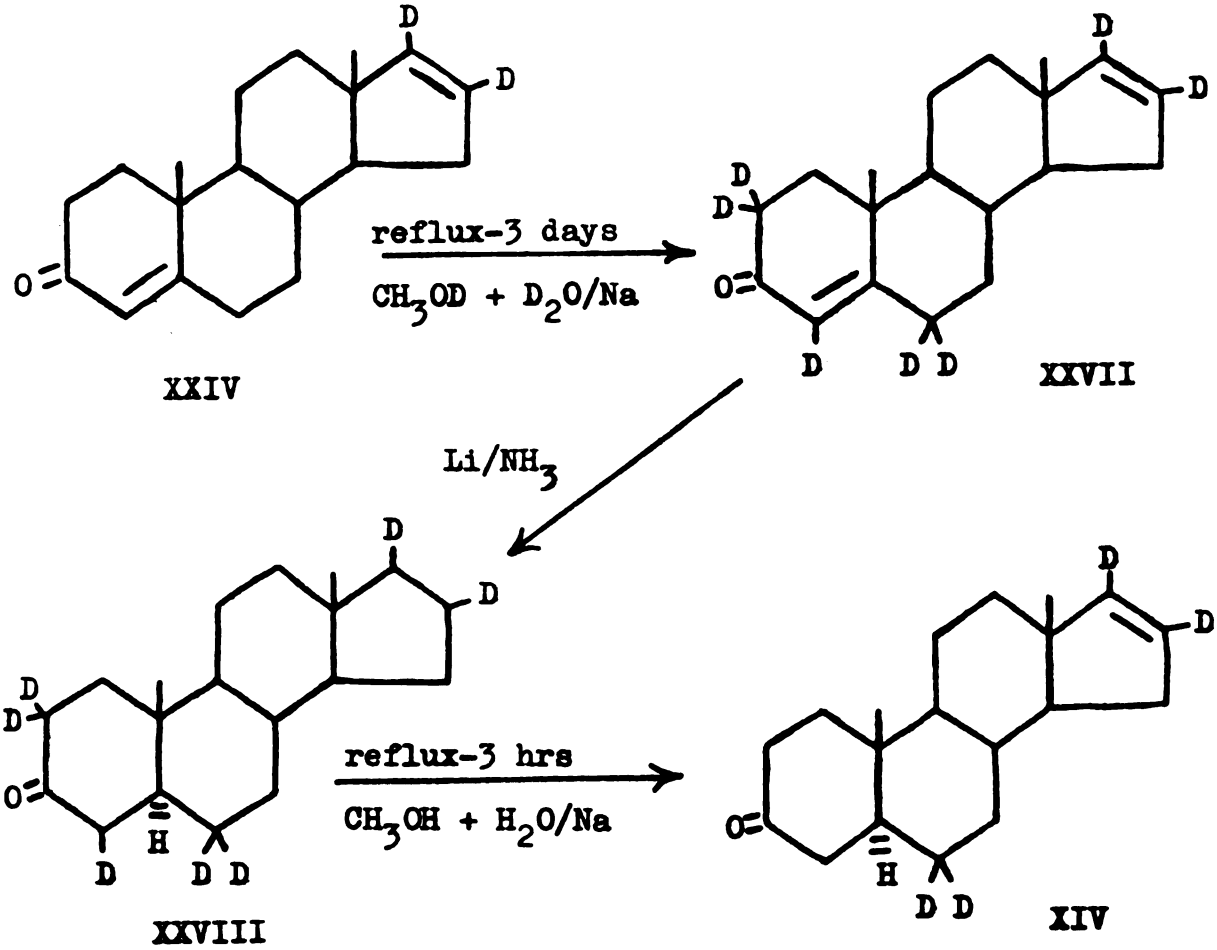
a) 16,17-d_2-4,16-Androstadien-3-one; b) 4,16-Androstadien-3-one

Operating conditions are given in the legend to Figure 7.


2
Σ
a
257 M+
272
D
D
O
3
Σ
b
255 M+
270
H
H
O
100
80
60
40
20
50
100
150
200
250
m/e
RELATIVE INTENSITY (%)

6,6,16,17-d_4-5α-Androst-16-en-3-one

The steps used to prepare this material are shown below:



The activated hydrogens in 16,17-d_2-androstadien-3-one (XXIV) were exchanged with deuterium by equilibration in methanol-OD/D_2O. The product (XXVII) was reacted with a solution of lithium metal in liquid ammonia which reduced the Δ^4-double bond and yielded 2,2,4,6,6,16,17-d_7-5α-androst-16-en-3-one (XXVIII). The exchangeable deuteriums at C-2 and C-4 were removed by back exchange in methanol-OH/H_2O. The final product, 6,6,16,17-d_4-5α-androst-16-en-3-one (XIV), was chromatographed on a column of silver nitrate

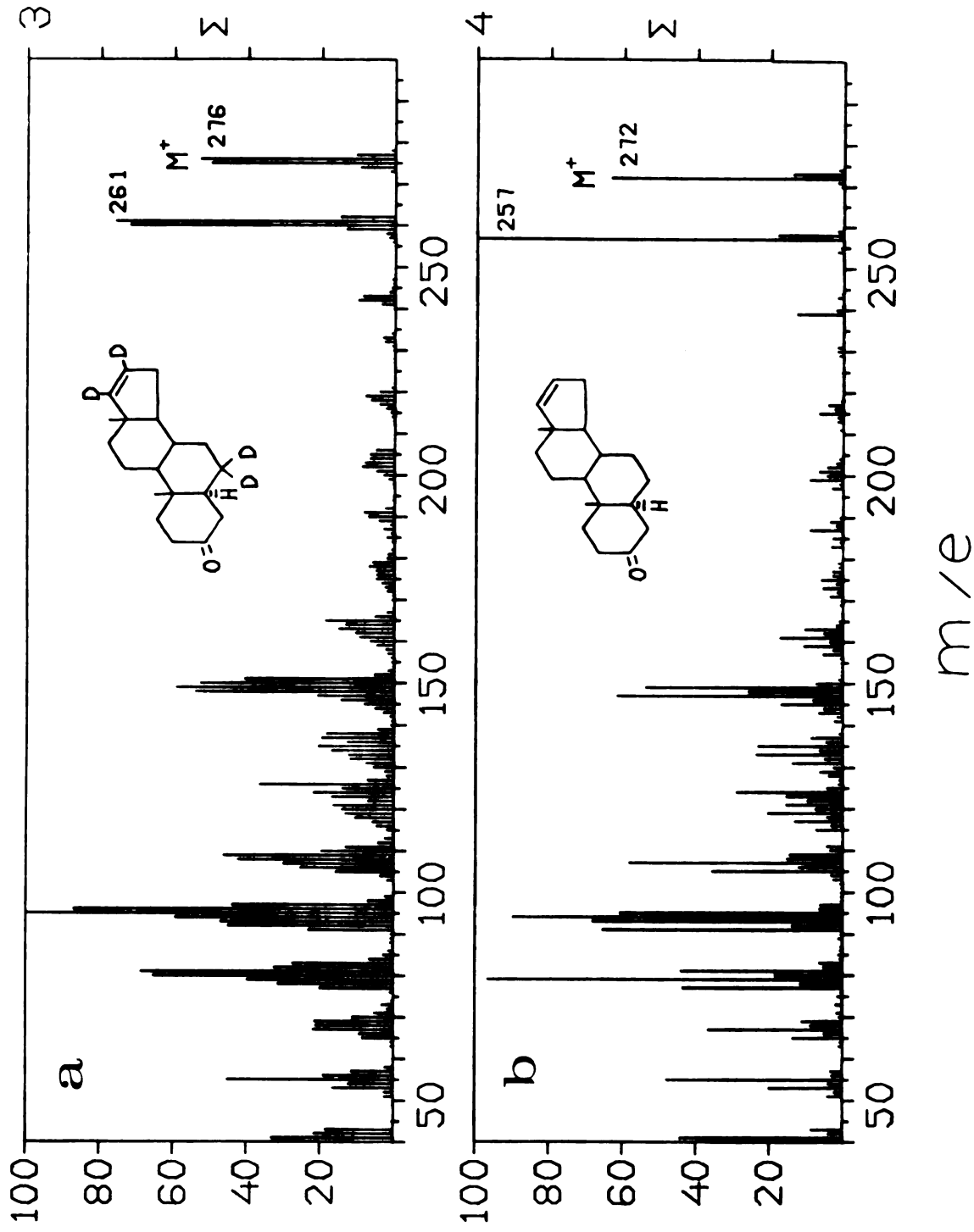
impregnated silica gel and crystallized from acetone-hexane. The procedure gave 0.7 g or 35% yield of the deuterated steroid. The mass spectra of the deuterium and protium forms of 5α-androst-16-en-3-one are shown in Figure 14a and b. The absence of an ion at m/e 272 (M^+ for protium form) in the bar graph for the deuterium form is a good indication that this material would be very useful as a carrier and internal standard for the assay of 5α-androst-16-en-3-one by mass spectrometric reverse isotope dilution.

Time did not permit analysis of the fat samples using all of the labeled steroids. However, the labeled compounds are now available for future studies.

Figure 14. Mass Spectra of Deuterium and Protium Forms of 5α-Androst-16-en-3-one

a) 6,6,16,17-d_4-5α-Androst-16-en-3-one; b) 5α-Androst-16-en-3-one

Operating conditions given in the legend to Figure 7.


3
Σ
M+
276
261
a
4
Σ
M+
272
257
b
100
80
60
40
20
50
100
150
200
250
m/e
RELATIVE INTENSITY (%)

SUMMARY

A reverse isotope dilution/carrier technique was adapted for determination of 5α-androst-16-en-3-one. After the addition of a deuterium labeled form of 5α-androst-16-en-3-one to the fat sample, the mixture of deuterium and protium forms was purified and the resultant fraction was analyzed by combined gas chromatography-mass spectrometry. The deuterium/protium ratios were obtained by multiple ion detection using the accelerating voltage alternator accessory of an LKB 9000 gas chromatograph-mass spectrometer.

The addition of a labeled form of the steroid enabled extremely small amounts of the compound to be partially purified and separated by gas chromatography without total loss by adsorption. Losses during the isolation were automatically accounted for since the ratio of the labeled and unlabeled species would remain constant during isolation and analysis. The amount of unlabeled 5α-androst-16-en-3-one was determined from the isotope ratio resulting after the addition of a known amount of the labeled species. This procedure was shown to be useful in analyzing for 5α-androst-16-en-3-one in fat samples.

Deuterium labeled forms of the five most common C_{19}-Δ^{16} steroids were synthesized to provide standards for quantitative analysis for these compounds in the pig. The labeled C_{19}-Δ^{16}steroids that were synthesized include

the following compounds: d_2-, d_3-, and d_4-5α-androst-16-en-3-one; d_4-5α-androst-16-en-3α-ol; d_4-5α-androst-16-en-3β-ol; d_2-4,16-androstadien-3-one and d_2-5,16-androstadien-3β-ol.

Several fat samples from cryptorchid (males with retained testicles) pigs were analyzed for 5α-androst-16-en-3-one by stable isotope dilution and the levels were correlated with odor intensity scores by both a trained laboratory panel and a meat industry panel. The results for the meat industry panel were not statistically significant. Although the trained laboratory panel scores were significantly ($P<.05$) correlated with the levels of 5α-androst-16-en-3-one, this only accounted for 25% of the variation in scores. This suggests that 5α-androst-16-en-3-one is not the only compound contributing to the undesirable odor in boar fat. Further support for this viewpoint is evident by the fact that several fat samples having high odor intensity scores were found on analysis to have only low levels of 5α-androst-16-en-3-one. It seems likely that the other C_{19}-Δ^{16} steroids may account for some of the discrepancies in the results.

Although the labeled C_{19}-Δ^{16} steroid standards were synthesized and are now available in our laboratory, time did not permit analysis. These labeled standard compounds will be useful in subsequent studies on the significance of the C_{19}-Δ^{16} steroids in both pigs and human beings.

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