STUDIES IN STEROL BIOSYNTHESIS

An investigation into the mode of cyclization of squalene during conversion *in vivo* to cholesterol

BY

FREDERICK PATRICK ROSS B.Sc. (Honours) M.Sc (Rand)

submitted in partial requirement for the degree of Doctor of Philosophy in Molecular Sciences at the University of Warwick.

This research was conducted at the Milstead Laboratory for Chemical Enzymology, Sittingbourne, Kent, England.

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DIAGRAMS AND TABLES

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DECLARATION

The work described in this thesis has not been used before. As mentioned in the text the conversion of { 2, 11 (12), 16, 23-$^{3}H_{4}$} cholesterol to 1, 4-androstadiene-3, 17-dione was performed by Dr. G.T. Phillips. Dr. Phillips was able to improve the yield of the microbiological transformation, an approach which was initiated by the author, who also performed all preliminary experiments in this area.

F.P. Ross

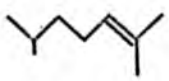
F.P. ROSS.

ABBREVIATIONS

The following abbreviations have been used in the text :-

DCCD	=	Dicyclohexylcarbodi-imide
FPP	=	Farnesyl pyrophosphate
THF	=	Tetrohydrofuran
DMSO	=	Dimethylsulphoxide
NADH	=	Nicotinamide Adenine Dinucleotide, reduced form.
NADPH	=	Nicotinamide Adenine Dinucleotide Phosphate, reduced form.
NSL	=	Non-saponifiable lipids
J	=	Nuclear Magnetic Resonance coupling constant
νmax	=	Wavelength of absorbtion maximum in the infra-red spectrum.
λ max	=	Wavelength of absorbtion maximum in the ultravioiet spectrum.
ε	=	Molar extinction coefficient
K_m	=	Michaelis constant
V_{max}	=	Maximal velocity of an enzymic reaction or process.
g. l. c.	=	Gas-liquid chromatography
t. l. c.	=	thin layer chromatography
p. l. c.	=	preparative thin layer chromatography.
R_f	=	Distance moved relative to solvent front.
N. M. R.	=	Nuclear Magnetic Resonance

In Figs. 3 and 4.

R_1	=	Homogeranyl
R_2^1	=	- Adenyl Dinucleotide
R_3	=	
R_4	=	bis-nor - R_3
R_5	=	dihydro R_3

SUMMARY

The pathways by which sterols are synthesized from acetate are now known in some detail, but numerous questions remain unanswered. One such question is whether squalene which is an intermediate in the sequence leading to all sterols, undergoes further metabolism with differentiation between the two halves of molecule.

In an attempt to answer this question a sample of (5S, 3RS)-{5-^{3}H} mevalonic acid was prepared by a combination of enzymic and chemical reactions. This material, when converted to squalene, yields a molecule which, at the moment of synthesis, has the two halves labelled differently. The further metabolism of this compound before the two non-equivalent halves become 'scrambled' will result in sterols having a non-uniform distribution of label. The determination of the labelling pattern in the steroids will therefore indicate whether or not differentiation of the two halves of squalene occurs. This problem has been investigated in non-mammalian systems and differentiation does not occur. In mammalian systems faulty experimental design, using *in vitro* incubations in which the process under study may have been disrupted, leaves the answer uncertain.

In this investigation the labelled precursor was converted to cholesterol *in vivo* ensuring that if differentiation occurs it will be detected. The labelled cholesterol was degraded by a combination of microbiological and chemical reactions. The pattern of labelling seen indicated that there is no differentiation of the two halves of squalene during its conversion in the rat to cholesterol.

CHAPTER I

INTRODUCTION

Mevalonic acid (II) is an important intermediate in the biogenesis of a large number of natural products [1]. It is formed via a pathway (Fig. 1) in which three molecules of acetyl Coenzyme A are condensed to give (S) *-3-hydroxy-3methylglutaryl Coenzyme A (I), which is then reduced using two equivalents of NADPH, to (II). Evidence has been obtained recently which implicates a hemithioacetal as an intermediate in this reduction [2]

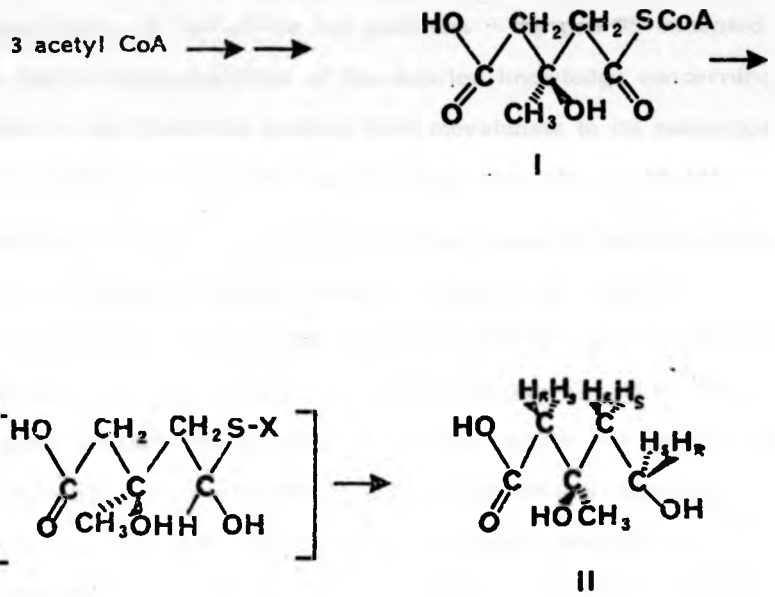


Fig. 1

The reduction is enzymatically irreversible, and so this step is an important site of metabolic control. The reaction is subject to feedback inhibition which limits the rate of enzyme synthesis [3], a process which also exhibits a natural diurnal variation [4].

* For definitions relating to chiral and pro-chiral groups and their stereochemical specification see reference number [5].

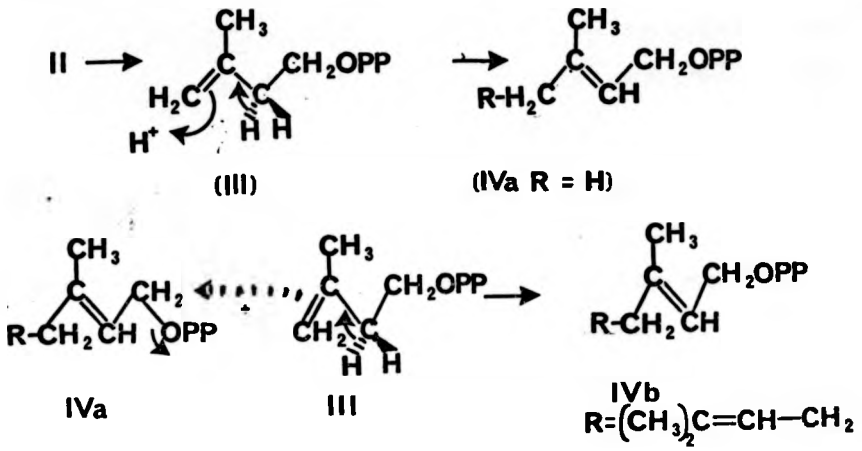
The (3R) and (3S) isomers of mevalonic acid have been synthesized [6] and it has been shown that in the rat [6], in the mouse [7], in a Mycobacterium [8] and in the fungus Claviceps purpurea [9] the (3R) insomer is used exclusively. These findings apparently constitute the experimental basis for the general assumption that (3S) mevalonate is inert in all biological systems.

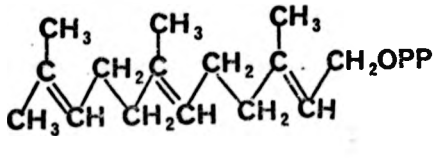
The structure of (II) includes three pairs of pro-chiral hydrogen atoms, at C-2, C-4 and C-5 (Fig. 1). The availability of mevalonic acid labelled stereospecifically at five of the six positions - the pro-5S excepted - has been a major tool in the acquisition of the detailed knowledge concerning the mechanism of the reactions leading from mevalonate to its numerous metabolic products. (For detailed reviews, see refs. 1, 10-12).

The positions C-2 and C-4 were labelled by chemical methods using reactions of known stereospecificity on reactants of known stereochemistry[13]. Each final product contained a pair of enantiomers, of which only the (3R) insomer is used (vide supra). Since the relationship between the chirality at C-3 and that at C-2 or C-4 was known from the earlier chemistry, the product was biologically homogeneous. The (5R) compound was prepared enzymically[14], using the stereospecificity of the enzyme mevaldate reductase to introduce the label. This enzyme did not distinguish between the enantiomers at C-3 in the substrate and so the final product was also racemic at C-3.

It was the purpose of the present study to synthesize (5S)-[5-^{3}H] mevalonic acid and to use this material to study certain aspects of sterol biosynthesis. A short report on the successful synthesis has been published simultaneously with two other syntheses of the same compounds[15], the latter two being based on the two reductive enzyme reactions discussed above.

The subject of sterol biosynthesis from mevalonic acid has been reviewed extensively and in depth[1, 10, 12]. Only those aspects which are directly relevant to the present work will be discussed in detail. The results obtained so far justify the predictions of the theoretical "isoprene rule"[16], but in addition they have revealed more details of the reactions involved than could have been predicted. The series of reactions leading from mevalonate to sterols may be considered logically in two sequences. The first of these is the synthesis of farnesyl pyrophosphate (FPP) (V) from mevalonic acid (II) by soluble enzymes, and appears to be common to all sterol-producing systems (Fig. 2).





(V)

Fig. 2

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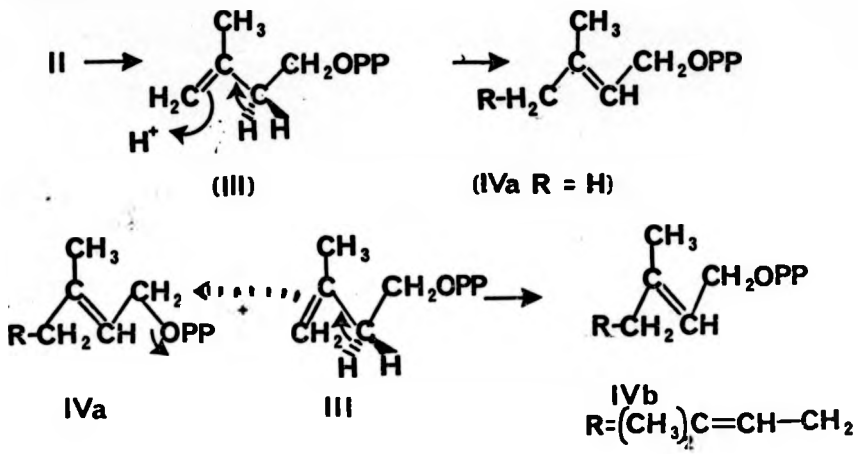

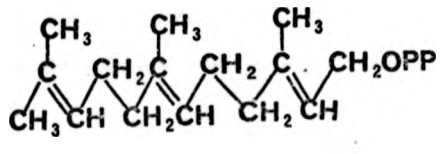

(V)

Fig. 2

Isopentenyl prophosphate (III) is formed from (II) by a series of well-documented reactions[17]. An isomerase converts (III) to (IVa), which alkylates (III) to produce (IVb). In an analogous reaction (IVb) reacts with (III) to give (V).

The recent report[18] on the stereochemistry of the protonation of (III) completes the knowledge of the stereospecificity of this set of reactions.

The second sequence of reactions (Fig. 3), from farnesyl pyrophosphate (V) to the individual sterols, is catalysed by membrane-bound enzymes. Two molecules of FPP (V) are condensed to produce presqualene pyrophosphate (VI) which is converted in consecutive reactions, dependent on NADPH and oxygen respectively, into squalene (VII) and (3S)-2,3-oxidosqualene (VIII). The enzyme oxidosqualene cyclase[19], initiates proton attack on (VIII), producing the carbonium ion (IXa), possibly as the stabilized intermediate (IXb)[20].

In a series of 1,2 shifts by hydrogen and methyl groups[21-25] the carbonium ion centre is transferred to C-8 (X), where it may be stabilized in one of two ways. Loss of the C-9 hydrogen leads directly to lanosterol (XI), the first free sterol in mammalian systems. Alternatively, this same hydrogen may shift to C-8, producing a new carbonium ion at C-9 (Xa), which may lead to two products. Loss of the proton from C-8 would yield (XI) once more, while a C-9 - C-19 cyclopropyl ring will result from loss of a C-19 proton, leading to cycloartenol (XIII), the first phytosterol. This latter pathway occurs in plants with retention of the hydrogen originally at C-18[26], but it is not possible to distinguish the alternatives in the biosynthesis of lanosterol. Further oxidative procedures, as yet only partly understood[27], produce the large variety of sterols and derived compounds, amongst which cholesterol (XIII) is the most abundant mammalian sterol.

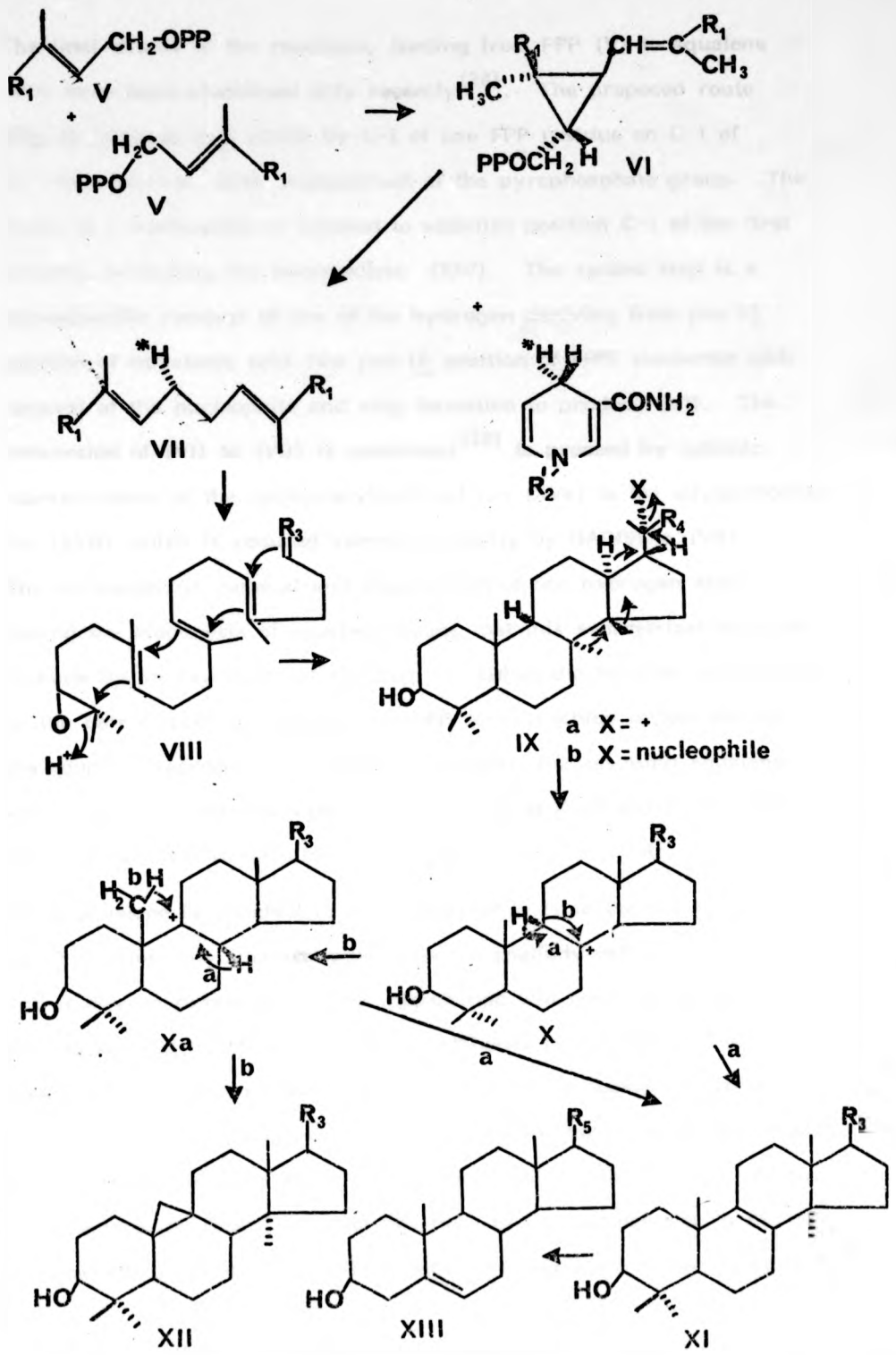
CH2-OPP
R1
V
H2C
PPO
R1
V
R1
CH=C
R1
CH3
H3C
PPOCH2 H
VI
*H
R1
R1
VII
*H H
CONH2
N
R2
R3
O
H+
VIII
X
R4
H
H
HO
IX
a X = +
b X = nucleophile
R3
H2C
b H
a
H
HO
Xa
b
R3
H
b
a
HO
X
a
a
b
R3
HO
XII
R5
HO
XIII
R3
HO
XI

Fig. 3

The final details of the reactions, leading from FPP (V) to squalene (VII) have been elucidated only recently[28]. The proposed route (Fig. 4) involves S_N2 attack by C-2 of one FPP residue on C-1 of the other residue, with displacement of the pyrophosphate group. The action of a nucleophile is invoked to stabilize position C-1 of the first residue, producing the intermediate (XIV). The second step is a stereospecific removal of one of the hydrogen deriving from pro-5S position of mevalonic acid (the pro-1S position of FPP) concerted with removal of the nucleophile and ring formation to produce (VI). The conversion of (VI) to (VII) is postulated[28] to proceed by cationic rearrangement of the cyclopropylcarbinyl ion (XVa) to the allylcarbonium ion (XVb) which is reduced stereospecifically by NADPH to (VII). The stereospecific removal and replacement of one hydrogen atom during the biogenesis of squalene means that this symmetrical molecule is made by an asymmetrical reaction. If either the hydride ion inserted or the pair of pro-1S hydrogens of FPP (one of which is lost during the coupling reaction) is isotopically labelled, the resulting squalene will contain two non-equivalent carbon atoms at C-12 and C-13, only one of which will be labelled (Fig. 4, (VII) H_S or H = *H).

While squalene is attached to the synthetase enzyme the two halves, which are formally identical, will exist in spatially different environments, and it may be presumed that no interchange will occur which would lead to scrambling of the two halves. When a single molecule of squalene is transferred from the synthetase to the epoxidase two types of pathway can be envisaged: (a) a random process in which the molecule exists unbound at some stage during the transfer, and in which the two ends have equal probabilities of attaching to a particular site in the new enzyme-substrate complex; (b) a spatially-controlled transfer in which

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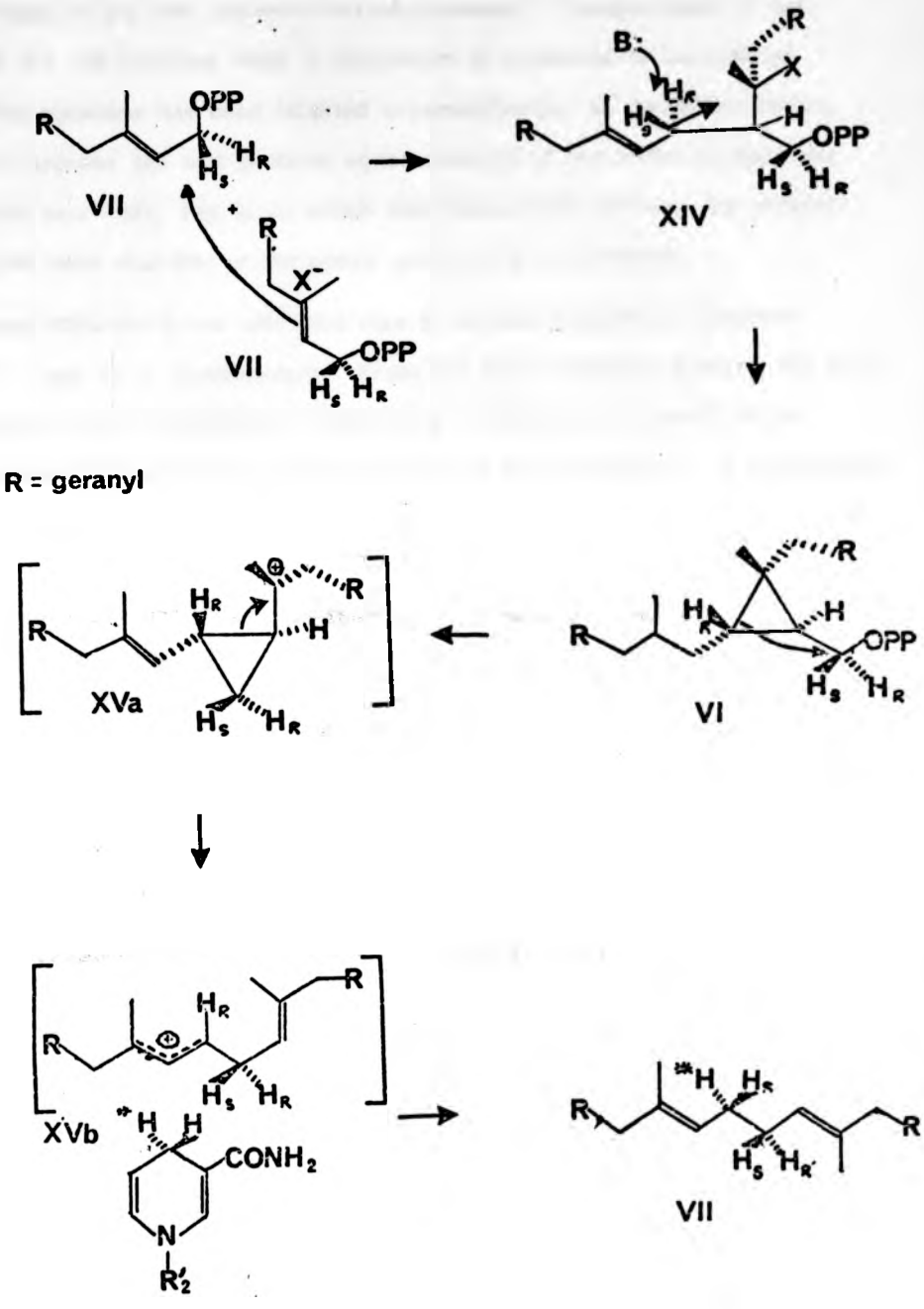

Fig. 4

a particular half of the molecule always occupies a particular spatial position in the new asymmetrical environment. Combinations of (a) and (b) are possible when a population of molecules is considered. If the squalene has been labelled asymmetrically, as described above, then process (a) will produce equal amounts of two different epoxides (VIIIa and VIIIb, Fig. 5) in which the relationship between the position of the label and that of the epoxy group will be different.

These different forms will give rise to sterols labelled at positions 11-β and 12 α respectively. From the same squalene process (b) will produce only one epoxide, (VIIIa or b, depending on which end of squalene is presented to the active site of the epoxidase). A combination of (a) and (b) will produce the two forms in unequal amounts.

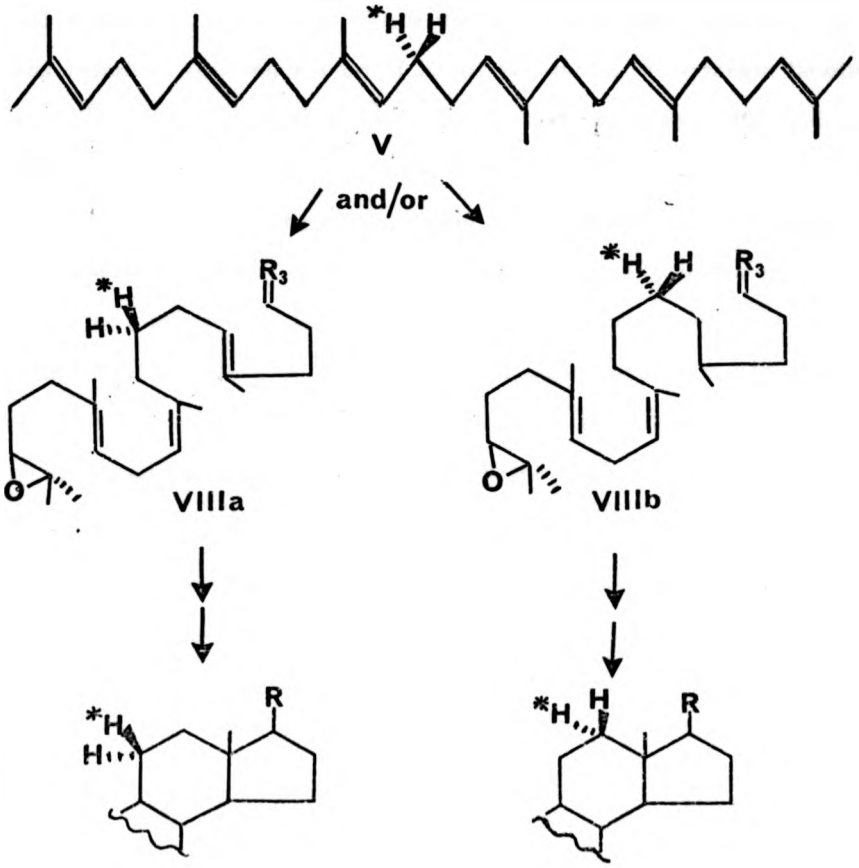

Fig. 5

An analysis of the labelling pattern of the resultant sterols will yield information on the specificity of the transfer of squalene from the synthetase to the epoxidase. If the process is random equal amounts of the 11β- and 12α-labelled sterols will be produced. Any divergence from unity in the ratio of label at C-11 to that at C-12 is a measure of the degree of specificity in the transfer reaction.

Studies with isotopic tracers suggest that, in the rat liver at least, two pools of squalene exist, one of which is metabolically more active than the other. Popjak[29] converted {1-^{14}C} sodium acetate to squalene and cholesterol using rat liver slices. Carrier squalene was added and the non-saponifiable lipids were separated into squalene and cholesterol. The specific activity of the endogenous squalene, calculated from the measured result and the known dilution factor, was about 0,1μ Ci/μmole. Since the cholesterol contained 3μCi of ^{14}C this required the conversion of about 30μ moles of squalene labelled with ^{14}C. This amount is much larger than the size of the endogenous pool, about 0,3μ moles under the conditions used, and might be explained by very rapid and complete turnover of the pool about 100 times. But this would inevitably mean that the specific activity of the squalene would be higher than that of the cholesterol, for there was much more endogenous cholesterol than endogenous squalene in the preparation (liver slices supplemented with egg-yolk). In fact the specific activity of the squalene was lower than that of the cholesterol.

The alternative hypothesis is that only a small fraction of the total squalene becomes labelled, at a much higher specific activity. This "metabolically active" squalene might "leak" slowly into the bulk pool (Fig. 6) with most of the newly biosynthesized squalene in the bound

form. If these conditions existed the experimental results could be explained by a very small turnover of the active form of squalene.

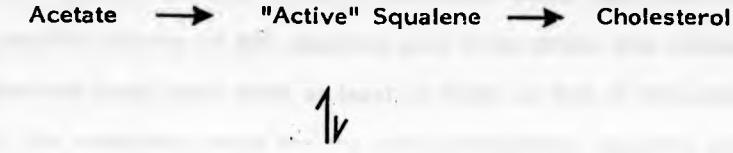

Fig. 6

Similar but more convincing results were obtained by Loud and Bucher[30] in a series of in vivo experiments. Intact rats were injected with {1-^{14}C} sodium acetate in tracer quantities and portions of each liver, exposed under anaesthesia, were removed at various times. Thus for each animal the time course of labelling could be determined and the results, in such a highly active metabolic system, are particularly useful since they are free from the error introduced by the pooling of experimental samples. The specific activity of the newly-formed cholesterol was calculated on the basis of the total counts incorporated into cholesterol together with the known rate of synthesis of the sterol in the intact animal. On the initial assumption that the squalene pool was metabolically homogeneous the specific activity of the squalene intermediate was measured by reverse isotope dilution. The figure obtained was about five percent of that for cholesterol as a result incompatible with the assumption of the nature of the squalene pool.

In a separate experiment animals were fed tritiated water for 21 days by which time their body water assumed a constant specific activity. By this time cholesterol would have been labelled to about 95% of the maximum specific activity which it can attain under such conditions [31]. The specific activity of the squalene pool from which this cholesterol was derived must have been at least as high as that of the cholesterol In fact the measured value for the total endogenous squalene was less than 50% of that for the cholesterol.

In summary these results indicate a distinct inhomogeneity in the squalene pool with perhaps five percent of the material in a metabolically more active form than the rest. Recent reports of the isolation of a protein from blood serum [32] and from the 105,000g supernatant of rat liver microsomal preparations [33, 34], named sterol carrier protein (SCP), which binds squalene and sterols avidly and which stimulates the conversion of squalene and other putative intermediates into cholesterol, provide information on the possible nature of this active pool. This suggests that squalene is transformed into sterols in the form of a protein complex, but says nothing as to whether or not the transfer to the epoxidase is a spatially-specific or a random process. Four attempts have been made to distinguish between these hypotheses. Lawrie et al. [35a] converted {1-$^3H_2$} FPP (V) into eburicoic acid (XVI) in the fungus Polyporus sulphureus (Fig. 7, Route a). The ratio of label at C-11 to that at C-12 was 1:1, indicative of random transfer. Caspi and co-workers [35b] converted (5S) - {5-^3H} mevalonic acid to fusidic acid using the organism Fusidium coccineum. Degradation of the molecule revealed that there was an equal distribution of label between C-11 and C-12.

In mammalian systems Samuelson & Goodman[35c] incubated {1-^{3}H} FPP (V) anaerobically with rat microsomes, thereby accumulating squalene (VII). After three hours oxygen was admitted and after further incubation the cholesterol (XIII) was isolated and converted to cholic acid (XVII) (Fig. 7b). The results obtained from the 12-oxo compound before and after base-catalysed equilibration indicated equal labelling at C-11 and C-12.

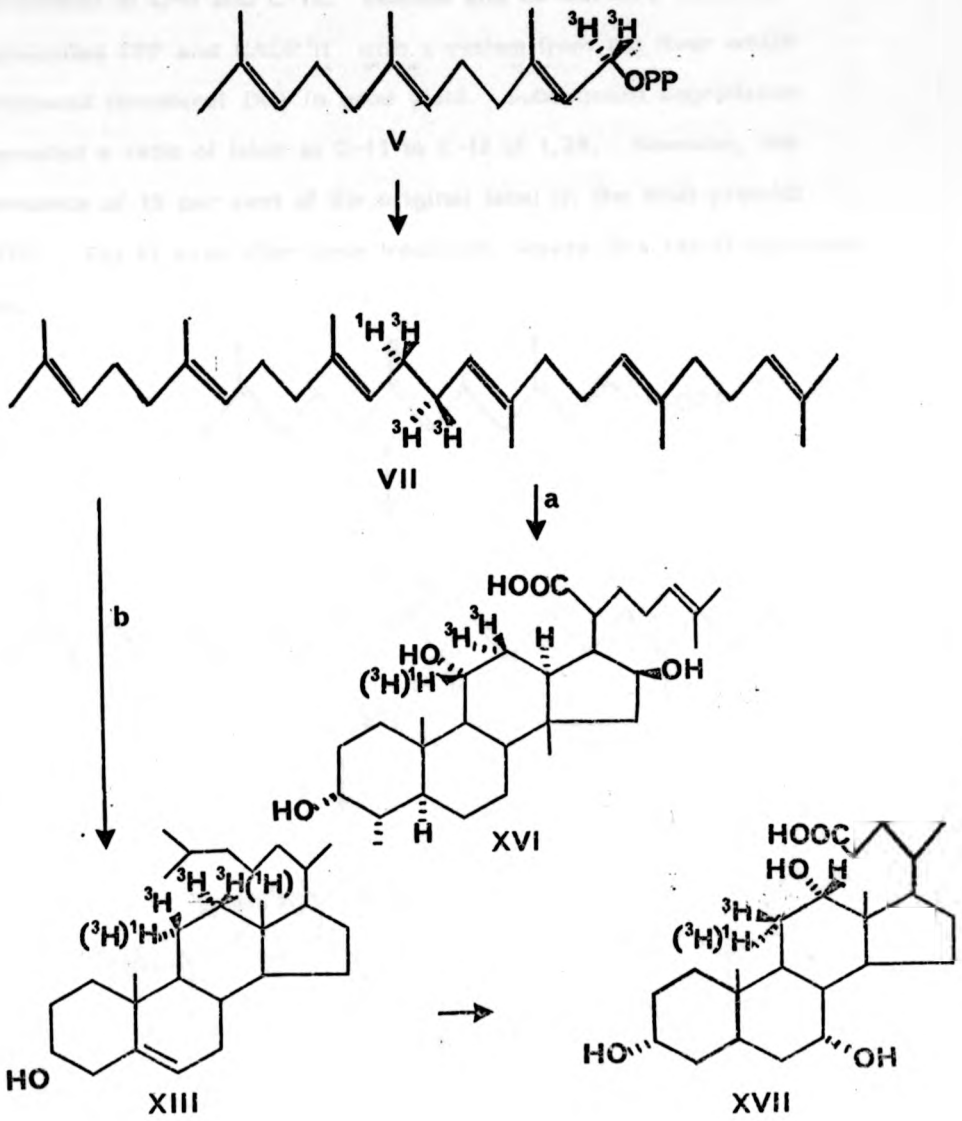

Fig. 7

Etemadi et al.[35d] criticized the latter experiment on the grounds that the absence of oxygen in the first stage of the incubation must have produced a build-up of labelled squalene. Since the normal pathway to sterols (Fig. 6) has been blocked the labelled squalene must have entered the putative non-bound pool. Thus any sterols produced subsequently would, if necessity, be scrambled at C-11 and C-12. Etemadi and co-workers incubated unlabelled FPP and NADP^{3}H with a system from rat liver which produced lanosterol (XI) in good yield. Subsequent degradation revealed a ratio of label at C-11 to C-12 of 1,28. However, the presence of 15 per cent of the original label in the final product (XIII), Fig. 8) even after base treatment, leaves this result equivocal too.

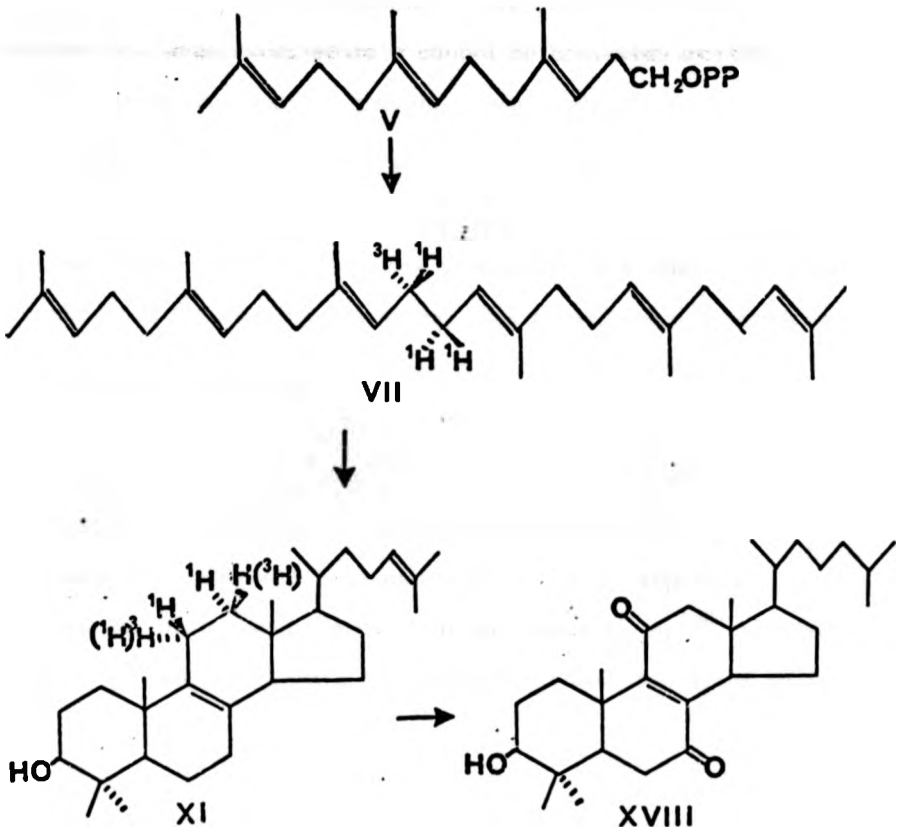

Fig. 8

The results for the biosynthesis of fusidic and of eburicoic acid, which indicate random transfer, cannot be doubted. It is possible that these systems do not contain the sterol carrier protein (SCP) implicated in the mammalian case since the oxidosqualene cyclase from yeast has been shown to be a soluble protein[36]. The results in the mammalian case are open to criticism and so it was decided to design an experiment to circumvent the previous flaws.
The use of sub-cellular fractions for sterol biosynthesis is always open to the criticism that the processes of cell rupture and fractionation destroy the degree of structural order which is being studied. It was decided therefore to use normal intact rats, since, in addition to eliminating the above objection, it is in these animals that the most convincing proof of the existance of more than one pool of squalene has been forthcoming. This decision precludes the use of FPP as a precursor, since it cannot be converted to sterols in intact animals (G. Popjak, private communication).

The logical precursor for the asymmetrical labelling of squalene in intact animals is (5S)-{5-^{3}H} mevalonic acid. On the basis of the schemes outlined above this will give rise to squalene and cholesterol labelled as shown in Fig. 9. The remaining problem will then be to determine the ratio of label at positions 11β and 12α in the cholesterol.

Numerous micro-organisms are known which will degrade and interconvert sterols by a combination of oxidative and reductive reactions[37]. Many of them use as substrates only C_{19} or C_{21} compounds. Those which catabolize C_{27} - C_{29} sterols are of greater interest for the present work. Van der Waard et al.[38] and Wix et al.[39] both described a Mycobacterium which converted cholesterol

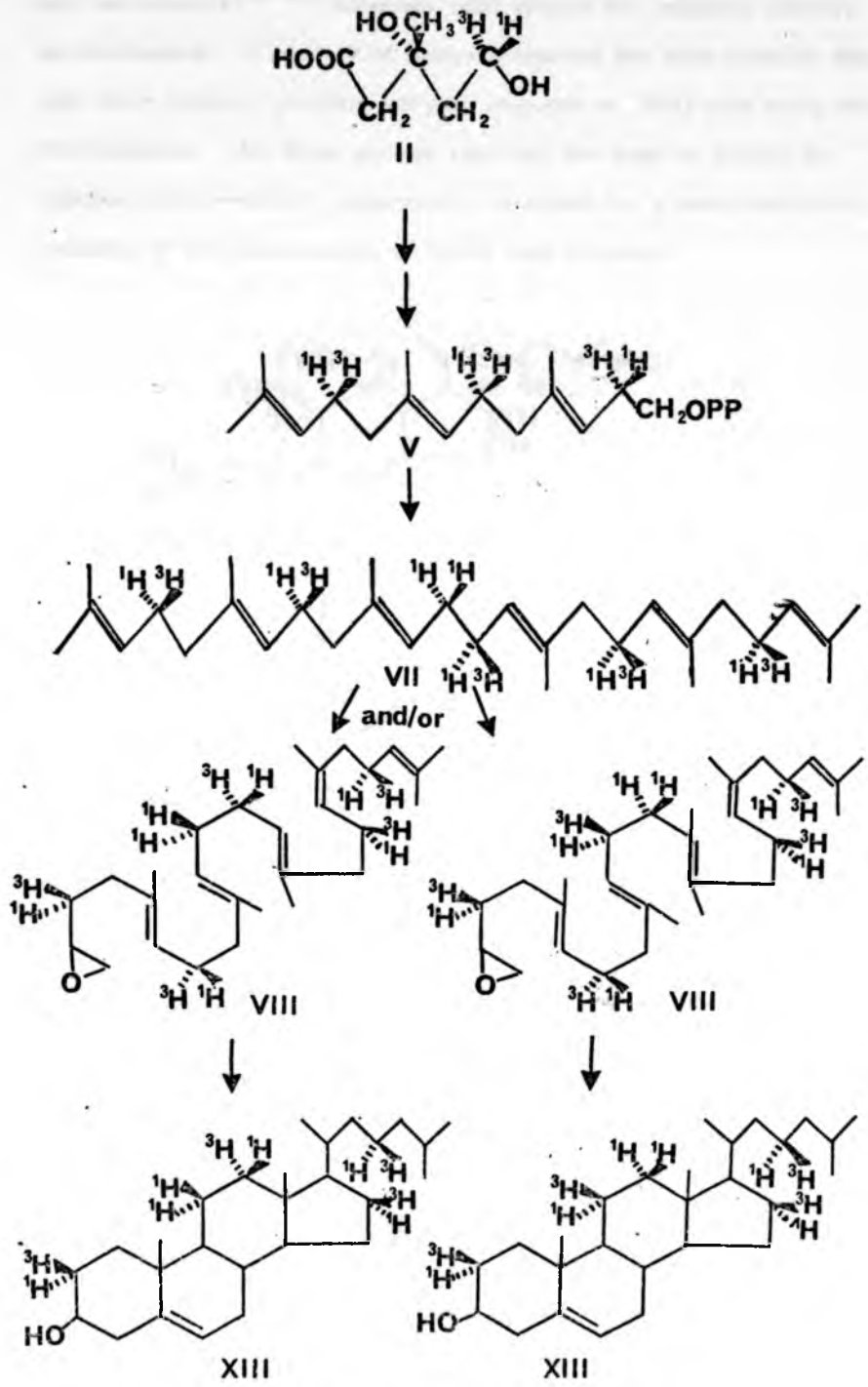
HO CH3 3H 1H
HOOC
C
C
OH
CH2 CH2
II
CH2OPP
V
VII
and/or
VIII
VIII
HO
XIII
XIII

Fig. 9

(XIII) to 1,4- androstadiene- 3,17-dione (XIX) (Fig. 10). Nagasawa and co-workers [40-43] screened 1600 strains for catabolic activity on cholesterol. A variety of bacteria cleaved the side-chain of this and other sterols, yielding varying amounts of (XIX) and many other intermediates. All three groups reported the need to inhibit the reaction (XIX)⟶(XX), apparently catalysed by a metal-containing oxidase, if the accumulation of (XIX) was required.

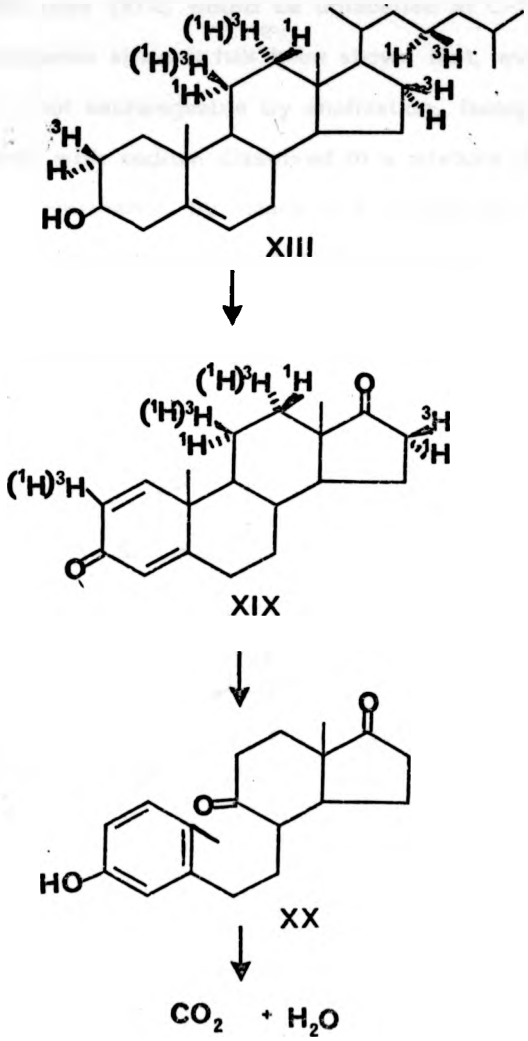

Fig. 10

The conversion of (XIII) labelled from (5S)-{5-^{3}H} mevalonate to (XIX) is attractive for the present study. Apart from removing the label in the side-chain, that at C-16 is exposed to base equilibration. The only undesired label which may be left is at C-2 of (XIX). The stereochemistry of the $\Delta^{1(2)}$ dehydrogenation has been studied, using chemically prepared substrates, in the cases of Bacillus sphaericus[44a], Nocardia corallina[44b] and Septomyxa affinis[44c]. The process was shown to involve loss of the 1α and 2β hydrogen atoms. If the same result applied in this case (XIX) would be unlabelled at C-2. The matter is of little consequence since it has been shown that, even though the proton at C-2 is not exchangeable by enolization, isotope may be introduced by reaction with sodium dissolved in a mixture of deuterium oxide and deuterio-methanol, by means of a Michael-type 1,4 addition[45] (Fig. 11). In a like manner treatment with protonated solutions will remove tritium from C-2. Thus, (XIX) can be obtained containing tritium only at positions 11β and/or 12α .

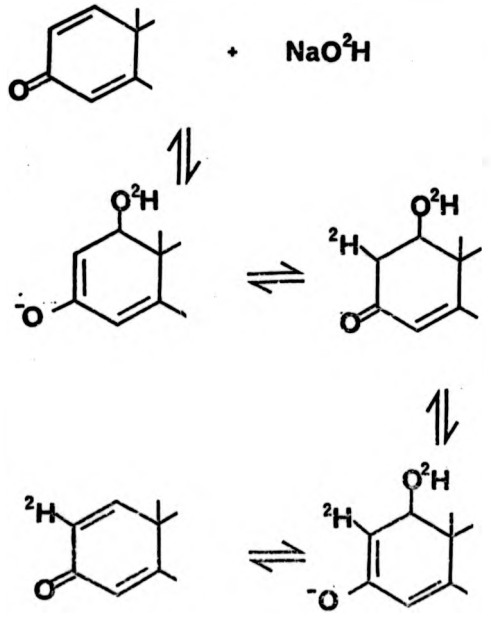

Fig. 11

1, 4-Androstadiene- 3, 17-dione (XIX) is a substrate for hydroxylation as performed by a wide range of organisms. In particular the fungus Aspergillus tamarii Kita is reported to convert (XIX) to a mixture of four compounds, (XXI) - (XXIV)[46] in which (XXI) and (XXIII) are the major components (Fig. 12). The key steps are the hydroxylations at the α and β positions of C-11. Microbial hydroxylations of steroids are said to be stereospecific[47], and in the few instances in which the subject has been investigated[48, 49], they have been shown to occur with replacement of a given hydrogen by a hydroxyl in the same stereochemical configuration.

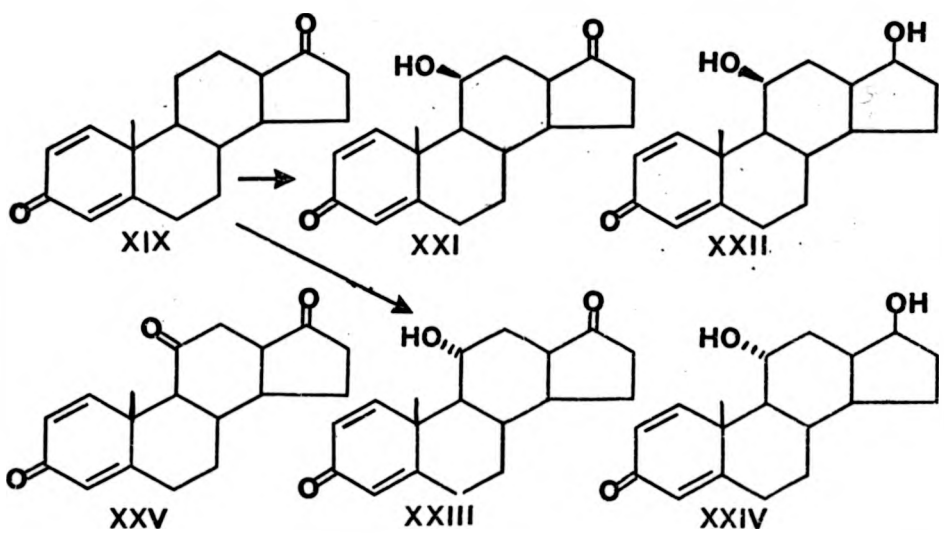

Fig. 12

The trione (XXV) is not converted to (XXII) or (XXIII), suggesting that it is not a free intermediate in the conversion of (XIX) to (XX) and (XXII). If the hydroxylation reactions are stereospecific in the sense predicted the product (XXI) will contain only those tritium atoms which were originally present at C-12α, and the product (XXIII), assuming the absence of an isotope effect, will have the specific activity of the starting material (XIX).

Oxidation of either compound under conditions in which 11-keto-group does not enolise will produce the trione (XXV) whose tritium content is again that of the molecules of (XIX) labelled at C-12. The label can be removed by base-catalysed exchange and should yield unlabelled product.

This thesis describes the synthesis of (5S)-{5-^{3}H} mevalonic acid, its conversion in vivo in the rat to cholesterol, and the microbiological degradation of this cholesterol to determine the labelling pattern at C-11 and C-12.

CHAPTER 2

RESULTS AND DISCUSSION PART 1

SYNTHESIS OF (5S, 3RS) - { 5-^{3}H } MEVALONIC ACID

Of the six compounds obtainable by labelling the pro-chiral positions of mevalonic acid, five had been prepared at the time that this study began. Four of the five compounds, those labelled at C-2 and at C-4, were available commercially and the fifth, (5R)- {5-$^3H_1$} mevalonic acid had been prepared by the reduction of mevalonic acid using labelled NADPH and a rat liver system to introduce the label[14]. Although it would have been feasible to prepare the required compound by reducing mevalonic acid which was labelled in the aldehydo position, it was decided to try a new approach.

The proposed scheme involved the use of horse liver alcohol dehydrogenase to reduce { 1-^3H} 3-methyl-3-butenal with NADH to the corresponding (1S)-{ 1-$^3H_1$} 3-methyl-3-butenol, followed by conversion of the alcohol to mevalonic acid.

It had already been shown[50] that the alcohol, trivially named isopentenol, is a good substrate for oxidation by alcohol dehydrogenase and so it could be expected that the reduction would proceed readily under suitable conditions. The essential conditions could be predicted to be a pH near 7, when the equilibrium for the reaction lies strongly in the direction of reduction, and an excess of NADH.

By measuring the decrease in absorbance at 340nm, which is characterestic of NADH, the extent of reaction could be followed readily. A sample of the aldehyde (prepared as discussed below) underwent reduction rapidly and the major product was shown by g.l.c. to be the required isopentenol (XXXV), contaminated only by the isomeric dimethylallyl alcohol (XXXVa). The contaminant was presumably formed by enzymic reduction of the conjugated aldehyde, which is formed very readily by isomerization of the labile substrate. The ease of isomerisation is seen in the results of the experiment outlined in Table 1 (p.69). The almost neutral pH at low temperature still caused considerable isomerization within 10 minutes.

In an attempt to minimize this problem all enzymic reductions were performed by adding aliquots of an ice-cold solution of the unconjugated aldehyde to the enzyme solution.

Although the required aldehyde and a derivative had been reported[51], the results reported here indicate that the compound prepared was probably the conjugated isomer. The aldehyde was first synthesized by acid hydrolysis of the diethyl acetal (XXVIII).

As could be expected considerable isomerization occurred under the conditions of hydrolysis, but by careful fractionation a sample of 3-methyl-3-butenal was isolated which was almost completely pure as measured by g.l.c. Even when stored at -20° under nitrogen the compound decomposed and isomerized slowly.

The identity of the aldehyde as 3-methyl-3-butenal rests largely on the spectral characteristics of the compound. The lack of absorbance in the region above 220nm argues strongly against the presence of a conjugated enal since such compounds exhibit strong absorbance in this region ($\varepsilon \sim 5\text{-}10 \times 10^3$)[52]. Both the i.r. and the N,M.R. spectra are interpreted best as indicating that the proposed structure is correct.

The i.r. spectrum showed all the peaks expected for such a compound[53]. Thus, the absorbtion of 1720 cm^{-1} is characteristic of the carbonyl group in an aliphatic aldehyde, as opposed to the band in the region 1680-1700 cm^{-1} for a conjugated aliphatic aldehyde. The presence of an aldehyde is confirmed by the C-H stretching at 2730 and 2820 cm^{-1}. Finally the bands at 1645 and 895 cm^{-1}, taken jointly, indicate the presence of a geminally substituted double bond.

Both the chemical shift values and the coupling contants seen in the N.M.R. spectrum were in accordance with predictions[54]. The signal for the methyl group was split into a triplet by cisoid allylic coupling, a process which has typical J values of about 1 Hz. The theoretical transoid coupling was not seen, probably because of the usually smaller coupling constant in this case. Similar allylic coupling was seen for the C-2 protons which were also

coupled to the aldehyde proton*. Whereas the i.r. spectrum of the semi-carbazone indicated that derivative formation had occurred without isomerization, the strong absorbance at 383nm and the m.p. of the 2-4-dinitrophenylhydrazone showed that this was the derivative of the conjugated isomer.

The elemental analyses of the aldehyde and its two derivatives, taken in conjunction with the route of synthesis and the spectral characteristics of the compounds involved indentified the compound (XXX) as the previously unknown C_5 aliphatic aldehyde.

The next problem to be considered was the production of the aldehyde labelled with ^3H at C-1. In contrast to the more normal oxidative and reductive procedures (for a review see ref. 55) the original attempt at labelling (with ^2H in model studies) involved the exchange of the C-2 proton in the appropriately mono-substituted 1, 3-dithiane as described by Carey and Seebach[56]. This C-2 carbon atom acts as a masked carbonyl group and the hydrogen is readily exchanged under basic conditions.

Since the experiments were performed a number of reports have appeared describing methods for aldehyde synthesis. Among these methods are a number which appear suitable for the synthesis of C-1 labelled aldehydes. This subject has been the subject of a recent review[57].

In the model experiments exchange was only 65% complete, but since labelling with ^3H involves the replacement of only a fraction of the total atoms, this was not considered a disadvantage. Attempts to convert the substituted dithiane to the aldehyde by hydrolysis under neutral conditions with mercuric salts as described[56], failed to yield the required product. When the reaction was performed at ambient temperature, the normal work-up procedure gave no product.

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* Similar i.r. and N.M.R. absorption bands were seen in all coumpounds containing the methallyl radical, when allowance was made for other groupings present. The spectra of such compounds are not analysed.

When the conditions were modified by performing the reaction at elevated temperatures, utilizing an analytical Kjeldahl apparatus, the products indicated that the cyclic thioacetal had been converted into a mixture consisting of the ethylene acetals of the isomeric aldehydes (XXX and XXXa, Fig. 14 p. 69). This assignment is based on an analysis of the N.M.R. spectrum of the distilled product, using the identifiable methyl peaks as points of reference for quantitative analysis of the spectrum. In the absence of a simple method for the hydrolysis of the dithiane (XXVII) to the aldehyde (XXX) this approach was abandoned. Subsequently it has been reported that Thallium(III) trifluoroacetate in THF will perform this conversion[58].

In a second approach the synthesis of the acetal (XXVIII), from which the aldehyde had been first prepared, was investigated. The published synthesis of the acetal[59] involved the use of excess triethylorthoformate. Since this material would be labelled in the projected sequence the reaction was re-investigated with a view to optimizing the yield with respect to orthoester. In the modified procedure excess Grignard reagent in THF was added to the orthoester maintained at a temperature such that the solvent distilled off continuously. The consequences of this manipulation were that the reaction was completed more rapidly, due to the higher temperature and greater concentration of reactants. Furthermore the yield, based on orthoester, increased by 25%, even when the reaction was performed on a small scale. Even though this approach was promising it was abandoned at this stage, largely because the overall process, including the synthesis of tritiated chloroform appeared unattractive as compared with a more conventional synthesis.

The third attempt at the synthesis of the labelled aldehyde involved oxidation of ^{3}H-labelled isopentenol. The preparation of the labelled alcohol by hydride reduction of a suitable compound was studied in model experiments. These were the methyl ester and the acid chloride derivatives of 3-methyl-3-butenoic acid and the aldehyde itself. The product from the acid chloride contained two unidentified impurities when examined by g.l.c.

and the yield was rather low. Reduction of the ester gave a good yield of alcohol, but when the reduction was performed using a solution of lithium borohydride which had been half hydrolysed with deuterium oxide as described by Cornforth[60], the isopentenol contained nearly 10% more label than expected. The presence of label at position other than C-1 would clearly invalidate the proposed experiment and the matter was not pursued. Reduction of the aldehyde gave a good yield of the required alcohol and results to be discussed (p. 65) showed that more than 99,5% of any label introduced was in the expected position.

Since the aldehyde was readily isomerized, conventional oxidation using an acidic medium appeared undesirable and the oxidation was performed using the mild system of DMSO - dicylohexylcarbodi-imide[61]. Under these conditions isomerization was minimal but an unidentified impurity could not be completely removed from the aldehyde, even by preparation g.l.c. The available information on the mechanism of the reaction suggested that the impurity was likely to be a thioether[62] obtained as shown in Fig. 13 . Since this compound did not inhibit the enzymic reduction of the aldehyde its presence was ignored, since the later chemical manipulations were likely to cause it to be removed.

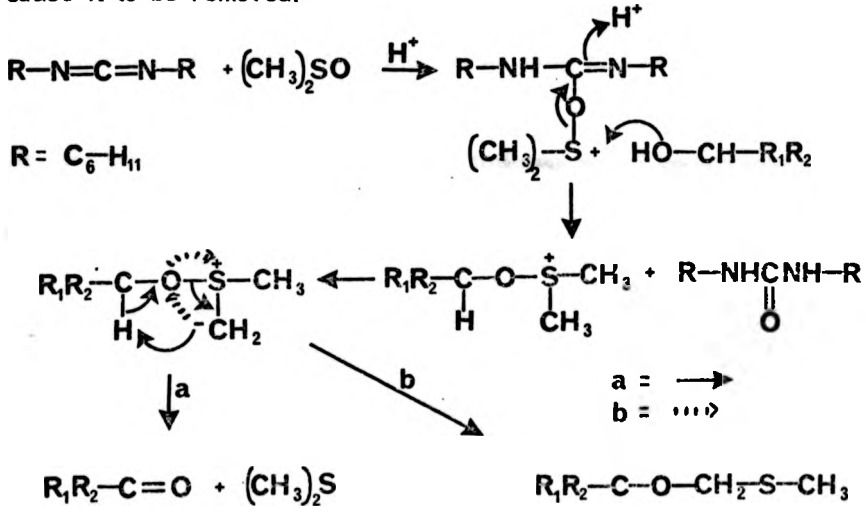

Fig. 13

Once it had been established that the synthesis of (1S)-{ 1-$^{3}H_{1}$} isopentenol was feasible attention was turned to the conversion of the alcohol to mevalonic acid. The original scheme proposed the protection of the alcohol function, epoxidation of the double band and opening of the oxiran ring with cyanide ion to give a nitrile which would yield mevalonic acid on acidic hydrolysis.

Conversion of the alcohol to the tetrahydropyranyl ether occurred smoothly. The analytical data and spectral characteristics of the product were consistent with the proposed structure. The complex N.M.R. spectrum derives from the presence of the acetal-like structure embodied in the molecule. It has been reported that ethyl acetals give complex N.M.R. spectra, owing to the non-equivalence of the ethyl protons[63]. The attempted epoxidation of this material was largely unsuccessful, either because of lack of reaction or because the oxiran ring was attacked by the free acid, yielding an ester. As an alternative means to preparing the epoxide the protected alcohol was reacted with N-bromosuccinimide and the product was ring closed by treatment with weak base. Once again the spectral properties and composition of the purified product were consistent with the structure. When this product was reacted with sodium cyanide in DMSO no reaction occurred, but when the process was repeated in a more polar solvent a product was obtained whose i.r. spectrum suggested that the oxiran ring had been opened and that a nitrile had been formed.

At this stage it was considered that the process of conversion of isopentenol to the final product could probably be shortened by two steps. Treatment of the unprotected alcohol with N-bromosuccinimide, followed by reaction with sodium cyanide in methanol, gave two products. When the major component was purified by p.l.c. its spectral characteristics were consistent with expected dihydroxy nitrile. Further proof of the correctness of the structure was given by the hydrolysis of this material to mevalonolactone. The product was characterized both by the indentity of its i.r. spectrum when compared with that of an authentic sample and by conversion to the crystalline benzhydrylamide.

It was then possible to link together the various stages of the synthesis to produce a sample of (5S)-{5-$^{3}H_{1}$} mevalonic acid. The reduction of 3-methyl-3-butenal was performed with a solution of lithium borohydride which had been treated with tritiated water. The resulting{ 1-$^{3}H_{2}$} isopentenol was oxidized back to the labelled aldehyde, which was reduced enzymatically to give the (1S)-{ 1-$^{3}H_{1}$} isopentenol which was diluted with unlabelled alcohol. The conversion to mevalonolactone was performed without purification. In order to check that the reduction of the aldehyde by borohydride had not resulted in non-specific labelling, as had been seen in the reduction of the ester, an aliquot of the lactone was oxidized to β-hydroxy, β-methylglutaric acid which was then converted to the methyl ester. The specific activity of this compound was less than 0,5% of that of the diluted isopentenol from which it derived. This figure represents the amount of non-specific label in the final mevalonolactone.

The sterochemical purity of the lactone and hence the degree of sterospecificity of the enzymic reduction was measured by combining an aliquot of the lactone with { 2-^{14}C} mevalonolactone. The double-labelled material was incubated with a rat liver homogenate as described[64] and the squalene was purified by t.l.c., formation of the thiourea clathrate and regeneration of the squalene. Based on an atomic ratio of ^{3}H: ^{14}C set at 6:6 for the precursor mevalonate (measured after conversion to the benzhydrylamide and triple crystallization after the addition of carrier), the squalene had an atomic ratio of 5,:6

This result and the subsequent incorporation of samples of this material into fusidic acid[35a], gibberellic acid[65a] and abscissic acid[65b], with in each case the resultant indicating complete loss or retention of the label, as predicted, confirms the sterochemical purity of the material.

The absolute stereochemistry of the product depends on the stereospecificity of the enzyme liver alcohol dehydrogenase. This has been demonstrated for the terpenoid alcohol geraniol[66a] On this basis it has been assumed that the enzyme removes the 1-pro-R hydrogen atom from an alcohol during oxidation. An apparent exception to this invariant rule is the report that

a cell-free extract of Andrographis panniculata removes the pro-S hydrogen while converting trans, trans-farnesol into the cis, trans isomer and the pro-R hydrogen in the reverse isomerisation[66b]. Since it is not known whether the aldehyde is an intermediate in this reaction it is not clear that an alcohol dehydrogenase of variable specificity has been discovered. Therefore a sample of (3RS, 5S) -{ 5-$^{3}H_{1}$ } mevalonolactone had been prepared in which both the position and stereospecifity of the label was known. This material was considered suitable for the next stage of this study, namely incorporation in vivo cholesterol and subsequently the determination of the labelling pattern.

CHAPTER 3

RESULTS AND DISCUSSION PART 2

THE CONVERSION IN VIVO OF (5S, 3RS)-{ 5^{3}H } MEVALONIC ACID TO CHOLESTEROL AND DEGRADATION OF THE STEROL.

The conversion of (3 R)-mevalonic acid into cholesterol is known to occur in a wide variety of mammals and in various organs within the mammals. The subject has been reviewed recently, from both the aspect of the detailed pathways involved in the biosynthesis of cholesterol[10] and from that of the factors regulating the rate of sterol biosynthesis.[67].

The decision to convert (3 RS, 5S)-{5-^{3}H } mevalonic acid to cholesterol in the intact rat was taken over several grounds. Since the aim of the experiment was to determine whether or not the two ends of squalene, which are non-equivalent at the time of synthesis from (3 R, 5 S)-{5-^{3}H } mevalonic acid by the enzyme squalene synthetase, remain non-equivalent during conversion to (2 S)-2,3-oxidosqualene, the use of an intact animal, rather than a tissue preparation, would remove all possible artifacts. The use of rat, in preference to other mammals, rested on two points. Firstly, the previous attemts[35c), (35d)] to resolve this problem used rat liver preparations and since the results were equivocal, either because of the design of the experiment or because of the introduction of non-specific label(as discussed on p. 13), the problem could be resolved completely for this animal. Secondly, the use of small, young rats would minimize the inevitable wastage of labelled material which must accompany whole animal experiments. The advantage of using mice, with possibly even better incorporation into cholesterol was considered and discarded, largely on the grounds discussed above.

Although numerous workers have used tissue homogenates to study sterol biosynthesis, very little work has been done on the intact animal. Goodman et al[68] attempted to delineate some of the precursors of cholesterol in the liver by injecting rats with (3 RS)-{2-^{14}C } mevalonic acid and isolating from liver labelled intermediates. These authors found that cholesterol of the highest specific activity was obtained about 30 minutes after injection of label. The results obtained in this study, namely highest specific activity obtained after 45 minutes, correlate well with the published data.

It must be considered that various organs may have different rates of synthesis, but that, unlike the liver which excretes most of the synthesized material, either as cholesterol or after conversion to bile acids, an essentially irreversible deposition of cholesterol might allow a higher radiochemical yield to be obtained by excision after a longer period. Dietschy and Siperstein[69] investigated the incorporation of {2-^{14}C } sodium acetate into seventeen tissues of the rat and reported significant rates of incorporation only in the intestine and liver. This result may only represent the efficient absorption of the precursor by the two organs concerned. Since acetate is a ubiquitous and very general precursor of sterols it was decided to use as a probe of the capacity of different organs for sterol synthesis more metabolically committed mevalonic acid.

The fact that the major portion of the labelled non-saponifiable lipids was found in the kidneys was initially surprising, since this organ gave very low rates of incorporation from acetate[69]. The results obtained in this study confirm the fear, expressed above, that the precursor, acetate, was possible not reaching all possible biosynthetic sites. The failure of the kidney to convert significant quantities of the precursor to cholesterol, but rather to halt the synthesis at squalene or lanosterol, ruled against the isolation of labelled material from this organ. Although lanosterol would have been a satisfactory product for the study of the cyclase-epoxidase system, the entire study had been designed with a view to the degradation of cholesterol rather than of squalene. Thus, cholesterol was isolated from rat livers subsequent to injection of (3 RS, 5S)-{5-^{3}H } sodium mevalonate.

Siperstein and co-workers have obtained similar results to those quoted above for the incorporation of mevalonate in renal tissue. In an in vivo study[70] they showed that {^{14}C } mevalonate has a two phase turnover rate in blood, an initial phase of T ½ = 5 minutes and a slower phase with T ½ = 40 minutes. When tracer amounts of mevalonate were given, either intravenously or intraperitoneally, the major site of synthesis of labelled

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sterols was the kidney, with squalene and lanosterol as the major components. In the same experiment the almost exclusive sterol in the liver was cholesterol. When the same precursors were administered orally the intestine and liver were the two organs exhibiting the highest incorporatior These results will confirm those found earlier for acetate. When substrate quantities of mevalonate were used to dilute the label prior to administration the labelling pattern changed in the experiments whether the mode of administration was either intravenously or intraperitoneally. The liver now became the major site of sterol synthesis, with cholesterol still the major sterol.

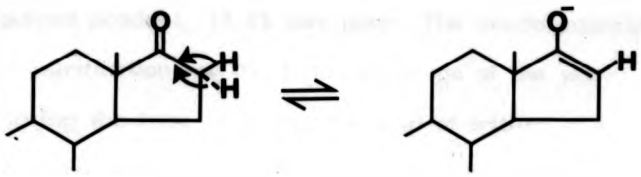
Subsequently[71] the ability of liver slices and of kidney slices and organ fractions to synthesize sterols was investigated. These experiments merely confirmed the in vivo results. The glomeruli were the dominant site of synthesis in the kidney, but with little capacity to convert squalene or lanosterol to cholesterol. The apparent Km and V_{max} values for the conversion of mevalonate to sterols were 1,48mM and 1430nmoles $g^{-1}hr^{-1}$ for liver and 0,19mM and 160 nmoles $g^{-1}hr^{-1}$ for the kidney. These results explain the different labelling patterns seen in the two organs when tracer and substrate quantities of mevalonate were administered.

The chromatographic purification of the biosynthesized non-saponifiable lipids using the method described by Frantz[72] gave {^{3}H} - labelled cholesterol which appeared to be radiochemically pure. The identity of the major peak seen on the elution profile of the chromatogram was proved by co-chromatography with authentic {4-^{14}C} cholesterol. The (^{3}H)-labelled material appeared in the same fractions as did the bulk of ^{14}C activity. The small ^{14}C peak, constituting about 2% of total ^{14}C eluted from the column, can be attributed to an impurity in the {4^{-14}C} cholesterol. The unchanged $^{3}H/^{14}C$ ratio in the cholesterol before and after the purification via the di-bromide is a good but not unequivocal proof of the radiochemical purity of the sample. This procedure has been shown to remove many of the compounds found on the biosynthetic pathway between squalene and cholesterol[72]. The sample of biosynthetic

cholesterol was now available for degradation in order to determine the position and degree of labelling with tritium. This sample of cholesterol will be labelled with tritium at C-2 α,β, C-11 β and/or C-12 α (depending on the mode of cyclization of the labelled squalene precursor), C-16 β and C-23. This labelling pattern is based on the known stereospecific pathway, the correctness of which with (5S)-{5-^{3}H} mevalonate has been confirmed (p. 71) for the biosynthesis of squalene[73] and on the stereochemistry of the cyclization of squalene to cholesterol, based on the results obtained with both (5R)- and (5S)-{5-^{3}H} mevalonate. Since the objective of this study is to investigate the labelling at C-11/C-12, what is required is that the cholesterol be degraded so that the only remaining label is at C-11 and/or C-12, followed by functionalization of either or both of these positions in order to determine the labelling pattern.

Numerous organisms are known which will effect partial or complete degradation of the steroid nucleus[37], [40]. The pathways have not been described in detail but the overall scheme appears to be very similar in those few cases which have been investigated.[38 - 40]. Cleavage of the side chain occurs to yield a ketone or hydroxy group at C-17, either before or after oxidation of the allylic alcohol at C-3 to a 4-ene-3-one. Dehydrogenation at C-1,2 yields the important intermediate 1,4-androstadiene-3,17-dione (XIX). Further metabolism involved oxidation at C-9 with cleavage of the C-9,10 bond and opening of ring B of the steroid nucleus. Thereafter oxidation continues in an as yet unknown manner to yield, presumably, carbon dioxide and water. Various workers[38-40] have shown that the enzyme system which catalyses the oxidation at C-9 can be inhibited by one of a number of organic metal-chelating agents, e.g. 8-hydroxyquinoline, 1,1'-bipyridyl, or by high concentrations of certain inorganic salts, e.g. $NiSO_4$. It is postulated by these authors that they enzyme system, probably a mixed function oxygenase, contains an atom of iron which is either chelated or replaced 'isomorphously', thereby inactivating the enzyme.

The product which accumulates under the conditions in which the 9-hydroxylase is inhibited, viz. 1,4-androstadiene-3,17-dione represents a suitable intermediate for further degradative studies. Of the carbon atoms in the original species of cholesterol labelled with tritium from (5S)-{ 5-^{3}H } mevalonate, C-23 has been removed along with the side-chain and the two other positions from which it is desired to remove tritium, C-2 and C-16 are both subject to base catalyzed exchange of the hydrogen atoms attached, by virtue of the adjacent C-3 and C-17 carbonyl groups. Treatment of the 1,4-diene-3,17-dione systems will cause exchange of both hydrogens attached at C-16 by a mechanism which involves a reversible enolization of the carbonyl group.



Although the hydrogen at C-2 appears at first to be in a non-enolizable position, 1,4 addition occurs across the 1-ene-3-one-system, thereby rendering the hydrogen atom exchangeable (p. 17).

In fact is has been shown[73] and the formation of the C-1,2 double occurs with stereospecific trans-diaxial removal of the 1 α and 2 β hydrogen atoms. Thus the 1,4-androstadiene-3,17-dione (XIX) obtained by degradation of the stereospecifically labelled cholesterol (XIII) will contain tritium only at C-11 and/or C-12 (1g atom) and at C-16. Following exhaustive base-catalysed exchange the only label remaining in the molecule will be at C-11/12.

The organism Arthrobacter simplex has been reported to convert cholesterol to 1,4-androstadiene-3,17-dione in high yield[40]. However, the strain obtained, at least under conditions used, failed to convert more than a few per cent added cholesterol to the required 1,4-androstadiene-3,17,dione and this particular organism was abandoned.

As an alternative the organism Mycobacterium phlei was tested for its capacity to carry out the requisite conversion. Early experiments showed that the organism was performing the conversion, but the yields were low and variable. Following attempted microbiological enrichment by growing the organism for some time on cholesterol as the sole source of carbon and the addition of a solution of trace elements to the culture medium the yields of 1,4-diene-3,17-dione improved and a culture of the organism was used to convert { 2,11(12), 16,23-$^3H_4$} cholesterol to { 11(12), 16-$^3H_2$} 1,4-androstadiene-3,17-dione. In this crucial experiment the organism proved capricious once more and the yield of required product, 12,6% was poor. The crude material was used without purification for the following stage of the work.

Before attempting the base-catalysed removal of tritium at C-16 β it was necessary to prove that exchange would indeed be complete. This was shown by treating unlabelled 1,4-diene-3,17-dione with base in the presence of deuterium oxide of high isotope excess. The degree of exchange can be estimated by mass spectrometry and nuclear magnetic resonance. When a sample of 1,4-androstadiene-3,17-dione (XIX) was subjected to thrice repeated exchange in sodium deuteroxide/deutero-methanol, changes were seen in both the NMR and mass spectra, measured under similar conditions for labelled and unlabelled material. In the NMR spectrum measured at 100 M Hz of unlabelled (XIX) the hydrogens attached at C-1, C-2 and C-4 were clearly defined and two other hydrogens, probably those at C-6, were also separated from the methylene envelope. The C-1 hydrogen exhibited as a doublet centred at δ = 7,05 with J = 10 Hz. It was coupled in a normal fashion[74] to the C-2 proton. The absorbance of this latter hydrogen was centred at δ = 6,24 and consisted of a pair of doublets with J_1 = 10 Hz and J_2 = 2 Hz. The second splitting is due to coupling through the C-3

carbonyl group with C-4 hydrogen. This hydrogen exhibited as a triplet, actually comprising overlapping doublets each split by about 2 Hz. The one splitting is clearly due to hydrogen at C-2 and the other is probably due to the C-6 hydrogen. The coupling constant between C-4 and C-6α is virtually zero in the case of 4-ene-3-ones[74]. A broad complex band above δ = 2,3 corresponds to two hydrogens and the absence of significant absorption in the methylene envelope above δ = 2,0 in the spectrum of androsterone suggests that the signal is due to the methylene group at C-6. In the spectrum of the fully deuterated material considerable simplification had occured. The only absorbance remaining above δ 2=1, was a singlet at δ = 7,05 integrating for one hydrogen. This is clearly the hydrogen at C-1, no longer coupled to C-2. The disappearance of all other lowfield signals is consistent with the above analysis, whether the multiplet corresponds to C-6 or C-16. The results for deuterium incorporation as measured by mass spectometry confirm the essentially complete nature of the exchange process. The degree of deuteration was measured by examining the spectrum in the region of the parent ion m/e = 284. Although the ion at m/e = 122 constitutes the base peak, the existence of a large m/e = 121 in unlabelled material complicates any attempted analysis in this region. The presence of 90% $\underline{d}_5$ + $\underline{d}_6$ material indicates that exchange was virtually complete. The shift in the base peak from m/e = 122 to m/e=126 is consistent with the cleavage suggested for the product of m/e = 122.

Before performing the removal of tritium from C-16 in (XIX) it was desirable to show that there was no radiochemical impurity in the crude compound. To this end the biosynthetic material was diluted with authentic 1,4-androstadiene-3,17-dione and a small sample taken for preparation of the 17-ethylene ketal. The specific activity of this nicely crystalline derivative of the sometimes difficult-to-crystallize parent compound

served as a reference point for all further studies. A total of $2,71 \times 10^4$ dpm ^3H in 15,0 mg were taken for derivatization. The apparent specific activity of this material was therefore 0,231 μCi mmol.$^{-1}$ The specific activity of the 17-ethylene ketal was 0,220 μ Ci mmol^{-1}, indicating that the radiochemical purity of the diluted 1,4-androsta-diene-dione was 95%.

The bulk of the material was then submitted three times to base-catalysed exchange and a sample of the unpurified material was converted to the 17-ethylene ketal. The specific activity, 0,114, μCi mmol.$^{-1}$, showed that 48,2% of the available tritium had been removed. One further exchange on a 10 mg sample, followed by derivatization failed to change the specific activity of the {11,(12), 16-$^3H_2$ } 1,4-androstadiene-3,17-dione, consistent with equal distribution of label between C-11(12) and C-16. This was achieved by microbiological transformation of the {11(12) ^3H } 1,4-androstadiene-3,17-dione to products containing an hydroxyl group at C-11.

Numerous organisms are described which cause hydroxylation of the steroid nucleus at virtually every carbon atom containing a C-H bond[75]. Amongst organisms which cause hydroxylation at C-11 the fungus Aspergillus tamarii[46] seemed particularly suitable. It was reported to convert 1,4-androstadiene-3,17-dione to a mixture of the 11α - and 11β - hydroxy derivatives in high yield.

Since it is known that microbiological hydroxylation of both steroids and aliphatic compounds occurs with retention of configuration[76], the mixture of 11α and 11β - hydroxy-1,4-androstadiene-3,17-diones should yield useful amount of information about the presence of tritium at C-11. In the absence of an isotope effect which may cause an alteration in the specific activity of the products, the specific activity of the 11α - hydroxy compound should be the same as that of the unhydroxylated diene-dione, while that of the 11β -hydroxy derivative should reflect the amount

of tritium which had been at C-11β and was removed by the stereospecific hydroxylation. Such an isotope effect has been reported during the microbiological hydroxylation of steroids, with very large changes, both in the amounts and specific activities of the metabolites[77].

When 1,4-androstadiene-3,17-dione was incubated with a culture of A. tamarii three products were usually isolated. In some cases, particularly when incubation was performed at an altitude of 1700 m., hydroxylation was incomplete after four days and unchanged starting material, which ran to the same R_f as one of the metabolites in the solvent system routinely used for separation by p.l.c. of the products, had to be removed by the use of another solvent system. The identity of two of the products followed from the physical constants reported for the compounds, while the third compound apparently differed in structure from any compound previously reported to be formed under these conditions.

The compound identified as 11β -hydroxy-1,4-androstadiene-3,17-dione exhibited spectral characteristics consistent with the proposed structure. The infra-red spectrum revealed the presence of two carbonyl groups, one in ring D at C-17, the other in conjugated ketone in a six-membered ring, and an hydroxyl group. It has been shown[78] that spectroscopy locates the position of an hydroxyl group in steroids only in special cases. The N.M.R. spectrum confirms the presence of 1,4-diene-3-one system since the C-1, C-2 and C-4 hydrogens give a pattern very similar to that for the unhydroxylated compound. The additional downfield absorption, centred at δ = 4,48 is indicative of a hydrogen atom attached to carbon to which is also attach an alcohol function. The equatorial 11α hydrogen atom will be coupled to the 9 α 12 α and 12β hydrogens. The resultant small axial-equatorial (9 α and 12 β) and equatorial-equatorial coupling constants (∿ 2,5 Hz) will yield the relatively narrow quartet seen in the spectrum. The application of Zurchner's rules[74] predict δ values for the C-18 and C-19 methyl groups of 1,21 and 1,51 resp., in

good agreement with the experimental values. By contrast (see below) the predicted nature of the C-11 hydrogen absorbance and the δ values for the angular methyl groups would be very different.

The mass spectrum is also consistent with the proposed structure. The loss of water (m/e = 282) is indicative of an alcohol and the base peak at m/e = 122 suggests the presence of the 1,4-diene-3-one system unsubstituted in rings A or B. Finally the elemental analysis and melting point confirm the structure of the 11 β hydroxy-1,4-androstadiene-3,17-dione (XXI).

The compound of lowest R_f was tentatively assigned the structure of the epimeric 11α -hydroxy compiund. The i.r. sprectrum again confirms the presence of the major functional groups. The N.M.R. shows considerable differences from that of the previous compound. The C-1 hydrogen still appears as a doublet, but it is now shifted far downfield ($\Delta\delta$ = 0,75). The closer proximity of the 11α - hydroxy group to C-1 would account for this considerable deshielding. The absorptions of the C-2 and C-4 hydrogens are similar to those in the previous case, although the C-2 hydrogen is now shielded relative to its position in the 11 β spectrum ($\Delta\delta$ = 0,13) while the C-4 hydrogen is slightly deshielded ($\Delta\delta$ = 0,08). More significantly, the band width of C-H-OH absorption is considerably greater (12 Hz as opposed to 9 Hz) and the position of the methyl resonances has shifted. The 11β -axial hydrogen atom would undergo two axial-axial splittings (9 α and 12β) and one equatorial coupling (12α). The large value of the diaxial coupling constants (∿ 10 Hz[74]) will result in a broad band, as seen. The application of Zurchner's rules leads to the prediction of δ = 0,90 for C-18 methyl and of δ = 1,38 for C-19 methyl. The experimental values of 0,94 and 1,34 fit these predictions closely. The mass spectrum again indicates the presence of an alcohol (m/e = 282) and a 1,4-diene-3-one system (m/e=122). Final proof of the correctness of structure as 11α -hydroxy-1,4-androstadiene-3,17-dione (XXIII) was provided by the indentity of i.r. spectrum and by a mixed melting point with an authentic sample.

The third and minor component has been assigned the structure 6 β hydroxy-1,4-androstadiene-3,17-dione. The i.r. spectrum revealed the same functional groups as in the two previous cases. Evidence as to the structure was again provided by the mass spectrum and by the 100 MHz N.M.R. spectrum. The base peak in the mass spectrum is at m/e = 79 instead of m/e = 122 as in the case considered so far. The peak m/e = 122 derives from ring A by cleavage of the C-6,7 and C-9,10 bonds with transfer of 2 hydrogen atoms from elsewhere in the molecule. Thus it can be decuded that the secondary alcohol function, the presence of which is suggested by the i.r. and N.M.R. spectra is in ring B, at either C-6 or C-7.

A consideration of the splitting pattern of the CH-OH proton gives further information about the stereochemistry of the alcohol group. Of the four possible positions the only one compatible with the narrow triplet with J_1 = 3 Hz and J_2 = 1 Hz is the 6α position. The equatorial hydrogen will undergo two approximately equal equatorial-equatorial splittings with the hydrogens at C-7 and a very small splitting with the hydrogen at C-4. In the case of the other three possibilities the existence of large axial-axial coupling constants (C-6 β with C-7α) and in the case of hydrogen on C-7 the additional coupling to C-8 would produce a much broader resonance. Calculations based on Zurchner's rules once again point to the C-6 hydrogen being 6α and the hydroxy group C-6β . The experimentally determined values for the C-18 and C-19 methyl resonances were 0,98 and 1,42 δ . In the four possibilities 6α , 6 β -, 7α and 7β - hydroxy the calculated values based on Zurchner's rules for C-18 and C-19 are 0,97 and 1,26; 1,00 and 1,46; 0,97 and 1,26 and 0,99 and 1,29 respectively. It is clear that the most reasonable structure for this compound is 6β -hydroxy-1,4-androstadiene-3,17-dione.

Once the structure of the products produced by the fungus A. tamarii had been elucidated it was possible to use this culture to convert {11(12)-$^3H_1$ } 1,4-androstadiene-3,17-dione to the 11-hydroxy derivatives.

Incubation and purification in the normal fashion yielded a distribution of metabolites differing from that seen in the model studies. The major component was a metabolite more polar than any seen before. On the basis that it might represent a 11 hydroxy compound in which the C-17 ketone had been reduced, as has been reported[46], authentic samples of 11 α and 11β - hydroxy-1, 4-androstadiene-3, 17-dione were reduced with sodium borohydride in tetrahydrofuran and the resulting 17-ols. were used as reference compounds in the chromatographic investigation of the new metabolite. Neither compound chromatographed with the unknown compound. The structure of this compound was not investigated further at this stage.

The only product present in significant amounts which had been functionalized at C-11 was the 11β - hydroxy derivative. Even though the compound was pure as measured by t.l.c. and N.M.R. it resisted all attempts at crystallization. It was decided to oxidize it to the 11-ketone compound which crystallises more easily. Since the insertion of the 11β -hydroxyl group should have removed all tritium at C-11, the specific activity of the resulting ketone should be the same as that as the present alcohol, provided that exchange did not occur at C-12. The absence of exchange under the conditions of Jones oxidation was proved by oxidizing a sample of authentic alcohol in acetone containing ^3H_2O. The amount of tritiated water added was sufficient to allow the detection of less than 0, 05% exchange into the resulting ketone. The oxidation of the axial 11β -hydroxy compound occurs more slowly than for its 11α -epimer and the oxidation, which was monitored by t.l.c., was stopped when it seen, by visual inspection under u-v light, that oxidation was 60-70% complete. The almost complete lack of exchange enabled the sample of { 11(12)-$^3H_1$} 11-hydroxy-1, 4-androstadiene-3, 17-dione to be converted to the 11-keto compound with no loss of any isotope at C-12.

The specific activity of the resulting 1,4-diene-3,11,17-trione, 0,058 μ Ci mmol.$^{-1}$ as compared with the value of 0,114μ Ci mmol.$^{-1}$ for the (11(12)-$^{3}H_{1}$} 1,4-androstadiene-3,17-dione showed that 51% of the tritium had been at C-11. It remained to be shown that the remaining label was C-12. Base-catalysed exchange of an aliquot of the 11-keto compound yielded a compound devoid of radioactivity.

Thus it can be seen that a sample of cholesterol, biosynthesized in vivo from (5S, 3RS)-{ 5-$^{3}H_{1}$ } mevalonic acid, when degraded by a combination of microbiological and chemical means, was shown to have an atom of tritium equally distributed between C-11 and C-12 in the cholesterol molecule. This proves definitely that, in the conversion of newly synthesized squalene to 2,3-oxidosqualene, both ends of a molecule which is chemically (and hence biologically) non-symmetrical at the time of biosynthesis, are epoxidized to the same degree. This can be envisaged as occurring if the newly-snythesized squalene is not transferred, either directly or by means of a (sterol)carrier protein, from the squalene synthetase to the squalene epoxidase, without being 'free' for some time, i.e. by passing through a 'pool' of unbound squalene where the asymmetry induced by an initial asymmetrical labelling pattern, will be lost. This work confirms the in vitro studies of Goodman and de Witt[35c] and suggests that the spurious label present in the final product of Etemadi et al.[35d] must represent non-specific labelling under the in vitro conditions. This could have arisen easily since the preparation contained transhydrogenases which could cause labilization of ^{3}H in NADPH into the water. Since the reduction of various double bonds during steroid biosynthesis involves addition of H^{-} (from NADPH) at one end of the bond and H^{+} at the other, unexpected and subsequently stable label may be introduced in this way.

CHAPTER 4

EXPERIMENTAL

A. General Procedures

Solvents used were of the highest commercial grade available. Ethereal solvents were dried where necessary by stirring them over calcium hydride, followed by distillation of the decanted material from lithium aluminium hydride.

Melting points were determined on a Kofler Micro Hot Stage and are corrected.

Elemental analyses were performed in these laboratories and are accurate to within $\pm$ 0,4%.

Refraction indices were determined at 20°with an Abbe refractometer made by Hilger-Watts, using as a reference freshly distilled water (n_D^{20} = 1,3329).

Infra-red (i.r.) absorption spectra were determined using a Perkin-Elmer 137 spectrophometer. Liquids were as films measured between plates of sodium chloride. Solids were mixed with potassium bromide at a concentration of about 1,5mg/100mg of potassium bromide and were run as pressed discs. Solution spectra were run in carbon tetrachloride or chloroform freed of ethanol by passage through active alumina, using matched 0,2mm sodium chloride cells.

Nuclear magnetic reasonance (N,M.R.) spectra were recorded on a Varian A60 spectrometer. Spectra were usually run on a solution of the material in deuteriochloroform containing 1-2% tetramethylsilane as an internal reference. In ceetain cases, spectra were run on the neat liquid. In order to resolve some problems which were unclear at 60MHz certain of the spectra were re-run in a Varian HA100 spectrometer. The chemical shift value for each signal is given in δ units (part per million downfield from tetramethylsilane). The number of protons associated with the signal, the multiplicity and, where possible, the coupling constant, are given parenthetically.

Thin-layer chromatography (t.l.c) was performed on 20 x 20 or 20 x 5 cm^2 plates coated with a 0,025cm layer of Kieselgel F_{254} supplied by Merck Industries. All plates contained a fluorescing indicator by which material could be seen by observation under a U.V. lamp at either 254 or 366nm. In those cases in which the material under consideration was not visible under U.V. light, it was visualized either by exposure to iodine vapour followed by examination under U.V., or, when the material was not needed subsequently, by spraying the whole plate with a 60% solution of sulphuric acid in ethanol and heating it in an oven at 130° C. All substances involved in these investigations charred under these conditions, sometimes with the production of characteristic colours.

Preparative thin-layer chromatography (p.l.c.) was performed on the same 20 x 20 cm^2 plates as used for t.l.c. It was found that these plates gave satisfactory resolution provided that no more than about 20mg of any mixture was applied as a streak across the plate. All plates which were used for p.l.c. were washed by developing them in chloroform/methanol. 1:1 All subsequent development was performed in the same direction as the original one and the solvent was never allowed to rise higher than 18cm from the lower edge of the plate. Radioactive material on t.l.c plates was detected by scanning in a Packard strip scanner in the case of the 20 x 5cm plates and in a Desaga plate scanner for 20 x 20cm plates.

Gas chromatography (g.l.c.) was performed on a Varian 1400 or 1520 gas chromatography equipped with a flame-ionization detector. The dimensions of individual columns and the temperature of operation are given in the text. The stationary phase for all packings was acid-washed Chromasorb W 100/120, obtained from Johns-Manville Ltd.

Columns were made either of metal or, in those cases where the compounds under investigation were decomposed on metal surfaces, of glass. The columns were so constructed that the material was injected directly into the column without contact with a metal surface. The carrier gas was nitrogen at a flow rate of approximately 20ml/min.

Preparative gas chromatography was performed on a Wilkens Autoprep 700 fitted with a thermal conductivity detector, and using helium as the carrier gas. The effluent was collected in commercially available collecting tubes at -78° C. Spectrometry (GC-MS) was performed on the same machine fitted with a Llewellyn adaptor[79] which admitted the effluent from a gas chromatograph to the ionization chamber of the mass spectrometer.

Gas radiochromatography was performed on an apparatus designed and built in these laboratories. A modified form of the original machine[80] was used in which a stream splitter conducted 10% of the effluent gas to a flame ionization dector while the remainder was channelled to the scintillation detector.

Samples containing ^{14}C and/or ^{3}H were assayed for radioactivity in a Packard Tri-Carb liquid scintillation counter, model 3375. The scintillation solution was prepared by dissolving 6g of 2,5-bis-(5-tert-butyl-benzoxazol-2-yl)-thiophene (BBOT), obtained from CIBA, per litre of a solution prepared by mixing 80g napthalene with 1 litre of 2-methoxy-ethanol/toluene (2:3 v/v). For routine monitoring of experiments no attempt was made to convert counts per min. (c.p.m.) to disintegrations per min (d.p.m.) Specific activities were determined by adding to the appropriate solution, a known volume "spike" of labelled toluene of known specific activity, following which the sample was re-counted and the efficiency of counting thereby determined. In doubly-labelled samples two "spikes" were added successively to each sample,

one containing { ^{14}C }- and the other { ^{3}H }-Toluene, with a re-measurement after each addition. With the setting used the method gave efficiencies of approximately 80% for ^{14}C alone and 32% for ^{3}H alone. When the ^{3}H and ^{14}C channels were used together the efficiencies were about 20% for both ^{14}C and ^{3}H/in the ^{3}H channel, and 50% for ^{14}C and 0,8% for ^{3}H in the ^{14}C channel.

B. Preparation of 3-Methyl 3-butenal

1,3-Dithiane

Propan-1,3-dithiol (20ml) and methylal (18,2ml) were reacted as described[81] to give 1,3-dithiane (XXVI) (12,5g), m.p. 53-54° (white needles from ether). The material was dried in vacuo over phosphoric anhydride.

N.M.R. δ : 2,1 (2H, multiplet), C-5 protons; ∿ 2,85 (4H, multiplet), C-4 and C-6 protons; 3,80 (2H singlet) C-2 protons

Analysis:

Found:	39,8%C	6,6%H
Calc. for $C_4 H_8 S_2$:	40,0%C	6,7%H

2-(2-Methyl-2-propenyl)-1,3-dithiane

Dithiane (XXVI) (6,0g) was dissolved in dry THF (250ml) in a dry flask which had been flushed with N_2 . A solution of butyl lithium in hexane (1,42M, 36ml) was added with syringe to the cooled, (-30°), stirring mixture, which was maintained at -30° for 90 minutes before adding in one portion 1-iodo-2-methyl-2-propene (9,78g). After stirring overnight at 4° the solvent was replaced with water which was brought to pH 7 with dilute hydrochloric acid. The aqueous layer was extracted with methylene chloride and the crude material, dissolved in a little benzene, was chromatographed on Silica gel (65g) in a column (10cm x 4,5cm).

The column was eluted with benzene and 10ml fractions were collected. Fractions Nos. 3-12 contained 2-(2-methyl-2-propenyl) -1,3-dithiane (XXVII) (6,5g), a light yellow oil, b.p. 85-88° /0,4mm. ν_{max}^{film} 3100cm^{-1} (RR'C=CH$_2$ stretching); 1640cm^{-1} (C=C stretching); 908cm^{-1} (RR'C=CH$_2$ deformation). N.M.R. δ: 1,78 (3H, triplet, J=1,0 Hz), methyl protons; ∿ 2,2 (2H, multiplet), C-5 protons; 2,48 (2H, doublet), J=7,7 Hz), C-1' protons; ∿ 2,8 (4H multiplet) C-4 and C-6 protons; 4,20 (1H, triplet, J=7,7Hz), C-2 proton; 4,8 (2H, multiplet), olefinic protons.

Analysis:

Found:	55,4%C	8,1%H
Calc. for $C_8H_{14}S_2$	55,2%C	8,1%C

Deuteration of (XXVII)

A solution of (XXVII) (0,174g) in dry THF was treated with butyl lithium in hexane (1,41M, 0,75ml) and stirred at -25° for 90 minutes. Deuterium oxide (0,5ml) was added and the solution was stirred overnight. The mixture was worked up as above to give a yellow oil (150mg) which was purified by p.l.c. using benzene as developer. The main band (R_f = 0,45) was eluted with ether to give a yellow oil (123mg).

N.M.R. δ : 1,78 (3H, triplet J <1,0 Hz), methyl protons: ∿ 2,1(2H, multiplet), C-4 protons: 2,46 (doublet, J=7,2 Hz) and 2,45 (singlet), total integral 2H, C-1' protons, undeuterated and C-2 deuterated species; ∿ 2,85 (4H, multiplet), C-4 and C-6 protons: 4,19 (0,5H, triplet J=7,2Hz), C-2 proton partially substituted with deuterium: ∿ 4,85 (2H, multiplet), olefinic protons.

Hydrolysis of (XXVII)

A mixture of ethylene glycol and water (9:1, 10ml) was used to wash mercuric oxide (650mg) and (XXVII) (520mg) into the inner chamber of a Kjeldahl apparatus. Steam was flushed through the outer chamber while a solution of mercuric chloride (1,76g) in the same solvent mixture (3ml) was added. A white precipitate formed immediately. The passage of steam through the outer jacket was continued with intermittent stirring of the reaction mixture by brief passage of steam through it, and the small amount of water plus oily droplets which distilled were collected. After one hour the mixture was steam-distilled and the combined distillates were extracted with pentane after saturating them with sodium chloride. After being dried ($MgSO_4$) the organic layer was concentrated to give a colourless liquid (390mg). By g.l.c. (150 × 0,3cm, 10% Carbowax, 75° C) two components were detected besides the solvent, in the ratio of about 3:1, with retention times of 8,5 and 3 minutes respectively.

N.M.R. (neat) δ : 0,9 and 1,3 not integrated, pentane signals: 1,75 (triplet, J <1,0 Hz), vinyl methyl protons; 1,95 and 2,18 doublets in the ratio 1:1, J <1,0 Hz, gem-dimethyl protons attached to α,β-unsaturated carbonyl function, (the signals at 1,75 and 1,95 were in the ratio 10:3), 2,36 (doublet J=5.0 Hz), alkyl methylene protons, ratio of signal at 1,75 to that at 2,36 was 3:2; $\sim$ 3,84 (multiplet, unresolved), four ring protons of an ethylene acetal, ratio to signal at 1,75 was 4:3; 4,8 (multiplet, unresolved), overlapping with 4,96 (triplet, J=5,0 Hz), olefinic protons plus acetal proton of ethylene acetal respectively, total signal relative to that at 1,75 was 2,90:3; 4,88 (doublet, J=8,0 Hz), each signal split further into multiplets, J $\sim$ 1-2 Hz, total signal relative to that at 1,95 was 1:3, vinyl proton; 9,95 (doublet J=8,0 Hz), signal relative to that at 1,95 was 1:3, aldehyde proton.

In a similar experiment performed at 70° followed by distillation at 60mm the organic material obtained exhibited similar g.l.c. and N.M.R. properties except that the ratio of the two components was reversed from 10:3 to 1:10.

Synthesis of 3-methyl-3-butenal diethyl acetal (XXVIII)

The compound was prepared as described[59], yielding a main fraction (40g) b.p. 71,5 -72,5/30mm. A redistilled sample had n_D^{20} 1,4151, b.p. 166° / 755mm (Lit.[59] n_D^{21} = 1,4155 b.p. 162° / 745mm)

ν_{max}^{film} : 3030cm^{-1}, 1040cm^{-1} (broad) (C-O-C-O-C stretching); 885cm^{-1} ($RR'C = CH_2$ deformation), N.M.R. (neat) δ : 1,15 (6H triplet, J=6,9 Hz), methyl protons of ethoxy group; 1,75 (3H, triplet, J <1,0 Hz), methyl protons; 2,30 (2H, doublet, J=6,0c.p.s. each split further with J < 1,0 Hz), C-2 protons; 3,55 (4H, 24-line multiplet, methylene protons of ethoxy group; 4,61 (1H, triplet, J=6,9 Hz), acetal proton; 4,81 (2H, multiplet, J < 1,0 Hz), olefinic protons.

At 100 MHz in $CDCl_3$ all assignments were confirmed but the signal at 3,55 δ now appeared as a 16-line multiplet.

Analysis:

Found	68,2%	11,6%H
Calc. for $C_9 H_{18} O_2$	68,3%C	11,5%H

Modified synthesis of (XXVIII)

(i) Preparation of 2-methyl-2-propenyl magnesium chloride (XXIX)

Dry THF (130ml) was distilled directly into a three-necked flask containing magnesium turnings (7,0g). The flask was equipped with a condenser and dropping funnel containing a solution of 2-methyl-2-propenyl chloride (23g) in dry THF (25ml). A portion of the halide (1ml) and a few cyrstals of iodine were added and the solution was warmed. After a few minutes the yellow colour disappeared. The mixture was cooled in ice and the solution of

halide in THF was added over six hours while the reaction was kept cool in ice. The solution was warmed to room temperature and filtered under compressed nitrogen into a dry flask, which had been flushed previously with dry nitrogen. The tightly-stoppered flask was stored in a desiccator over phosphoric anhydride. The solution was assayed by hydrolysis of an aliquot and titration of the alkai with 0,1N hydrochloride acid. The solution was 1,5M in Grignard reagent.

(ii) Reaction of Grignard reagent with triethyl orthoformate.

A dry two-necked flask was equipped with a dropping funnel and a 15cm Vigreux column connected to a distillation head and condenser set up to collect the distillate. Thiethyl orthoformate (2ml) was pipetted into the flask which was then heated at 110° in an oil bath. The Grignard reagent (XXIX) (10ml) was transferred under nitrogen pressure into the funnel and added over one hour to the orthoformate, and the distillate was collected and analysed by g.l.c. (150 x 0,3cm column, 10% SE30, 100°). Only one peak was detected, (retention time < 10 seconds) and it was assumed to be THF. Under these conditions the acetal has a retention time of 2,5 minutes and the orthoester one of 1,5 minutes. The residual gummy solution was treated with saturated aqueous ammonium chloride.

Initially gas was evolved, and when excess aqueous solution had been added, a two-phase system was produced. The upper layer was removed, yielding a liquid (1,74g), shown to be 95% pure acetal by g.l.c. using the conditions. Extraction of the acqueous layer yielded, after removal of solvent, more material (30mg) of similar composition. The combined products were redistilled to give the acetal (1,4g) identical with an authentic sample by g.l.c. and refractive index. Yield based on orthoformate, 75%. On a larger scale (450m.mol orthoester) the yield of acetal was 55,0g, 77%.

When the halide (2ml) and excess orthoester were reacted as described by Cornforth & Firth[59] the yield of purified acetal was 1,2g, or 60% based on halide.

Hydrolysis of (XXVIII).

The acetal (5,53g) was shaken for 35 minutes with saturated aqueous oxalic acid (55ml). The turbid solution was extracted with ethyl chloride (4x 10ml). The organic layer was washed four times with saturated aqueous calcium chloride and twice with brine, before being dried over sodium sulphate. The solvent was removed through a 15 x 1cm fractionating column with Helipak coils, leaving a mixture (2,5g).

By g.l.c. (150 x 0,3cm, 10% Carbowax 20M, 75° C)three components were detected together with traces of solvent. One was identified as the acetal (retention 14 minutes), the other two had retention times of 7 minutes and 9 minutes respectively. The former of these was the major component of the mixture (about 60%). Careful distillation at reduced pressure (100mm) from a small Claisen flask immersed in a bath at 45-50° into a cooled receiving vessel gave a liquid (1,2g) which contained only the two components of shorter time in the ratio of about 9:1. A re-distilled sample was about 99% pure 3-methyl-3-butenal (XXX). n_D^{20} = 1,4158; ν_{max}^{film} 3440 cm^{-1}, (overtone of band at 1720 cm^{-1}, C = CH_2 stretching); 2820 cm^{-1} and 2730^{-1} (aldehyde C-H stretching); 1720 cm^{-1} (C=0 stretching); 1645 cm^{-1} (C=C stretching); 895 cm^{-1} (RR'C = CH_2 deformation). N.M.R. δ ; 1,79 (3H, triplet, J=1,0 Hz), methyl protons; 3,12 (2H, two pairs of doublets, J=2,5 Hz and 1,0 Hz), C-2 protons; 4,84 and 5,02 (2H, two sets of Octets J ~ 1,0 Hz), olefinic protons; 9,70 (1H, triplet, J=2,5 Hz), aldehyde proton. The compound in methanolic solution exhibited no absorption above 220nm.

Analysis:

Found	71, 6%C	9, 6%H
Calc. for $C_5 H_8 O$	71, 4%C	9, 6%H

Preparation of semicarbazone of (XXX)

A solution was prepared of semicarbazide hydrochloride (100mg) and sodium acetate (200mg) in water (2ml). The aldehyde (50mg) was added to this solution in a test-tube, which was allowed to stand at 4° for 1 hour. The white crystalline precipitate was collected, recrystallized from water, and dried over phosphoric anhydride to give 3-methyl-3-butenal semicarbazone (XXXI) (27mg) as short needles, m.p. 128-130°.

ν_{max}^{KBr} 3440cm^{-1} (bonded N-H_2 stretching); 3280cm^{-1}, 3040cm^{-1} (N-H stretching) 1690cm^{-1} (C=O stretching); 1678cm^{-1} (C=O stretching); 1600cm^{-1} (N-H deformation); 1660cm^{-1} (C=N stretching); 890cm^{-1} (RR'C = CH_2 deformation).

Analysis:

Found	51, 0%C	8, 2%H	30, 1%N
Calc. for $C_6H_{11}O N_3$	51, 0%C	7, 9%H	28, 8%N

Preparation of 3-methyl-2-butenal 2, 4-dinitrophenylhydrazone.

The aldehyde (XXX) (25mg) was added to a saturated solution of 2, 4-dinitrophenylhydrazine in 2N hydrochloric acid (5ml). After 30 minutes addition of more reagent to the solution produced no further opalescence. The dark red precipitate as collected and recrystallized from ethanol, giving brick red flakes m.p. 178-179°. Lit.[51] m.p. 179-181°

λ_{max}^{MeOH} 383nm (ε = 28900)

Analysis:

Found	50,0%C	4,4%H	21,2%N
Calc. for $C_{11}H_{12}O_4N_4$	50,0%C	4,6%H	21,1%H

C. Preparation of Deuterio-triethyl orthoformate

Preparation of sodium ethoxide.

Sodium shot was prepared from sodium (5,75g) as described[82]. Dry ethanol (14,6ml) distilled from magnesium ethoxide[83], was added in one portion to the shot suspended in dry ether (150ml) and the solution was refluxed for 5 hours exclusion of moisture. The solvent was distilled off and the flask was heated at 150° for 1 hour under rotary pump vacuum to remove all ethanol. The flask was filled with nitrogen and the dry powder (14,3g) was stored over phosphoric anhydride.

Reaction of sodium ethoxide with chloroform.

Sodium ethoxide (4,63g) was suspended with stirring in dry ether (40ml) in a flask fitted with condenser and drying tube, and a self-equilibrating dropping funnel. Chloroform (2,0ml), freed of ethanol by passage through active alumina, was dissolved in dry ether (5ml) in the dropping funnel. This solution was added to the ethoxide over one hour and the resulting mixture was refluxed overnight. Total transfer of liquid into a receiver cooled to -75° was performed at 20mm pressure. Ether was removed with a fractionating column and the remaining liquid was examined by g.l.c. (250 x 0,3cm, 10% Carbowax M20, 100°). Apart from traces of ether the compounds detected were triethyl orthoformate and ethanol. These were fractionated in a one piece Claisen flask fitted with a Vigreux column to give the orthoester (1,86g, 52% yield). The product was identical with authentic material by g.l.c. and N.M.R.

n_D^{20} 1,4300, (Lit.[84] b.p. 69 °/5mm, n_D^{20} 1,4308). On cooling a solid, m.p. 21 °, formed. N.M.R. δ : 1,80 (3H, triplet J < 1,0 Hz), methyl protons; 2,06 (2H, multiplet, J< 1,0 Hz), methylene protons, 4,90 (2H, multiplet, J < 1,0 Hz), olefinic protons; 9,55 (1H, singlet), J < 1,0Hz), olefinic protons; 9,55 (1H, singlet), carboxyl proton.

Preparation of 3-Methyl-3-butenoyl chloride (XXXIII)

The acid (XXXII) (5g) was mixed with freshly distilled thionyl chloride (4,0ml) in a 25ml Vigreux flask. After one hour excess reagent was removed by heating the flask to 90 °. Careful fractionation yielded a colourless liquid (3,6g), b.p. 105-107° / 754mm.

ν_{max}^{film} 3080cm^{-1} (RR'C = CH_2 stretching); 1790cm^{-1} (C=O stretching); 1650cm^{-1} (C=C stretching); 1600cm^{-1}, small (conjugated C=C stretching); 900cm^{-1} (RR'C = CH_2 deformation); 765cm^{-1} (C-Cl stretching). A small extraneous peak at 825cm^{-1} was assigned to traces of thionyl chloride. N.M.R. δ : 1,82 (3H, triplet, J < 1,0Hz), methyl protons; 3,56 (2H, triplet, J < Hz c.p.s.), C-2 protons: 5,0 (2H, doublet, J=5,0 Hz, further split into quartets, J < 1,0 Hz), C-4 protons. Small peaks at 1,95 and 2,18 δ revealed traces of the conjugated isomer. By integration this represented less than 5% contamination.

Preparation of Methyl 3-methyl-3-butenoate (XXXIV)

An ethereal-alcoholic solution of diazomethane was prepared from N-methyl-N-nitrose toluene-p-sulphonamide (23g) as described[85] This was added to an ethereal solution of the acid (XXXII) (3,5g) until a faint yellow colour persisted. The solvent was removed on a rotary evaporator and the remaining liquid was distilled to give a clear liquid (1, 82g) b.p. 127/760mm, n_D^{20} = 1,4180 (Lit.[86] b.p. 126° /760mm, n_D^{20} = 1,4171).

ν_{max}^{film} 2980 and 2930 cm^{-1} (CH_3 stretching); 2880 cm^{-1} (CH_2 stretching); 1470cm^{-1} (CH_2 bending); 1440cm^{-1} (CH_3 asymmetrical bending); 1370cm^{-1} (CH_3 symmetrical bending); 1333cm^{-1} (C-H bending); broad band around 1090cm^{-1} (C-O-C stretching).

N.M.R. δ : 1,23 (9H, triplet, J=7,0 Hz), methyl protons; 3,64 (6H, quartet, J=7,0 Hz), methylene protons; 5,12 (1H, singlet) methine proton.

Analysis:

Found	56,7%C	11,1%H
Calc. for $C_7H_{16}O_3$	56,7%C	10,9%H

Reaction of sodium ethoxide with deuteriochloroform

The reaction was performed as above, but using deuterichloroform (2,1ml, isotopic purity 99,7%) in place of chloroform. The yield of distilled material was 1,64g, 45%.

N.M.R.: The signal at 5,12 δ had not disappeared completely. The ratio of signals at δ 1,25, 3,64 and 5,12 was 9:6:0,34.

D. Preparation of 3-Methyl-3-buten-1-oic acid (XXXII) and derivatives.

Carbonation of 2-methyl-2-propenyl magnesium chloride (XXIX)

The Grignard reagent was prepared in THF as described previously (p.47), from the halide (50ml) and magnesium (14g). The total product was poured onto excess crushed solid carbon dioxide. When all the CO_2 had disappeared concentrated hydrochloric acid (60ml) was added, the solution was saturated with sodium chloride and extracted with ether. The extract was dried ($MgSO_4$) and evaporated to give a viscous oil (29g). Distillation yielded 3-methyl-3-buten-oic acid (XXXII) (24g), b.p. 93°/35mm

n_D^{20} 1,4300, (Lit.[84] b.p. 69 /5mm, n_D^{20} 1,4308). On cooling a solid, m.p. 21 °, formed. N.M.R. δ : 1,80 (3H, triplet J <1,0 Hz), methyl protons; 2,06 (2H, multiplet, J< 1,0 Hz), methylene protons, 4,90 (2H, multiplet, J <1,0 Hz), olefinic protons; 9,55 (1H, singlet), J 1,0Hz), olefinic protons; 9,55 (1H, singlet), carboxyl proton.

Preparation of 3-Methyl-3-butenoyl chloride (XXXIII)

The acid (XXXII) (5g) was mixed with freshly distilled thionyl chloride (4,0ml) in a 25ml Vigreux flask. After one hour excess reagent was removed by heating the flask to 90 °. Careful fractionation yielded a colourless liquid (3,6g), b.p. 105-107° / 754mm.

ν_{max}^{film} 3080cm^{-1} (RR'C = CH_2 stretching); 1790cm^{-1} (C=O stretching); 1650cm^{-1} (C=C stretching); 1600cm^{-1}, small (conjugated C=C stretching); 900cm^{-1} (RR'C = CH_2 deformation); 765cm^{-1} (C-C1 stretching). A small extraneous peak at 825cm^{-1} was assigned to traces of thionyl chloride. N.M.R. δ : 1,82 (3H, triplet, J< 1,0Hz), methyl protons; 3,56 (2H, triplet, J < Hz c.p.s.), C-2 protons: 5,0 (2H, doublet, J=5,0 Hz, further split into quartets, J < 1,0 Hz), C-4 protons. Small peaks at 1,95 and 2,18 δ revealed traces of the conjugated isomer. By integration this represented less than 5% contamination.

Preparation of Methyl 3-methyl-3-butenoate (XXXIV)

An ethereal-alcoholic solution of diazomethane was prepared from N-methyl-N-nitrose toluene-p-sulphonamide (23g) as described[85] This was added to an ethereal solution of the acid (XXXII) (3,5g) until a faint yellow colour persisted. The solvent was removed on a rotary evaporator and the remaining liquid was distilled to give a clear liquid (1m82g) b.p. 127/760mm, n_D^{20} = 1,4180 (Lit.[86] b.p. 126° /760mm, n_D^{20} = 1,4171).

ν_{Max}^{film} : 3050cm^{-1} (RR'C = CH_2 stretching); 1740cm^{-1} (C = O stretching); 1660cm^{-1})C = C stretching; 890cm^{-1} (RR'C = CH_2 deformation).

N.M.R. δ: 1,80 (3H, triplet, J=1,0 Hz), olefinic methyl protons; 3,00 (2H, triplet, J <1,0 Hz), methylene protons; 3,68 (3H, singlet), methyl ester protons; 4,86 (2H, multiplet, J <1,0 Hz), olefinic protons.

Analysis:

Found	63,1%C	8,9%H
Calc. for $C_6H_{10}O_2$	63,1%C	8,8%H

E. Reduction of derivatives of 3-Methyl-3-butenoic acid and of 3-Methyl-3-butenal to 3-Methyl-3-butenol (isopentenol) (XXXV)

Preparation and assay of solutions of Lithium borohydride and Lithium borodeuteride.

Lithium borohydride (1g) was stirred overnight in dry THF (50ml) The solution was filtered under N_2 into a dry measuring cylinder fitted with a tight stopper. Aliquots were assayed periodically by reaction with standard potassium iodate at alkaline pH, followed by titration of the liberated iodine when the solution was acidified. The original concentration (0,50M) remained unchanged during three months. In later experiments a 2,05M solution was used. A solution of lithium borodeuteride prepared similarly assayed at 0,63M.

Reduction of Methyl 3-methyl-3-butenoate (XXXIV).

A solution of lithium borohydride (0,5M, 1,0ml) was pipetted into a dry test-tube. The ester (XXXIV) (0,12ml) in dry THF (0,5ml) was added in one portion and the mixture was refluxed with exclusion of moisture for 1 hour. All liquid was removed at 50° /15mm, the distillate being trapped at -78° . By g.l.c. (150 × 0,3 cm, 10% SE 30, 50°) the distillate was shown to contain only THF. The residual white solid in the test-tube was hydrolysed with water (2ml) followed

by hydrochloric acid (2N, 1ml) and the solution was extracted with ethyl chloride. The extract was dried, the bulk of the solvent was removed and the residual liquid was made to 1,0ml with ether in a volumetric flask. This was compared under identical conditions with a standard solution { 100mg of (XXXV) made up to 1,0ml with ether} by g.l.c. (150 x 0,3cm, 10% Carbowax M20, 90°). From the relative peak areas obtained by averaging fine consecutive traces for each sample the yield of isopentenol (XXXV) was 78mg, 91%.

Reduction of (XXXIV) with half-hydrolysed $LiBD_4$ [60]

Three aliquots of $LiBD_4$ in THF (1,0ml) was pipetted into separate dry, testltubes, each fitted with a condenser and Bunsen valve. Each sample was treated with deuterium oxide (11,5 µ l, isotopic purity 99,5%) and then refluxed for one hour. One sample was then assayed for residual power and the other two were treated with the ester (XXXIV) (60µ 1) added in one portion. These two samples were then refluxed for another hour. A second sample was then assayed in the same manner as the first while the third was worked up as in the previous experiment. The ethereal solution was analysed for deuterium content of the isopentenol by combined gas chromatography-mass spectrometry (150 x 0,3cm), 10% Carbowax 20m, 80°).

The solution assayed after hydrolysis with deuterium oxide retained 0,37m.eq. of reducing power and that used to reduce the ester 0,125m.eq. A comparison of the peaks in the molecular ion region m/e = 86-89 for the isotopically labelled sample with those in the region m/e 86-88 in authentic isopentenol, determined under identical conditions, revealed the following isotopic composition for the labelled alcohol :

d_0 = 1,3%, d_1 = 10,5%, d_2 = 79,6%, d_3 = 8,6%

Reduction of 3-Methyl-3-butenoyl chloride (XXXIII) with lithium borohydride

A solution of lithium borohydride in THF (0,5M, 1,0ml) was pipetted into a dry stoppered test-tube cooled to 0° . A Hamilton syringe was used to introduce the acid chloride (XXXIII) (80µ 1) in one portion. The solution, which became warm, was allowed to stand for 15 minutes, by which time white precipitate had formed. Distillation of the total liquid contents (20mm) from an oil-bath at 50° left a white residue in the test-tube. The distillate, as examined by g.l.c. (150 x 0,3cm, 10% SE 30, 50° C), contained THF and very slight traces of (XXXIII). The white residue was dissolved by addition of water (1ml) followed by dropwise addition of hydrochloric acid (2N) until the pH was below 4. The aqueous phase was dried and the bulk of the solvent was removed through a 15 cm fractionating column. The residual liquid was washed with ether into a volumetric flask (1,0ml) and was made up to the mark. A standard solution of the alcohol (XXXV) in ether (50mg made to 1,0ml) was prepared and the two solutions were compared by g.l.c. as in the previous experiment. The yield of alcohol was 37mg { 61% based on (XXXV} . The g.l.c. trace of the prepared alcohol also contained peaks corresponding to ethyl chloride and THF, as well as two other components with retention times intermediate between that of the aldehyde (XXX) and of the alcohol (XXXV).

Reduction of 3-Methyl-3-butenal (XXX) with half-hydrolysed lithium borohydride.

A solution of lithium borohydride in THF (2,05M, 1ml) was pipetted into a dry test-tube fitted with condenser and Bunsen valve, and water (36µ 1) was added. The solution was refluxed for 30 minutes and then cooled to -78° , and the aldehyde (XXX) (0,35ml), previously cooled to -78° , was added in one portion. The mixture was allowed to warm to room temperature and to stand for 20 minutes. Solvent was

removed on a rotary evaporator and the white residue was hydrolysed with saturated aqueous amnonium sulphate followed by a cold sulphuric acid (10N) until gas evolution ceased, and the solution was extracted with ether. The extract was dried, the bulk of the solvent was removed through a 15 cm fractionating column, leaving a liquid (751mg). Careful application of a vacuum (130mm) while the flask was rotated slowly decreased the weight (to 264mg). Total distillation gave isopentenol (XXXV) (251mg) n_D^{20} = 1,4327, pure by g.l.c. Lit.[87] n_D^{20} = 1,4331)
Yield from aldehyde 83%.

Preparation of Isopentenyl- 3, 5-dinitro-benzoate (XXXVI)

A solution of the alcohol (XXXV) (86mg) in dry benzene was treated with pyridine (100µl) and 3,5-dinitrobenzoyl chloride (251mg). Water was added after three hours and the organic phase was washed successively with aqueous acetic acid, aqueous sodium hydroxide, water until neutral and then with saturated brine. The solvent was dried and the solvent was removed under vacuum, yielding an oil (254mg). Recrystallization from ether yielded (XXXVI) (129mg) long needles, m.p. 55-56° . ν_{max}^{KBr} : 3100cm^{-1} (RR'C = CH_2 stretching); 3030cm^{-1} (aromatic C-H stretching); 1710cm^{-1} (C=O stretching); 1640cm^{-1} (C=C stretching); 1610cm^{-1} (aryl C=C stretching); 1540cm^{-1} and 1340cm^{-1} (C-NO_2 stretching); 1280cm^{-1} (C-O stretching); 1965cm^{-1} (1,3,5 substitution); 980cm^{-1} (aromatic C-H deformation).

λ_{max}^{MeOH} 230mm ($\varepsilon = 2,3 \times 10^4$)

N.M.R. δ : 1,86 (3H, singlet), methyl protons; 2,57 (2H, triplet J=3,5 Hz, broadened, so that $W_{\frac{1}{2}}$ = 1,9 c.p.s.) C-2' prontons; 4.57 (2H, triplet J=3,5 Hz), C-1' protons; 4,83 (2H, quartet J < 1,0 Hz) olefinic protons; 9,01 (2H, distorted doublet J=1,0 hZ), C-2 and C-6 protons; 9,11 (1H, distorted triplet J=1,0 Hz), C-4 proton.

Analysis:

Found	51,4%C	4,3%H	9,8%N
Calc. for $C_{12}H_{12}O_6N_2$	51,4%C	4,4%H	9,8%N

F. Conversion of Isopentenol (XXXV) to (RS) - Mevalonolactone (XXXIII)

Reaction of Isopentenol (XXXV) with 2,3-dihydropyran (XXXVII)

A mixture of the alcohol (XXXV) (5g), dihydropyran (XXXVII), (10g) freshly distilled off KOH, and toluene-p-sulphoric acid (2mg) was stirred at room temperature. After 30 minutes no trace of alcohol could be detected when a small sample was analysed g.l.c. (150 x 0,3cm, 3% SE 30, 60°). A small amount of potassium acetate was added and the excess dihydropyran was removed by distillation at atmospheric pressure. The remaining liquid was distilled at water-pump pressure to give 2-(3-methyl-3-butenoxy)-tetrahydpyran (XXXVIII) (9,5g) b.p. 100° /21mm. n_D^{21} = 1,4500.
ν_{max}^{film} 3035cm^{-1} (RR'C = CH_2 stretching); 1645cm^{-1} (C=C stretching); 1200cm^{-1} - 1000cm^{-1}, numerous bands, (C-O-C-O-C stretching); 900cm^{-1} (RR'C = CH_2 deformation). N.M.R. (neat) δ 1,58 (6H, multiplet), C-3 C-4 and C-5 protons; 1,74 (3H, triplet, J < 1,0 Hz), protons; 2,24 (2H, triplet, J = 7,0 Hz), C-2' protons; $\sim$ 3,6 (4H, multiplet, band width 50 Hz), C-1' and C-6 protons; 4,58 (1H, broad triplet, J=3,0 Hz), anomeric proton; 4,72 (2H, poorly resolved octet, J $\sim$ 1,0 Hz), olefinic protons.
Analysis:

Found	70,7%	10,6%H
Calc. for $C_{10}H_{18}O_2$	70,5%	10,7%H

Epoxidation of (XXXVIII) by treatment with peracids

The ether (170mg) in methylene chloride (2ml) was treated with 1,1 equivalents of (a) m-chloro-perbenzoic acid, (b) monoperphthalic acid, (c) trifluoro-peracetic acid, in the same solvent. After four hours the unused peracid was reduced with sodium sulphite, the acid was removed by extraction with aqueous sodium bicarbonate

and the dried ($MgSO_4$) solution was examined by i.r. and t.l.c. (4:1 benzene/ethyl acetate).

The results for each oxidant were qualitatively the same, but the amounts of each product differed in the three cases :

(a) gave largely starting material (R_f0,48), with two components (R_f0,39 and 0,60). The total sample was purified by p.l.c. and the three bands were isolated by eluting with ether. The compound of lowest R_f had no peaks at 1640cm^{-1} but exhibited absorption at 3030cm^{-1} and 837cm^{-1}, characteristic of the oxiran ring. The peaks 1265cm^{-1} and 920cm^{-1}, also seen in epoxides[53], were masked. The N.M.R. spectrum of this compound was identical with that of a sample of the epoxide (XXXX) prepared independently via the bromohydrin (see below). The compound of highest R_f absorbed strongly in ultra-violet light and had characteristic peaks at 3500cm^{-1} (-OH) and 1740cm^{-1} (C=O, ester), with no absorptions for a double bond. This suggested that the structure of this compound was that of the diol mono-ester.

(b) hardly reacted at all, and even prolongation of the reaction time to four days resulted in the detection of about 90% of starting material.

(c) consumed all the starting material, but the major component, (R_f0,58) had an absorption in the i.r. (film) at 1785cm^{-1}, characteristic of a trifluoroacetyl ester. A small amount of epoxide (R_f0,38) remained.

Preparation of 2-(4-Bromo-3-hydroxy-3-methylbutoxy)-tetrahydropyran (XXXIX) by reaction of (XXXVIII) with N-bromosuccinimide.

A suspension of (XXXVIII) (170mg) in water (4ml) was shaken with N-bromosuccinimide (180mg) until all the solid had dissolved. The solution was saturated with sodium chloride and extracted with ether. The dried extract was concentrated to give a colourless oil (175mg)

ν_{max}^{film} 3500cm^{-1}, broad (-O-H stretching); 1720cm^{-1}, weak (C=O succinimide) 1200cm^{-1} -1000cm^{-1}, (C-O-C-O-c stretching).

N.M.R.δ : 1,35 (3H broad singlet), methyl protons; 1,65 (6H broad envelope), C-3, C-4 and C-5 protons; 1,98 (2H, triplet, J=6,0 Hz), C-2' protons; 3,20 (1H, broad singlet), -OH proton; 3.45 and 3,48 (2H, pair of singlets), C-4' protons; $\sim$ 3,75 (4H, broad multiplet), C-1' and C-6 protons; 4,6 (1H, broad signal, $W_{\frac{1}{2}}$ = 6,4 Hz), anomeric proton. A small peak at δ = 4,5 confirmed the presence of succinimide in the speciment.

Reaction of (XXXIX) with calcium hydroxide to produce 2-(3,4-epoxy-3-methylbutoxyl-)-tetrahydropyran (XXXX)

The product from the previous reaction was suspended in water and treated with calcium hydroxide (128mg). After shaking for 10 minutes the aqueous solution was saturated with sodium chloride and extracted to give oil (173mg). On a larger scale (XXXIX) (7,04g) gave 2-(3,4 epoxy-3-methyl butoxyl)-tetrahydropyran (XXXXI) (6,2g), b.p. 64 °/0,25mm, n_D^{21}= 1,4532. ν_{max}^{film} 3030cm^{-1} H_2 - C - C/stretching); 1200cm^{-1} to 1000cm^{-1} broad band (C-O-C-O-C stretching); 840cm^{-1} (oxiran ring deformation). N.M.R.δ : 1,37 (3H, broad singlet), methyl protons; $\sim$ 1,8 (8H envelope in which two downfield signals of a triplet, J=5,5 Hz can be seen, centred at 1,91 δ. These two signals are split further, J < 1,0 Hz) C-3, C-4 and C-5 protons plus triplet for C-2' protons; 2,5 and 2,7 (2H AB quartet J=5,0 Hz). The four signals are split into triplets in which $W_{\frac{1}{2}}$ for the downfield pair is 4,0 c.p.s. and into triplets in which $W_{\frac{1}{2}}$ for the downfield pair is 4,0 c.p.s. and for the upfield pair is 2,0 c.p.s.) oxiran protons: $\sim$ 3,7 (4H, envelope with band width 65 Hz), C-1' and C-6 protons; 4,60 (1H, broad singles, $W_{\frac{1}{2}}$ = 6,5 Hz), anomeric proton. At 100 MHz the signal centred on 3,7 gave two complex bands centred at 3,51 38,2. The bands were approximately symmetrical about 3,67.

Analysis:

Found	64,8%C	10,0%H
Calc. for $C_{10}H_{18}O_3$	64,5%C	9,7%H

Attempted reaction between (XXXX) and sodium cyanide

A solution containing (XXXX) (100mg) in DMSO (3ml) was refluxed for 16 hours with sodium cyanide (49mg). The solution was diluted with water and extracted with ether. The colourless liquid obtained was identical by t.l.c. and i.r. with the starting material.

When the reaction was repeated using ethanol as a solvent the brown oil (87mg), obtained after refluxing for 3 hours, had strong bands at 3500cm^{-1} (-OH stretching) and 2245cm^{-1} (-C ≡ N stretching) in the infra-red. This product was not purified further.

Preparation of 4-Bromo-3-hydroxy-3-methylbutanol (XXXXI) from Isopentenol (XXXV) and N-bromosuccinimide

The alcohol (430mg) in water (20ml) was shaken with N-bromosuccinide (900mg). After 15 minutes the solution was extracted with ether to give a colourless liquid (458mg).

ν_{max}^{film} : 3500cm^{-1}, broad band (-C-OH stretching); 1710cm^{-1} weak (traces of succinimide, C=O stretching).

N.M.R. δ : 1,34 (3H, singlet), methyl protons; 1,89 and 1,92 (total 2H, pair of triplets with J=6, 3 Hz and 5,8 Hz respectively), C-2 protons; 2,74 (0,1H, sharp singlet), protons of succinimide; 3,48 (2H, singlet), C-4 protons; 3,87 (2H, triplet, J=6,0 c.p.s.) C-1 protons; a singlet (2H, hydroxyl protons) exhibited a chemical shift value which varied with concentration.

The difference in coupling constant and chemical shift for the C-2 protons was confirmed by re-running the spectrum at 100 MHz with an offset of 165 Hz and a sweep width of 50 Hz.

Preparation of 4-Cyano-3-hydroxy-3-methylbutanol (XXXXII) by reaction of (XXXXI) with potassium cyanide

A solution of (XXXXI) in methanol (5ml) was stirred with potassium

cyanide (140mg) at room temperature for 4 hours and at reflux for one hour. A white precipitate had formed by this time. The solvent was removed in a stream of nitrogen and the residue was extracted exhaustively with ether.

The colourless liquid (80mg) which remained after removal of the solvent was examined by t.l.c. and i.r. A strong absorption appeared at 2240cm^{-1} in the infra-red. Thin-layer chromatography (chloroform/acetone, 4:1) indicated two components, (R_f 's 0,18 and 0,3) with the lower component the major spot.

The major component was purified by p.l.c. and eluted off the silica gel with ether, yielding a brown oil (70mg) ν_{max}^{film} 3400cm^{-1} broad band (-O-H-stretching); 2245cm^{-1} (-C ≡ N stretching); 1710cm^{-1} (C=O, succinimide); 1160cm^{-1}, broad R_3- C - OH stretching); 1040cm^{-1} (R-CH_2-OH stretching).

Mass spectrum: m/e 114 (M+ - CH_3); 111 (M+ - H_2O);)M+ - CH_2CN); 84 (M+ -CH_2CH_2OH); 71 (M+ - CH_2CB - H_2O); 43 (CH_2C ≡O)

Hydrolysis of (XXXXII) to Mevalonolactone (XXXXIII)

The crude product of the above reaction was hydrolysed with sodium hydroxide (2N, 2ml) under reflux for 24 hours. The solution was acidified (pH3) with sulphuric acid and extracted with chloroform for 24 hours. The organic phase was washed with water, dried and concentrated to give a slightly brown oil (98mg). Thin-layer chromatography (chloroform/acetone 4:1) showed one major spot (R_f0,40) co-chromatographing with authentic (XXXXIII), and one other component remaining at the origin. After purification of a portion of the oil by t.l.c. using the same solvent system the major product gave an infra-red spectrum identical with that of authentic (XXXXIII).

ν_{max}^{film} - 3400cm^{-1} (-O-H stretching); 1730cm^{-1} (1 actone C=O stretching); 800cm^{-1} (characteristic of mevalonolactone).

Preparation of Mevalonobenzyhydrylamide (XXXXIV)

The remaining (XXXXIII) (80mg) was heated with diphenylmethylamine (130mg) in dry benzene (0,5ml) in a tightly-stoppered test tube maintained for one hour at 80 °. The solution was extracted exhaustively with aqueous ortho-phosphoric acid, followed by sodium hydroxide and then water until the aqueous phase was neutral. The organic phase was dried and the solvent was removed to give a semi-crystalline mass (63mg). Recrystallization from benzene afforded (XXXXIV) as fine white needles (40mg), m.p. 99-100°. The material and the authentic compound exhibited identical infra-red and mass spectral data.

Mass spectrum: m/e 313 (M^+); 295 ($M^+ - H_2O$); 278 ($M^+ - 2H_2O$); 208{ $(C_6H_5)_2 CNCO^+$ }; 182 { $(C_6H_5)_2 CHNH^+$ } 167 { $C_6H_5)_2CH^+$ }; 77 ($C_6H_5^+$).

The infra-red spectrum (KBr) was complex with no highly-characterestic peaks.

Synthesis of (3RS, 5RS)- { 5- ^{3}H} Mevalonolactone (XXXXIII)

The reduction of (XXX with half-hydrolysed lithium borohydride (see p. 56) was repeated on the same scale, but using tritiated water (5mC/mmol, 36 µ l), in place of deuterium oxide. Distillation gave 1-RS- { 1- ^{3}H}}sopentenol (210mg). The specific activity was determined by scintillation counting of a weighed sample of the crystalline 3,5-dinitrobenzoate derivative { (XXXVI), p. 58 }. Specific activity 7,5 mCi/mol.

The labelled alcohol (6mg) was diluted with unlabelled compound (97mg) and the total sample was converted to mevalonolactone as described above. The major product, purified by t.l.c. (benzene/methanol, 1:1) followed by elution with acetone of the major radiochemical component detected by scanning the plates, R_f = 0,5) was the lactone (87mg). Small amounts of this material were chromatographed in three solvent systems, (benzene/methanol, 1:1, chloroform/acetone, 4:1, ethyl acetate/hexane, 4:1), and in each case scanning of the plates revealed only one radiochemical component (R_f 0,5, 0,4 and 0,2 respectively), co-chromatographing with authentic lactone.

Oxidation of (3RS, 5RS)- {5-^{3}H }- Mevalonolactone (XXXXIII) to (3RS)-3-hydroxy-3-methyl-glutaric acid (XXXXV) with aqueous zinc permanganate

The labelled lactone (XXXXIII) from the above experiment was diluted with authentic material (45mg) and a small sample removed for determination of specific activity (0,3 mC/mol.). The remainder was brought to pH 8 with aqueous sodium hydroxide using phenolphthalein indicator. Zinc permanganate (10% molar excess) was added and the solution was stirred for two hours. The addition of sulphuric acid to lower the pH (<3) followed by hydrogen peroxide dissolved the brown precipitate and the aqueous solution was extracted continuously with ether for 10 hours after concentrating it to about 5ml. The ethereal solution was dried and the solvent was removed to give a semi-solid material (123mg), which was triturated with chloroform to remove traces of lactone present. The residual solid was recrystallized twice in a Craigi tube from acetone/benzene, giving 3-hydroxy-3-methylglutaric acid (XXXXV) (71mg)

Specific activity: 2×10^{-3} mCi/m.mol.

Preparation of Dimethyl (3RS)-3-hydroxy-3-methylglutaric acid (XXXXVI)

An ethereal solution of diazomethane was used to esterify a sample of the above acid (XXXV) (2,5mg).. The total reaction product was separated by t.l.c. (4:1 chloroform/acetone) and two radioactive components (R_f 0,25 and 0,48) were detected, corresponding in R_f to mevalonolactone (XXXXIV) and the ester (XXXXVI) respectively. The material of higher R_f was eluted from the plate with ether, giving the ester (1,4mg). The specific activity of the ester was determined by counting aliquots of a methanolic solution of the above purified material and found to be $7,5 \times 10^{-4}$ mC/m.mol.

G. Synthesis of (5S, 3RS) -{ 5-^{3}H }Mevalonolactone and determination of the stereospecificity of labelling

Synthesis of 3-Methyl-3-butenal (XXVIII) by oxidation of isopentenol (XXXV)[61]

A mixture of isopentenol (1,03g) dicycohexylcarbodiimide (DCCD) (7,42g), dry DMSO (15ml), and a solution of phosphoric acid in DMSO (2M, 2ml) was stirred for three hours at room temperature. The reaction contents were distilled at 0,1mm from an oil bath at about 30° into a container cooled to -170° C. An equal volume of brine was added to the distillate and the solution was extracted with ethyl chloride. The extract was dried and concentrated and the remaining liquid examined by g.l.c. (75 x 0,3cm 10% Carbowax, 75 °C and 180° C). Five components were present, none of which was DMSO. The major product was the aldehyde (XXVIIII) with about 2% of the conjugated isomer. The third and fourth components were dimethyl sulphide and dimethyl disulphide, as shown by comparison of the N.M.R. and g.l.c. data with authentic samples. The fifth component appeared as a shoulder on the main peak. The total contents were purified by preparative g.l.c. (250 x 12cm, 12,5% Carbowax M20, 90° C) giving (XXVIIII) free of its isomer and sulphide compounds, but still

containing about 5 per cent of the unknown impurity. The structure of the aldehyde was confirmed by i.r., N.M.R. and analytical g.l.c. The impurity was examined by combined g.l.c. - mass spectrometry. No definitive information as to its structure was forthcoming from the mass spectrum.

Synthesis of { 1- ^{3}H }3-Methyl-3-butenal (XXVIII)

1-{1-$^{3}H_{2}$ }isopentenol was oxidized as described above with DCCD/DMSO The solution obtained after removal of the solvent was re-distilled at 45-50° C/15mm as described for the unlabelled compound (p.66), yielding a colourless liquid (87mg), about 90% pure by g.l.c.

Synthesis of (1S) - 1-^{3}H Isopentenol (XXXV) by enzymic reduction of { 1- ^{3}H }3-methyl-3-butenal (XXVIIII) and conversion to (3RS. 5S)-{ 5-^{3}H } Mevalonolactone (XXXXIV)

A sample of (XXVIII) (5, 5mg) obtained by oxidation of { 1- ^{3}H } isopentenol was diluted with 15, 5mg unlabelled aldehyde and reduced enzymically as described below. The alcohols were separated by preparative g.l.c. on a 6ft x ¼in. 12½% Carbowax column at 90° , giving 12mg of (1S)-{1-^{3}H }isopentenol. This was diluted to 1m.mol. with unlabelled alcohol and converted to(3RS, 5S)- {5-^{3}H} mevalonolactone by the methods described above. Yield of purified lactone was 84mg (0, 7m.mol). The specific activity was determined by conversion of 0, 5mg to the benzhydrylamide (p. 64) which was purified by t.l.c. (benzene/ethyl acetate, 7:3), and re-crystallized from benzene in a drawn-out capillary. The total sample, 230 µ g, was used for counting. Specific activity 0, 20mC/mol. The bulk sample of the lactone was stored in benzene.

Preparation of a solution of liver alcohol dehydrogenase and its use in reduction of 3-Methyl-3-butenal (XXVIII)

A solution of crystailine liver alcohol dehudrogenase in saturated ammonium sulphate (10mg/ml, Boehringer) was centrifuged at 2° C at 10, 000g for 30 minutes in an MSE 40 high-speed centrifuge. The

supernatant was discarded and the precipitate was dissolved in glycine/KOH buffer pH 9,5 (0,1M, 1ml). The slightly turbid solution was dialysed against phosphate buffer (0,1M, 1 Litre), pH 7,0, for three days, with daily change of dialysing fluid. The dialysate was centrifuged at 25,000g at 2° C for 30 minutes and the supernatant was removed from a small smount of insoluble protein. The solution (containing 9,4mg/ml of protein as measured by the Buiret method) was stored at 4° C for not longer than two weeks.

A solution was prepared in a 10ml volumetric flask containing the following compounds: 8,4mg aldehyde (XXVIII) and 190mg of NADH (disodium salt, 87% pure by weight, Boehringer) dissolved in about 5ml of 0,1M phosphate buffer pH 7,0 and brought back to pH 7,0 using 1N sodium hydroxide. The flask was stoppered and incubated in a water bath at 37° . After 15 minutes it was made to the mark with buffer at the same temperature. The optical density of the solution at 340nm was determined vs a water blank by removing an aliquot (10µ1) with a Hamilton syringe and mixing it with 3,00ml of water in a spectrophotometer cell. Duplicate results were within 5%. A total of 50µ1 of solution was removed from the flask and the same volume of the enzyme solution was added. Aliquots (10µ1) were removed every few minutes and the optical density of each sample was determined similarly. When the reduction was complete as indicated by no further decrease in O.D. at 340nm the product was isolated by distillation of the solution from a small Claisen flask after saturation with ammonium sulphate. The distillate (4ml) was saturated with sodium chloride and extracted with ethyl chloride (3 x 1ml). When examined by g.l.c. (150 x 0,3cm, 10% Carbowax 20M, 100° C) the extract contained two peaks apart from the solvent, in a ratio of about 3:2 with retention times of 8 and 10,5 minutes respectively. They were identified as isopentenol (XXXV) and the isometric dimethylallyl alcohol (XXXVa) by the retention times of the authentic alcohols and by co-injection of the sample with each alcohol in turn.

Enzymic reduction of 3-methyl-3-butenal (XXVII)

Four flasks were set up in a water-bath at 37 , with contents as shown in Table No. 1.

Flask	O.1M phosphate buffer pH7	Water	Enzyme Solution	Na_2 - NADH
1	3,00ml	-	-	-
2	2,95ml	40 µl	10 µ l	10 µmoles
3	2,95ml	40 µl	10 µ l	-
4	2,90ml	90 µl	10 µ l	10 µmoles

Table No. 1

Flask No. 3 was immersed in a boiling water bath for 5 minutes. A Hamilton syringe was used to introduce 2 µ l of the aldehyde (XXVIII) into each flask. After 15 minutes the contents of each flask was assayed for percentage isomerization of (XXVIII) to (XXVIII, by measuring the O.D. at 237nm of an aliquot in methanol before and after treatment with aqueous base. The conjugated isomer has λ_{max}^{MeoH} 237nm ($\varepsilon = 1,1 \times 10^4$), [88] and the other compound has no appreciable absorption at 237 nm. (p 49). Flasks 113 all showed about 80% isomerization. Flask 3 had about equal quantities of (XXX) and (XXXa) , and by g.l.c. of an etheral extract the corresponding alcohols were present in a similar ratio in flask 4.

XXX XXXa

Fig. 14

Synthesis of { 1- ^3H } 3-Methyl-3-butenal (XXVIII) by oxidation with periodate of { 2-^3H }4-Methyl-4-penten-1,2-diol (XXXXVIII) [89]

A sample of 4-methyl-2-oxo-pentenyl formate (XXXXVII) synthesized in these laboratories was provided by Dr. C. Wakselman. A redistilled

sample containing the conjugated isomer (5% as measured by NMR) was used in these experiments.

The ester (XXXXVII), (71mg) was stirred with sodium bicarbonate (44mg) in water (2ml). After 1 hour sodium borotritide (0,17m.mol, specific activity 590mC/m.mol) was added to the solution, followed 12 hours later by sodium borohydride (5mg). After 1 hour the pH was brought to 6 with sodium dihydrogen phosphate and sodium pericdate (120mg) was added.

Distillation at 15mm was begun immediately from the unheated reaction vessel into a cooled Receiver. A total volume of about 5ml was distilled and kept at 0 ℃ until used shortly afterwards.

Enzymic reduction of {1-^{3}H }3-Methyl-3-butenal (XXVIII) to (1S)-{1-^{3}H } Isopentenol (XXXV)

A solution containing the disodium of NADH (86% pure, 820mg) and liver alcohol dehydrogenase (15mg) in phosphate buffer, pH 6,9, was made up to 10,0ml and incubated at 37°. An aliquot was assayed for NADH by spectrophotometry. The cold, aqueous solution of (XXVIII), as prepared above, was added in small amounts (0,5ml) to the enzymic solution which had been transferred to a 25ml volumetric flask. When the total sample had been reduced the solution was made up to volume and re-assayed for NADH. A total of 0,46m.mol of NADH had been consumed. An aliquot of the solution (10μ 1) was added to an ethereal solution containing authentic isopentenol (XXXV) and dimethylallyl alcohol (XXXVa) and a portion of this latter specimen was subjected to g.l.r.c. (150 x 0,3cm, 10% Carbowax M 20 80° C). Two radioactive components were seen in a ratio of about 6:1, at the retention times of (XXXV) and (XXXVa) respectively. The bulk of the solution was treated as before for isolation of the two alcohols. Unlabelled isopentenol was added to make 1 m.mol total of this alcohol and the mixture was converted to mevalonolactone as described above. (In unpublished work, Cornforth et al. have shown that the product from (XXXVa) under these experimental

conditions was well separated from mevalonolactone in the final purification by t.l.c.). The material was purified as usual giving the lactone (46mg, $7,2 \times 10^9$ d.p.m.). The specific activity was not determined.

Conversion of Potassium (3RS, 5S) - { 5-^{3}H 2-^{14}C }mevalonate to squalene using a rat liver homogenate

A portion of the (3RS, 5S)- 5- ^{3}H mevalonolactone was mixed with a solution of (3RS)- 2-^{14}C mevalonolactone in benzene. (The Radiochemical Centre, Amersham), and a small amount of this doubly-labelled material was diluted with lactone (2mg) and converted to the benzhydrylamide (p. 64). After t.l.c. (benzene/ethyl acetate, 7:3) the radioactive component was eluted, mixed with unlabelled mevalonobenzhydrylamide (25mg) and crystallized to a constant $^3H/^{14}C$ ratio of 10,03. The solvent was removed from the bulk of the doubly-labelled sample under N_2, potassium hydroxide (0,2N, 1ml) was added and the solution was kept at 40° for 3 hours. The total sample was incubated anaerobically with an S_{10} homogenate from rat liver (5g), prepared and used as described[64]. After three hours an equal volume of 2N ethanolic potassium hydroxide was added and the sample was hydrolysed for 1 hour at 60°. Total non-saponifiable lipids were extracted with hexane and separated by t.l.c. (hexane/ethyl acetate 7:3). The major radioactive component, (R_f 0,78), was eluted into a Craigi tube with ether and diluted with squalene (25mg))Koch Light Ltd.). The solvent was replaced with methanol (2ml) and an equal volume of saturated methanolic thiourea was added. The white needles which formed immediately were recrystallized twice and the mother liquors were discarded. The clathrate was decomposed by the addition of water and the squalene was extracted with hexane. Samples were counted for radioactivity.

$^3H/^{14}C$ ratio = 8,31 (average of 4 separate determinations, 8,29, 8,28 8,34, 8,34).

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H. In vivo Incorporation of Mevalonic Acid into Cholesterol by the rat

Incorporation of {2-^{14}C } mevalonic acid by different organs of the rat

A solution of (3 RS) - {2-^{14}C } mevalonolactone (50 µCi, 81, µ moles, Radiochemical Centre, Amsterdam) in benzene was blown to dryness under nitrogen and the lactone hydrolysed by incubation at 60° C for 30 minutes following the addition of aqueous potassium hydroxide (1ml, 10µmoles). Sodium chloride solution (9ml, 1%) was added and the resulting solution was stored at 4 C. One ml aliquots {(2,5 µCi, (3 R) - {2-^{14}C) }potassium mevalonate) were injected intraperitoneally into each of four female Wistar rats (40-45g). After one hour the animals were slaughtered by cervical dislocation and the livers and kidneys were dissected out and freed of all surrounding tissues. The organs were diced with a pair of dissecting scissors and hydrolysed with aqueous sodium hydroxide (5ml, 60%) at 90° C for two hours. The cooled solutions were extracted four times with hexane (5ml) and the hexane layer was dried ($MgSO_4$) and concentrated under nitrogen. An aliquot (10%) of the total non-saponifiable lipids (NSL) was applied to a 20 x 20cm Silica gel G plate together with markers for squalene, lanosterol and cholesterol. The plate was developed with hexane/diethyl ether (7:3) as solvent and the markers visualized by exposure to iodine vapour. The areas corresponding to squalene (R_f 0,95), lanosterol (R_f 0,65) and cholesterol (R_f 0,30) were scraped off and eluted with diethyl ether into scintillation vials. The eluant was blown off and the sample counted for radioactivity. On average the kidneys ($\pm$ 500 mg) contained a total of 9 x 10^5 dpm in NSL. Of this the majority occurred as squalene (70%) and lanosterol (25%) with only small amounts of cholesterol (5%). The livers ($\pm$ 1,4g) contained about 3 x 10^5 dpm in NSL with the majority as cholesterol (84%). Squalene (10%) and lanosterol (3%) comprised nearly all the other labelled lipid.

Time course of the incorporation by the rat of {2-^{14}C}mevalonate into cholesterol in vivo

One ml aliquots of potassium (3 RS) mevalonate (2,5 μ Ci) as prepared above were injected into six pairs of female Wistar rats (40-45g). The animals were sacrificed as before at 15, 30, 45, 60 and 90 minutes and the livers were removed, hydrolysed and extracted to obtain total nonsaponifiable lipids. The total extract from each animal was dissolved in hexane (200 μl) and streaked onto a preparative TLC plate and developed with hexane/diethyl ether (7:3). The cholesterol band was visualized, scraped off and extracted in a small Soxhlet apparatus for three hours, using ethyl acetate as solvent. The solution was blown to dryness in a counting vial and the sample was counted in the normal way. The peak for incorporation occurred aroud 45 minutes.

Conversion of (5S, 3RS) - {5-^{3}H } mevalonolactone to cholesterol in vivo

An aliquot (2 x 10^{10} dpm) of the (5S, 3RS) - {5-^{3}H }mevalonolactone was hydrolysed with potassium hydroxide, diluted with saline and the resulting solution (2 ml) was divided equally and injected into two female rats (50g). After 45 minutes the animals were sacrificed, their livers were removed, hydrolysed with aqueous sodium hydroxide (60%) and the nonsaponifiable lipids were extracted with hexane as described above. The extracts were combined, dried ($MgSO_4$) and the solvent was removed under hexane. The material (25,1mg) was re-dissolved in benzene and applied to a column (1,8m x 10mm) of silicic acid/ Celite (2:1) packed in benzene. The column was eluted with benzene[72], 3ml fractions being collected. Aliquots (5μl) of each fraction were removed for scintillation counting. Fraction numbers 120-140 were pooled and evaporated to dryness under nitrogen giving a white crystalline material (4,7mg). This was re-dissolved in hexane (5,0ml) and an aliquot (100 μl) was removed for further analysis. The bulk solution was stored at 4° C in the presence of an anti-oxidant.

The aliquot (~ 8×10^4 dpm) was diluted with a solution of {4-^{14}C} cholesterol (5mg, $1,4 \times 10^4$ dpm, purified via the dibromide[90]) in benzene (1ml) and the solution was applied to the same 2:1 silicic acid/Celite column (1,8m x 10mm) which was eluted again with benzene. Dual-label liquid scintillation counting showed a major peak of radioactivity containing both ^{3}H and ^{14}C. The combined fractions, when dried, yielded a white crystalline material (4,8mg). In addition there was one small (5%) peak eluted before the major peak which contained only ^{14}C-labelled material.

J. Conversion of Cholesterol (XIII) to 1,4-Androstadiene-3,17-dione (XIX)

Incubation of {4-^{14}C} Cholesterol with Arthrobacter simplex[40]

A solution of {4-^{14}C} Cholesterol in acetone was prepared by diluting the labelled material (5μCi 30μmoles, Radiochemical Centre, Amsterdam) with a solution of cholesterol (400mg, purified via the dibromide) in acetone (10ml). One ml aliquots were added under sterile conditions to a culture (100ml) of Arthobacter simplex IAM 1660 which had been grown for three days at 30° C on a defined medium (0,5% yeast, 0,1% NH_4NO_3, 0,25% K_2HPO_4, 0,025% $MgSO_4$, $7H_2O$, 0,001% $FeSO_4$, $7H_2O$ pH 7,0[81]. After a further seven days the solution was extracted with ethyl acetate (3 x 50ml) and an aliquot of the extract was applied as a streak to a 200 x 200 mm TLC plate together with markers for cholesterol, cholest-4-ene-3 one and 1,4 androstadiene-3,17-dione. The plate was developed with hexane/ethyl acetate (1:1) and, after drying, was viewed under U-V light. The position of the cholestenone and diene-dione markers was noted and the corresponding area of the streaked portion of the plate was scraped off and eluted with ethyl acetate (3 x 1ml). The eluate was blown to dryness in a counting vial and counted in the normal way. The cholesterol band was visualized with iodine and eluted in a similar fashion. The results were variable from one incubation to another, but always showed very little conversion to 1,4-androstadiene-3,17-dione (1% on a radiochemical basis) and considerable conversion to cholest-4-ene-3-one (15-30%). Recovery of added radioactivity was usually above 90%.

Incubation of {4-^{14}C }cholesterol with Mycobacterium phlei[39]

A solution of {4-^{14}C }cholesterol in dimethylformamide was prepared by diluting the labelled material (50μ Ci, 350μg, Radiochemical Centre, Amersham) with a solution of cholesterol (2g, purified via the dibromide) in dimethyl formamide (4ml). The solution was sterilized by autoclaving for 30 minutes at 15 p.s.i.

A freeze-dried shape of the organism Mycobacterium phlei CBS39336 was streaked onto an agar slope (corn steep liquor 10g l^{-1}, Na_2HPO_4 1,25 g l^{-1}. Tween 80 2,5 g l^{-1}, pH=6, agar 10g l^{-1}) and the slope was maintained at 30°C. The same medium was used for regular sub-culturing of the organism.

A sample from this slope was used to inoculate a yeast extract (Yeast extract (Difco) 5g l^{-1}, Tween 80 2,5 g l^{-1}, Na_2HPO_4 1,25 g l^{-1}, pH=6) which was shaken at 30° C. at 180 rpm on a New Brunswick shaker. After three days an aliquot (5,0ml) was used to inoculate a sterile corn steep solution (100ml) of the same composition as that above but without the agar, an aliquot of the {4-^{14}C} cholesterol solution in dimethylformamide (0,1 ml, 50 mg cholesterol) and a solution of $NiSO_4 . 7H_2O$. (50mg), in water (1ml) was added and skahing was continued for a further three days. The solution was extracted with dichloromethane (3 x 10ml) and the dried extract was concentrated, chromatographed and analysed as described above (p 74). Initial results indicated poor conversion (< 10%) of cholesterol to 1,4-androstadiene-3,17-dione.

A sample of the sub-cultured organism maintained on yeast was streaked onto an agar plate of the same composition as above except that the yeast extract was replaced by cholesterol (0,500g l^{-1} added in 10ml dimethylformamide per litre) as the sole source of carbon. Individual colonies which grew after incubation at 30° C for three days were transferred repetitively to similar plates and after 10-15 such transfers the resulting colonies were grown in yeast extract and then in corn steep solution as described before and the conversion of {4-^{14}C }

cholesterol to {4-^{14}C }1, 4-androstadiene-3, 17-dione was assayed in the usual way. No consistent or considerable increase in yield was obtained When final incubation medium was supplemented with a solution (1ml per litre) of trace elements ($CaCl_2$. $2H_2O$ 0, 66g l^{-1}, $ZaSO_4$. $7H_2O$ 0, 18 g l^{-1}; $CuSO_4$. $5H_2O$ 0, 16g l^{-1}, $MnSO_4$. $4H_2O$ 0, 15g l^{-1}; $CoCl_3$. $6H_2O$ 0, 18g l^{-1}; H_3BO_3 0, 1g l^{-1}; Na_2MoO_4 $2H_2O$ 0, 3g l^{-1} and $FeCl_3$. $6H_2O$ 16, 7g l^{-1}) the yield of material which co-chromatographed with 1, 4-androstadiene-3, 17-dione was much higher in some experiments (50% on a radiochemical basis) but the results were still variable from one experiment to another (10-60% yield).

Conversion of {2, 11, 12, 16, 23-$^3H_4$}cholesterol to{ 11, 12, 16-$^3H_2$ } 1, 4-Androstadiene-3, 17-diene-3, 17-dione by Mycobacterium phlei

The cholesterol (37mg, 7, 45 x 10^6 dpm) recovered from the column used to purify the sterols synthesized in vivo was incubated in two flasks with cultures of M. phlei as described above. The extracted material was purified by p. 1. c. and the diene-dione (5, 6 x 10^5 dpm) and material in the upper region of the plate, probably cholesterol and cholestenone (1, 25 x 10^6dpm), were isolated. The yield of crude { 11, 12, 16 -$^3H_2$} 1, 4 androstadiene-3, 17-dione was 12, 6%, allowing for removal of ^3H in the side-chain (5, 6 x 10^5 dpm, 3, 5 mg).

Preparation of { 2, 4, 6, 6, 16. 16 -$^2H_6$} 1, 4-Androstadiene-3, 17-dione by base-catalysed exchange of 1, 4-Androstadiene-3, 17-dione

A piece of freshly-prepared sodium metal (46mg) was added to methanol-1-d (99% isotope excess, 2ml) in a flask fitted with a condenser. When the sodium had dissolved, deuterated water)99, 5%, 1ml) was added (to give a solution of sodium deuteroxide (0, 67 M) in 66% deutero methanol) followed by 1, 4-androstadiene-3, 17-dione (100mg). The solution was refluxed under nitrogen for two hours, deuterated water (5ml) was added and the solution was extracted with dichloromethane (3 x 5ml). The organic phase was dried to yield an off-white mass (97mg) which was subjected to the same conditions a further twice. In the latter experiments one ml of methanol-1-d was reacted with sodium and the

second ml was used for transfer of the steroid into the flask. The crude product after three exchanges was dissolved in benzene (0,5ml) and applied to a small column (40 mm x 5 mm) of alumina (Woelm, Brockmann I) packed in benzene. Elution with benzene yielded a sample (72mg) of { 2,4,6,6,16,16-$^2H_6$} 1,4-Androstadiene-3,27 dione. Recrystallization from benzene/hexane gave pure material (46mg), m.p. 138-140,(Lit.[91] m.p. 141-142). An analysis of the mass spectrum showed that the material had the following isotopic composition do (1,4%), d_1(1,4%), d_2(1,1%), d_3(1,6%), d_4(2,9%), d_5(5,2%), d_6(86,4%). The base peak in the unlabelled compound, at m/e=122 had moved to m/e 126 and the parent at m/e=284 to m/e=290.

{ I am grateful to Dr. G.T. Philips for performing the conversion and initial purification.}

Preparation of 1,4-Androstadiene,3-one-17-ethylene ketal.[92]

A solution of 1,4-androstadiene-3,17-dione (100mg) in dry benzene(8ml) was refluxed with ethylene glycol (0,05ml) and toluene p-sulphonic acid(2mg) in a Dean and Stark apparatus for three hours during which time benzene (2ml) distilled off. The solution was cooled, washed with water (2 x 10 ml) dried ($MgSO_4$) and evaporated to dryness(130mg). The material was re-dissolved in benzene and applied as a streak to three preparative t.l.c. plates which were eluted with hexane/ethyl acetate (1:1). The major u-v absorbing band (R_f=0,74) was scraped off and eluted with ethyl acetate to yield a non-crystalline solid(94mg). This was purified by passage through a short column (40 mm x 5 mm) of alumina (Woelm, grade I) using benzene as the solvent throughout. The product was crystallized twice from hexane, yielding needles m.p. 170-172°C (Lit.[92] m.p. 171-172°C).

Analysis:

Found	76,8%C	8,5%H
Calc. for $C_{21}H_{28}O_3$	76,8%C	8,6%H

Preparation of { 1, 12, 16-$^{3}H_{2}$ }1, 4-Androstadiene-3-one-17-ethylene ketal

{ 11, 12, 16-$^{3}H_{2}$ } 1, 4-Androstadiene-3, 17-dione (5, 6 x 10^{5} dpm, 4mg) was diluted with unlabelled material (310 mg, Koch-Light) and an aliquot (15mg, 1ml) was used for the preparation of the 17-ethylene ketal as described above. The resulting recrystallized material (10, 4mg) had a specific activity of 0, 22 μCi/m.mol. (measured in triplicate on approximately 0, 8 mg samples), which did not change after two further recrystallisations.

Preparation of {11, 12-$^{2}H_{1}$ }androst-1, 4-diene-3, 17-dione by base-catalysed exchange of { 11, 12, 16-$^{2}H_{2}$ } androst-1, 4-diene-3, 17-dione.

The remaining { 11, 12, 16- $^{3}H_{2}$ } 1, 4-androstadiene-3, 17-dione (198 mg) was subjected to exchange in 50% methanolic potassium hydroxide (1N, 10ml). The process was repeated three times, without purification between each exchange and the resulting crude material (280mg) was divided into two portions. The one portion (20mg) was used for the preparation of sample { 11, 12-$^{3}H_{1}$ } 1, 4-androstadiene-3-one-17-ethylene ketal. The sample obtained (9, 9 mg) after three crystallizations from hexane had m.p. 170-171° C and a specific activity of 0, 114 μCi/m.mol (triplicate measurements on about 1 mg samples). The remaining portion was retained for further microbiological incubation (see below).

K. MICROBIOLOGICAL HYDROXYLATION OF 1, 4-ANDROSTADIENE-3, 17-DIONE BY INCUBATION WITH ASPERGILUS TAMARII.[46]

A freeze dried sample of Aspergilus tamarii Kita QM 1223 was initially grown, and then maintained with regular sub-culturing, on a slope of defined constitution (glucose 50g l^{-1}, 1ml of the same trace elements as described previously (p.76) and agar 10 g l^{-1}. Spores were used to inoculate six 1 litre Erlenmeyer flasks containing 250 ml of the above solution., minus the agar, and the flasks were shaken at 180 rpm at 30° C on a New Brunwick skaker for four days. An autoclaved solution of 1, 4-androstadiene-3, 17-dione (50 mg) in dimethylformamide (0, 5 ml) was added to each flask and shaking was continued for a further four days.

The contents of all flasks were pooled and the mycelium filtered off and washed on the sintered filter with dichloromethane (2 x 100 ml). More dichloromethane (200 ml) was added to the filtrate which was extracted with this first and two further aliquots (400 ml each) of organic solvent. The intractible emulsion which sometimes formed was broken by pouring through phase-separating paper. The total organic phase was washed with saturated brine solution (100 ml), dried ($NaSO_4$) and concentrated to give a residue (220 mg). The residue was re-dissolved in dichloromethane and applied as a streak to four p.1.c. plates, 200 mm x 200 mm. Triple elution of the plate to within 50 mm of the top using diethyl ether/methanol (19:1), followed by viewing under u-v light, usually produced three major bands. Only very small quantities of other material were ever visible. Each band was scraped off, the silica gel from the different plates corresponding to the same band was pooled and extracted continuously in a Soxhlet using ethyl acetate as solvent. The small amount of fine silica gel found in each resulting solution was removed by concentrating to dryness, re-dissolving in benzene (0,5ml) and passing the resulting solution through a small column (40 mm x 5mm) of alumina (Woelm, grade I) using benzene as eluant.

The compound obtained (85mg) from the area of highest R_f (0,70) was recrystallized from benzene/hexane giving fine needles of 11β - hydroxy-1,4-androstadiene-3,17-dione (XXI) m.p. 178-181 °C (lit.[93] 176-179 °C. KBr max 3350 cm^{-1}, broad band, (-O-H stretching); 1730 cm^{-1} (ring D C=O stretching); 1660 cm^{-1} (ring A C=O stretching); 1610 cm^{-1} (C=C stretching). N.M.R. δ : 7,26 (1H, doublet, J=10,0 Hz), C-1 proton; 6,28 (1H, pair of doublets, J_1 = 10,0 Hz, J_2 = 1,0 Hz), C-2 proton; 6,04 (1H, pair of overlapping doublets, J_1=2 Hz, J_2 = 1 Hz), C-4 proton; 4,5 (1H, multiplet, $W_{\frac{1}{2}}$ = 9 Hz), C-11 proton; 1,48 (3H, singlet), C-19 methyl group; 1,18 (3H, singlet), C-18 methyl group; 2,7 - 0,8 (1 H, broad envelope), remaining ring protons.

Mass spectrum: m/e 300 (M^+); 282 ($M^+ - H_2O$) 122 (Ring A + 2H).

Analysis:

Found	76,10%C	8,00%H
Calc. for $C_{19}H_{24}O_3$	75,97%C	8,05%H

In several experiments the material crystallized poorly, gave a low m.p. (75-90°C) and an incorrect elemental analysis. Analytical t.l.c. on silica gel using benzene/ethyl acetate (1:1) as eluant revealed the presence of unchanged starting material. When this was removed by preparative t.l.c. using the same conditions as for analytical t.l.c. the recovered compound exhibited the correct physical properties. The middle band on the original t.l.c. plates was always present in small amounts (5-10 mg from 300 mg starting material). It ran very close to the band of higher R_f and on occasions it was necessary to rechromatograph the material under the same p.l.c. conditions to remove completely the upper band. Recrystallization from acetone yielded white needles of 6β - Hydroxy-1,4-androstadiene-3,17-dione m.p. 200-202° C (Lit.[94] m.p 203,5-204,5°C.

The i.r. spectrum differed slightly from that of the previous compound only in the fingerprint region.

N.M.R. δ : 7,05 (1H, doublet 5=10 H_3), C-1 proton;

6,19 (1H, pair of doublets, J_1 = 1,0 Hz) C-2 proton;

6,14 (1H, singlet incorporating upfield half of C-2 desorption), C-4 proton;

4,55 (1H, triplet split again, J_1 = 3Hz; J_2 1Hz), C-6 proton;

1,42 (3H, singlet), C-19 methyl protons;

0,98 (3H, singlet), C-18 methyl protons.

Mass spectrum: m/e 300 (M^+) 282 ($M^+ - H_2O$);

Analysis:

Found	76,20%C	8,22%H
Calc. for $C_{19}H_{24}O_3$	75,97%C	8,05%H

The compound of lowest R_f crystallized readily from diethyl ether to yield crystals of 11α - hydroxy-1, 4-androstadiene-3, 17-dione (XXIII) (70mg) m.p. 210-211 °C (Lit.[95] m.p. 212-214°C).

The i.r. spectrum above 1500 cm^{-1} was the same as that of (XXI) and the whole spectrum was identical with that of an authentic sample of (XXIII)

N.M.R. δ : 7,83 (1H, doublet, J=10 Hz), C-1 protons; 6,13 (1H, pair of doublets, one of which is hidden under absorption at 6,09, J_1= 10 Hz, J_2 = 2Hz), C-2 proton; 6,09 (1H, overlapping doublets, plus a doublet from C-2, J_1=2 Hz, J_2 1Hz), C-4 proton.

4,11 (1H, broad multiplet, $W_{\frac{1}{2}}$ = 13 Hz) C-11β proton;

1,33 (3H, singlet), C-1 methyl group; 0,98 (3H, singlet), C-1 methyl group.

Mass spectrum: m/e 300 (M^+); 282 (M - H_2O); 122 (Ring A + 2H).

Analysis:

Found	75,81%C	8,19%H
Calc. for $C_{19}H_{24}O_3$	75,97%C	8,05%H

Incubation of {11, 12-$^3H_1$} 1, 4-Androstadiene-3, 17-dione with Aspergillus tamarii.

The crude {11, 12-$^3H_1$} 1, 4-androstadiene-3, 17-dione (280mg) described above (p) was dissolved in dimethyformamide (3ml) and, after autoclaving, added to a grown culture of asperigullus tamarii Kita QM 1223 as described above. After four days the culture was filtered and extracted as described and the products separated by p.l.c. on silica gel plates, using diethyl ether/methanol (19:1) as eluant.

The major band had a lower R_f than any of the known components. Elution from the silica gel yielded a white mass (89mg). A small sample was chromatographed with markers of 11α - and 11β-1, 4-androstadiene-3-one-17-ol (prepared by reduction of the respective 17-ones (5mg) in methanol (1ml) by the addition of sodium borohydride (5mg) followed by stirring for one hour, methanolic solution used without further purification. The R_f after triple elution on silica gel G plates, using methanol/diethyl either (1:19) as eluant for the five compounds were 0,72 (unchanged 11β-

hydroxy-1, 4-androstadiene-3, 17-dione) 0, 66 (11β-hydroxy-1, 4-androstadiene 3-one-17-oℓ, 0, 54 (unchanged 11α -hydroxy 1, 4-androstadiene-3, 17-dione) 0, 46 (unknown compound) and 0, 42 (11α -hydroxy-1, 4-androstadiene-3, one-17-oℓ). The three normal bands were isolated as previously described and yielded, in order of decreasing R_f, 1-4-androstadiene-3, 17-dione plus 11β -hydroxy-1, 4-androstadiene-3, 17-dione (122, 4mg), 6β -hydroxy-1, 4-androstadiene-3, 17-dione (4, 9mg) and 11α -hydroxy-1, 4-androstadiene-3, 17-dione (2, 1mg) respectively. The mixed band was separated by p. l. c. on silica gel using benzene/ethyl acetate (1:1) as eluant to give 1, 4-androstadiene-3, 17-dione (86, 0mg) and 11β -hydroxy-1, 4-androsta-diene-3, 17-dione (29, 4mg). The latter compound failed to crystallize from a number of single and mixed solvents.

Oxidation of 11β -Hydroxy-1, 4-Androstadiene-3, 17-dione in the presence of tritiated water.

A sample of 11β -hydroxy-1, 4-androstadiene-3, 17-dione (5, 2mg) prepared by incubation of 1, 4-androstadiene-3, 17-dione with A-tamarii was dissolved in acetone (0, 2ml) to which was added tritiated water (5μℓ 1, 2 x 10^9 dpm). A solution of Jones reagent[96] (5 μℓ 2, 67 M CrC_3 in 1:3 H_2SO_4/H_2O) was added to the cooled (4° C) solution which was stirred at 4° C for one hour. Water (1ml) was added and the solution was extracted with diethyl ether (2 x 1ml). The organic layer was washed with aqueous sodium hydrogen carbonate (0, 1 M, 1ml), dried ($MgSO_4$) and concentrated to yield white crystals (5, 0 mg). The material was dissolved in benzene (0, 5ml) and applied to a column (40mm x 5mm) of neutral alumina (Woelm, grade I) in benzene. Elution with benzene (4ml) yielded unchanged 11β -hydroxy-1, 4-androstadiene-3, 17-dione (1, 9mg). Further elution with benzene/ethyl acetate (3:1, 5ml) yielded { 12, $-^3H_2$ } 1, 4-androstadiene-3, 11, 17-trione (2, 9 mg) M.P. 193-194° C (Lit.[97] mp 197-198° C). Liquid scintillation counting (in triplicate on about 0, 8mg samples), showed that the trione had a specific activity of 0, 008 μCi/m. mol.

Oxidation of 11α -Hydroxy-1, 4-androstadiene-3, 17-dione in the presence of tritiated water.

A sample of authentic 11α -hydroxy-1, 4-androstadiene-3, 17-dione (5, 6mg, Upjohn Chemical Co.) was dissolved in acetone (0, 2ml) and tritiated water (5μℓ 1, 2 x 10^9 dpm) was added. A solution of Jones reagent[96] (5μℓ 2, 67 M CrO_3 in 1:3 H_2SO_4/H_2O) was added to the cooled solution which was stirred at 4° C for 20 minutes. The reaction was worked-up as described above for the oxidation of 11β -hydroxy-1, 4-androstadiene-3, 17-dione, yielding { 12, -$^3H_2$} 1, 4-androstadiene-3, 11, 17-trione, mp. 192-194° C. The specific activity of the trione was 0, 002μCi./m.mol.

Oxidation of { 11, 12-$^3H_1$ } 11β -Hydroxy-1, 4-Androstadiene-3, 17-dione

A sample of non-crystalline { 11, 12-$^3H_1$ } 11β -hydroxy-1, 4-androstadiene-3, 17-dione (2, 51 mg. 1, 08 x 10^4 dpm) was dissolved in acetone (0, 4ml) cooled to 4°C and treated with Jones reagent (25μℓ 2, 67 M). After one hour reaction was quenched with water (2ml) and the solution was extracted with diethyl ether (3 x 2ml). The ethereal extract was washed with sodium hydrogen carbonate (0, 2M, 2ml) dried ($MgSO_4$) and concentrated (24, 2mg). Chromatography on alumina yielded { 12-$^3H_1$ } 1, 4-androstadiene-3, 11, 17-trione. Recrystallization from acetone gave white needles (19, 4mg) mp 194-195° C. The specific activity of the material (measured in triplicate on approximately 2mg samples) was 0, 058μ Ci/m.mol.

Base-catalysed exchange of (12-$^3H_1$) 1, 4-Androstadiene-3, 11-17-trione.

A sample of { 12, -$^3H_1$} 1, 4-androstadiene-3, 11117-trione (10, 5mg, 0, 058/m.mo was dissolved in methanol (2, 0ml) to which was added aqueous sodium hydroxide (2M, 1, 0ml). The solution was refluxed under nitrogen for eight hours after which water (3ml) was added and the resulting milky solution was extracted with dichloromethane (3 x 1ml). The organic phase was dried ($MgSO_4$) and the crude material was subjected to two further treatments with base. The final crude material (8, 4mg) was purified

by chromatography on neutral alumina (Woelm, grade I) followed by three-fold crystallization from acetone to yield 1,4-androstadiene-3,11,17-trione (6,1mg) mp. 193-195° C. Liquid scintillation counting (in triplicate on about 1,6mg samples) revealed that the material was essentially unlabelled (fewer than 10 dpm above background).

CHAPTER 5

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