Studies on the Mechanism and Regulation of C-4 Demethylation in Cholesterol Biosynthesis

THE ROLE OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE

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(Received 12 July 1971)

1. An assay for demethylation has been developed based on the release of tritium from 4,4-dimethyl[3 α -³H]cholest-7-en-3 β -ol (II). 2. The maximum release of ³H from 3 α -³H-labelled compound (II) in a rat liver microsomal preparation occurs in the presence of NADPH and NAD⁺ under aerobic conditions. 3. Incubation of 3 α -³H-labelled compound (II) with NADPH under aerobic conditions leads to the formation of a 3 α -³H-labelled C-4 carboxylic acid. This compound undergoes dehydrogenation on subsequent anaerobic incubation with NAD⁺. 4. The ³H released from the steroid was located in [4-³H]nicotinamide and the medium. Incubation with synthetic [4-³H₂]NADH gave a similar result. 5. In the presence of glutamate dehydrogenase and α -oxoglutarate part of the ³H released from the steroid was transferred to glutamate. 6. A series of 3-oxo steroids were reduced equally well by [4-³H₂]NADH and [4-³H₂]NADPH. The reduction of 5 α -cholest-7-en-3-one was shown to use the 4B H atom from the nucleotide. 7. 3':5'-Cyclic AMP was shown to be a competitive inhibitor of the 3 β -hydroxy dehydrogenase enzyme in the demethylation reaction.

We have shown that adenosine 3':5'-cyclic monophosphate (cyclic AMP) inhibits cholesterol biosynthesis in both cellular and cell-free preparations from rat liver (Akhtar & Bloxham, 1970; Bloxham & Akhtar, 1971; D. P. Bloxham & M. Akhtar, unpublished work) and also in Chang human liver cell cultures (D. C. Wilton, unpublished work). The inhibition of cholesterol synthesis is accompanied by the accumulation of a C-4 carboxylic acid derivative of the type (I) (Bloxham & Akhtar, 1971). The present work is concerned with the removal of the 4 α -methyl group from 4,4-dimethylcholest-7-en-3 β -ol (II). A general scheme for C-4 demethylation of steroids is shown in Scheme 1 (Bloch, 1965; Swindell & Gaylor, 1968; Miller & Gaylor, 1970*a,b*). Demethylation involves the oxygen-dependent formation of a carboxylic acid (III; reaction 1) before oxidation of the 3 β -ol to the 3-ketone (IV; reaction 2), which facilitates decarboxylation (reaction 3), giving compound (V). Finally nicotinamide nucleotide-linked reduction of the 3-ketone (V or VI) yields the 3 β -alcohol (VII or VIII; reaction 4).

The present paper describes an assay of the overall reaction in Scheme 1 with 3 α -³H-labelled compound (II) as a substrate and provides evidence

to show that the dehydrogenation (reaction 2) involves the transfer of the 3 α -hydrogen atom from the steroid to NAD⁺. Cyclic AMP is shown to be a competitive inhibitor of this step.

RESULTS AND DISCUSSION

Release of ³H from 4,4-dimethyl[3 α -³H]cholest-7-en-3 β -ol (II). Removal of the carboxyl group at C-4 involves the conversion of the 3 β -alcohol into a 3-ketone with a concomitant loss of the 3 α -hydrogen atom (reaction 2). The loss of ³H from a 3 α -³H-labelled steroid would provide a simple assay for the demethylation process if a convenient method to remove the steroid from aqueous solution was available.

Several assays for the incorporation of radioactive precursors into tissue lipids are based on the fact that the precipitation of protein under acid conditions also precipitates water-insoluble lipids (Hechter, Yoshinaga, Halkerston & Birchall, 1967). Presumably lipids bind to the hydrophobic regions of the protein in this process. We have used the precipitation of microsomal protein with perchloric acid to remove the steroid from aqueous solution. Under these conditions any ³H that appears in the

Scheme 1. Intermediate steps in the demethylation at C-4.

aqueous phase (acid supernatant) represents the transfer of ^3H from the steroid to a water-soluble compound.

Fig. 1 shows a time-course for the liberation of the $3\alpha\text{-}^3\text{H}$ atom from $3\alpha\text{-}^3\text{H}$ -labelled compound (II) into the acid supernatant. In the presence of an NADPH-generating system and NAD^+ the loss of ^3H was generally found to be approximately linear for the first 30 min. The rate of loss of ^3H under these conditions was shown to be almost proportional to the amount of microsomal preparation added (Fig. 2).

A more rigorous experiment was then undertaken to determine the exact cofactor requirements for the release of ^3H into the acid supernatant (Table 1). The addition of either NAD^+ or NADP^+ causes only a small release of ^3H . The inclusion of an NADPH-generating system in the incubation increases the labilization of tritium, but the maximum release of ^3H only occurs when both the NADPH-generating system and NAD^+ are

present simultaneously. The reason for the release of ^3H in the presence of NADPH alone is not clear; however, we interpret this result as being caused by contamination of the microsomal preparation with endogenous NAD^+ . Experiments have shown that only rigorous treatment will remove the last traces of NAD^+ from the microsomal preparation (Miller, Kalafer, Gaylor & Delwiche, 1967).

In all cases the release of ^3H is decreased when the incubation is performed in an atmosphere of N_2 , indicating that there is an oxygen-linked process in the labilization of ^3H (i.e. reaction 1).

Conventional studies of demethylation have measured the liberation of $^{14}\text{CO}_2$ from $[^{14}\text{C}]$ methyl-labelled steroids. The ability of microsomal preparations to demethylate the monomethyl substrate $[4\alpha\text{-}^{14}\text{C}]$ methylcholest-7-en- 3β -ol (IIa) was investigated and it was shown that the cofactor requirements for $^{14}\text{CO}_2$ liberation and the release of ^3H were identical. The rates of demethylation measured by $^{14}\text{CO}_2$ evolution and release of ^3H

were 2.4 and 3.24 nmol/30 min per mg of protein respectively. Considering that two different steroids were used, these rates of release compare very favourably.

Release of ^3H from 3α - ^3H -labelled compound (II) in a two-step incubation. If the sequence of events shown in Scheme 1 is correct then it should be

Fig. 1. Time-course for the release of ^3H from 3α - ^3H -labelled compound (II). Microsomal preparations (0.5 ml) were incubated with 3α - ^3H -labelled compound (II) ($0.17\ \mu\text{mol}$) and GSH ($5\ \mu\text{mol}$) at 38°C in a final volume of 1 ml. ○, NADP^+ ($1.25\ \mu\text{mol}$), glucose 6-phosphate dehydrogenase ($7.5\ \mu\text{g}$), glucose 6-phosphate ($5\ \mu\text{mol}$) and NAD^+ ($0.5\ \mu\text{mol}$); △, no additions of cofactor. At appropriate times release of ^3H into the acid supernatant was measured.

possible to demonstrate an NAD^+ -dependent release of ^3H from the 3α - ^3H -labelled C-4 carboxylic acid derivative (III) under anaerobic conditions.

Fig. 2. Effect of protein concentration on release of ^3H from 3α - ^3H -labelled compound (II). Various concentrations of microsomal fraction were incubated with 3α - ^3H -labelled compound (II) ($0.17\ \mu\text{mol}$) for 30 min at 38°C in the presence of GSH ($5\ \mu\text{mol}$), NADP^+ ($1.25\ \mu\text{mol}$), glucose 6-phosphate dehydrogenase ($7.5\ \mu\text{g}$), glucose 6-phosphate ($5\ \mu\text{mol}$) and NAD^+ ($0.5\ \mu\text{mol}$). The incubation volume was 1.0 ml. At the end of the incubation the total ^3H released into the acid supernatant was measured.

Table 1. Release of ^3H from 3α - ^3H -labelled compound (II) in a microsomal preparation

Microsomal preparations (0.5 ml) were incubated with 3α - ^3H -labelled compound (II) ($0.17\ \mu\text{mol}$; 1.6×10^3 c.p.m./nmol) and GSH ($5\ \mu\text{mol}$) for 30 min at 38°C in a final volume of 1 ml. NADPH , NAD^+ ($0.5\ \mu\text{mol}$) and NADP^+ ($1.25\ \mu\text{mol}$) were added as indicated. NADPH was generated with NADP^+ ($1.25\ \mu\text{mol}$), glucose 6-phosphate ($5\ \mu\text{mol}$) and glucose 6-phosphate dehydrogenase ($7.5\ \mu\text{g}$). The ^3H released into the acid supernatant was measured. After this fraction had been freeze-dried the ^3H in the water-soluble residue and the water of the medium was analysed.

Gas phase	Addition	Total ^3H in acid supernatant (nmol/30 min)	After freeze-drying	
			^3H in water-soluble residue (nmol/30 min)	^3H lost to medium (nmol/30 min)
Air	—	0.22	0.22	0
N_2	—	0.31	—	—
Air	NAD^+	2.38	—	—
N_2	NAD^+	0.61	—	—
Air	NADP^+	1.25	—	—
N_2	NADP^+	0.57	—	—
Air	NADPH	6.35	2.37	3.98
N_2	NADPH	0.57	—	—
Air	$\text{NADPH} + \text{NAD}^+$	20.5	11.6	8.90
N_2	$\text{NADPH} + \text{NAD}^+$	2.16	—	—

To generate the carboxylic acid substrate *in situ* the method of Miller & Gaylor (1970a,b) was used. In this technique aerobic incubation of the methyl substrate with NADPH causes accumulation of the carboxylic acid. The dehydrogenation step (reaction 2) can then be measured in a second incubation under anaerobic conditions.

Initially it was confirmed that under our experimental conditions the incubation of 3α - ^3H -labelled compound (II) with NADPH in air caused accumulation of a polar intermediate that is detectable by t.l.c. and which presumably corresponds to the 3α - ^3H -labelled carboxylic acid because of its other properties (Fig. 3).

Fig. 3. Time-course for the synthesis of 4α -carboxy- 4β -methyl[3α - ^3H]cholest-7-en- 3β -ol. Microsomal fractions (0.5 ml) were incubated with 3α - ^3H -labelled compound (II) (0.17 μmol) in the presence of GSH (5 μmol), NADP $^+$ (1.25 μmol), glucose 6-phosphate (5 μmol) and glucose 6-phosphate dehydrogenase (7.5 μg) under aerobic conditions in a final volume of 0.75 ml. At appropriate times the formation of the carboxylic acid was assayed.

The release of ^3H into the acid supernatant was then measured after an anaerobic incubation. Table 2 shows that the bulk loss of ^3H only occurs when NAD $^+$ is present in the second incubation mixture. The process is also dependent on the prior formation of the carboxylic acid substrate, since omission of NADPH in the first incubation mixture markedly diminishes the release of ^3H in the second incubation mixture. These results are consistent with the dehydrogenation step shown in Scheme 1, which occurs after the formation of the carboxyl group at C-4.

Attempted isolation of [4 - $^3\text{H}_1$]NADH from 3α - ^3H -labelled compound. The dehydrogenation step shown in Scheme 1 (reaction 2) should result in the synthesis of [4 - $^3\text{H}_1$]NADH. The direct isolation of the nucleotide was attempted by using the two-step incubation system. Since NADH is unstable under acid conditions the reaction was terminated with potassium hydroxide and microsomal protein was separated from the nicotinamide nucleotide fraction by chromatography on Sephadex G-25.

When 3α - ^3H -labelled compound (II) was incubated in a two-step reaction involving an NADPH-generating system in the aerobic incubation and NAD $^+$ in the anaerobic incubation, a peak of radioactivity was associated with the nicotinamide nucleotide fraction, which was absent if the cofactors were omitted. Fig. 4 shows that when this fraction was analysed by paper chromatography only 16% of the radioactivity was associated with NADH whereas the remainder of the radioactivity was located in a compound with the same mobility as nicotinamide.

At this point it seemed possible that most of the [4 - $^3\text{H}_1$]NADH that might be formed was being degraded by either a chemical or an enzymic process. In a model experiment to evaluate this [4 - $^3\text{H}_2$]NADH (0.01 μmol , 35×10^3 c.p.m.) was incubated with a microsomal preparation in an

Table 2. Release of ^3H into the acid supernatant in a two-step incubation

In the first incubation microsomal fraction (0.5 ml) was incubated aerobically with 3α - ^3H -labelled compound (II) (0.17 μmol), GSH (5 μmol) and NADPH for 30 min at 38°C in a final volume of 0.75 ml. NADPH was generated with NADP $^+$ (1.25 μmol), glucose 6-phosphate (5 μmol) and glucose 6-phosphate dehydrogenase (7.5 μg). For the second incubation a further 0.5 ml of the microsomal preparation was added, and NAD $^+$ (0.5 μmol) was added where appropriate. The volume was adjusted to 1.5 ml with water. The incubation was continued for another 30 min in an atmosphere of N_2 , then the total release of ^3H into the acid supernatant was determined.

Additions		Total ^3H in acid supernatant (nmol/30 min)
Aerobic incubation	Anaerobic incubation	
—	—	0.45
—	NAD $^+$	2.89
NADPH	—	6.25
NADPH	NAD $^+$	20.0

Fig. 4. Attempted isolation of $[4\text{-}^3\text{H}_1]\text{NADH}$ from $3\alpha\text{-}^3\text{H}$ -labelled compound (II). Microsomal fractions were incubated in a two-step incubation (Expt. 3; Table 4). The nicotinamide nucleotide fraction was separated from the microsomal fraction by chromatography on Sephadex G-25 and analysed by paper chromatography in ethanol-1M-ammonium acetate, pH 7.5 (7:3, v/v).

Fig. 5. Degradation of $[4\text{-}^3\text{H}_2]\text{NADH}$ by microsomal fractions. $[4\text{-}^3\text{H}_2]\text{NADH}$ ($0.01\text{ }\mu\text{mol}$, 35×10^3 c.p.m.) was incubated for 30 min under anaerobic conditions with a sample of the microsomal fraction (0.5 ml) containing GSH ($5\text{ }\mu\text{mol}$). The final volume was 1 ml . The nicotinamide nucleotide fraction was isolated by chromatography on Sephadex G-25 and analysed by paper chromatography for distribution of ^3H . O, Incubation at 4°C ; Δ , incubation at 38°C .

atmosphere of N_2 . The nicotinamide nucleotide fraction was isolated and analysed by paper chromatography. Fig. 5 shows that when the

incubation was performed at 4°C most of the $[4\text{-}^3\text{H}_2]\text{NADH}$ was recovered intact, showing that the NADH was stable to the isolation procedure. However, when the incubation was performed at 38°C (Fig. 5) radioactivity was only recovered as nicotinamide. This result indicates that any NADH formed in this preparation must be subject to enzymic degradation.

Microsomal preparations are known to contain a very active NAD glycohydrolase enzyme capable of degrading NAD to nicotinamide (Swislocki, Kalish, Chaselow & Kaplan, 1967; Bock, Siekevitz & Palade, 1971). If $[4\text{-}^3\text{H}_1]\text{NADH}$ was oxidized to $[4\text{-}^3\text{H}_1]\text{NAD}^+$ (see below) then the action of NAD glycohydrolase on this substrate will result in the liberation of $[4\text{-}^3\text{H}_1]\text{nicotinamide}$ as a final product. The observation that labelled nicotinamide is formed from either synthetic $[4\text{-}^3\text{H}_2]\text{NADH}$ or from $3\alpha\text{-}^3\text{H}$ -labelled compound (II) provides evidence for the intermediate role of $[4\text{-}^3\text{H}_1]\text{NADH}$ in the decarboxylation of the steroid.

Release of ^3H from $3\alpha\text{-}^3\text{H}_1$ -labelled compound (II) in the presence of a trapping system. Incubations performed in the presence of a glutamate dehydrogenase trap or a lactate dehydrogenase trap are particularly pertinent both to the identification of $[4\text{-}^3\text{H}_1]\text{NADH}$ as an intermediate in the loss of ^3H from $3\alpha\text{-}^3\text{H}$ -labelled compound (II) and to the stereochemistry of the location of ^3H in NADH.

Microsomal preparations were incubated in a two-step system in the presence or absence of a trapping system. Table 3 shows the distribution of ^3H between the medium water and the water-soluble residue. In the absence of a trapping system there is a considerable loss of ^3H into the water of the medium. This may be attributed to the transfer, in a redox reaction, of part of the ^3H in $[4\text{-}^3\text{H}_1]\text{NADH}$ to form an ionizable group, which allows equilibration of ^3H with the medium (reaction 6, Scheme 2). The loss of ^3H into the medium is decreased by the addition of a trapping system consisting of either lactate dehydrogenase and pyruvate or glutamate dehydrogenase and α -oxoglutarate. The oxidation of $[4\text{-}^3\text{H}_1]\text{NADH}$ by either of these enzymes will result in the location of ^3H in a bond that cannot ionize and hence will not equilibrate with the medium (reaction 5, Scheme 2).

This interpretation was substantiated by incubating $[4\text{-}^3\text{H}_2]\text{NADH}$ (0.05 mol ; 80×10^3 c.p.m.) with microsomal preparations under anaerobic conditions. It was found that the ^3H release into the water of the medium ($17.4\text{ nmol}/30\text{ min}$) was greatly decreased by either a glutamate dehydrogenase or a lactate dehydrogenase trap ($4.5\text{ nmol}/30\text{ min}$).

The ^3H released from $3\alpha\text{-}^3\text{H}$ -labelled compound (II) into the water-soluble residue from incubations

Table 3. *Distribution of ^3H released from $3\alpha\text{-}^3\text{H}$ -labelled compound (II) between the medium water and the residue after a two-step incubation*

The incubation conditions were as described in Table 2. The lactate dehydrogenase trap consisted of pyruvate ($20\mu\text{mol}$) and lactate dehydrogenase (0.1mg). The glutamate dehydrogenase trap contained α -oxoglutarate ($20\mu\text{mol}$) and glutamate dehydrogenase (0.1mg). The acid supernatant was obtained by the usual procedure and freeze-dried.

Expt. no.	Additions		^3H in water-soluble residue (nmol)	^3H in water of the medium (nmol)
	Aerobic incubation	Anaerobic incubation		
1	—	—	0.45	0
2	NADPH	—	1.63	4.62
3	NADPH	NAD ⁺	10.0	10.0
4	NADPH	NAD ⁺ (lactate dehydrogenase trap)	18.3	2.5
5	NADPH	NAD ⁺ (glutamate dehydrogenase trap)	16.7	2.1

Table 4. *Stereochemistry of $[4\text{-}^3\text{H}_1]\text{NADH}$ intermediate formed from $3\alpha\text{-}^3\text{H}_1$ -labelled compound (II)*

A two-step incubation was performed in the presence of either a lactate dehydrogenase trap (incubation no. 4, Table 3) or a glutamate dehydrogenase trap (incubation no. 5, Table 3). A sample of the water-soluble residue from freeze-drying of the acid supernatant was analysed by chromatography. Paper chromatography in ethanol-1M-ammonium acetate, pH 7.5 (7:3, v/v), resolves glutamate (R_F 0.4) and nicotinamide (R_F 0.82), but lactate (R_F 0.72) and nicotinamide (R_F 0.82) are not clearly separated. These two compounds were purified by a second chromatography in ether-formic acid-water (7:2:1, by vol.), which enables resolution of lactate (R_F 0.90) and nicotinamide (R_F 0.75). The radioactivity associated with glutamate in the first solvent was shown to have the same mobility as authentic glutamate in the second solvent system (R_F 0.75). Compounds were eluted from the paper with water before the counting of radioactivity.

Enzyme trap	Compound	^3H (c.p.m.)
Lactate dehydrogenase	Nicotinamide	4450
	Lactate	212
	Glutamate	0
Glutamate dehydrogenase	Nicotinamide	3000
	Lactate	0
	Glutamate	1879

in the presence of a trapping system was analysed by paper chromatography. Table 4 shows that ^3H was only transferred to glutamate in the presence of a glutamate dehydrogenase trap. With a lactate dehydrogenase trap only a very small percentage of the ^3H was transferred to lactate. Only 30–40% of the available ^3H was transferred to glutamate which must indicate the effectiveness of the two pathways (reactions 5 & 6) shown in Scheme 2 in competing for the oxidation of $[4\text{-}^3\text{H}_1]\text{-NADH}$.

The fact that it is possible to transfer ^3H from the steroid into glutamate is strong evidence for the existence of $[4\text{-}^3\text{H}_1]\text{NADH}$ in the decarboxylation reaction. Further, the observation that ^3H is not transferred to lactate suggests that the steroid-derived ^3H may occupy the B-position in NADH.

Reduction of 3-ketone substrates with $[4\text{-}^3\text{H}_2]\text{-NADH}$ or $[4\text{-}^3\text{H}_2]\text{NADPH}$. The final stage in the demethylation process should be the reduction of a 3-ketone to regenerate the 3β -alcohol. The ability to reduce 4,4-dimethylcholest-7-en-3-one (IX), 4 α -methylcholest-7-en-3-one (V) and 5 α -cholest-7-en-3-one (VI) in a microsomal preparation was measured under anaerobic conditions. The results in Table 5 show that both NADH and NADPH may be used to reduce the 3-ketone substrates to their corresponding alcohols. Compound (IX), which is not a normal intermediate in cholesterol biosynthesis, was consistently found to be the poorest substrate.

It is difficult to compare the efficacy of either $[4\text{-}^3\text{H}_2]\text{NADH}$ or $[4\text{-}^3\text{H}_2]\text{NADPH}$ in this reduction, as it is necessary to know the specific radioactivity

Scheme 2. Postulated scheme showing the loss of ^3H to the water of the medium and the stabilization of ^3H in the presence of a trapping system. Glutamate, $\text{R} = [\text{CH}_2]_2\text{CO}_2\text{H}$, $\text{Y} = \text{NH}$; lactate, $\text{R} = \text{CH}_3$, $\text{Y} = \text{O}$. The observation that ^3H is found in both the medium water and the residue suggests that in reaction 6 there must be enzymes with both A and B stereochemistry capable of reducing an oxidized species such as X^+ to XH , the bond XH being able to dissociate into X^- and H^+ .

Table 5. Reduction of steroid 3-ketones by $[4\text{-}^3\text{H}_2]\text{NADH}$ or $[4\text{-}^3\text{H}_2]\text{NADPH}$

Microsomal fractions (0.5ml) were incubated in an atmosphere of N_2 with the appropriate steroid (0.25 μmol) and GSH (5 μmol) for 30 min at 38°C . $[4\text{-}^3\text{H}_2]\text{NADH}$ (1.07 μmol ; 2.6×10^6 c.p.m.) or $[4\text{-}^3\text{H}_2]\text{NADPH}$ (1.07 μmol ; 1.3×10^6 c.p.m.) was added as indicated. The incorporation into the steroid was corrected for the blank value in the absence of steroid.

Steroid	Nucleotide	Total ^3H radioactivity in alcohol (c.p.m.)
4,4-Dimethylcholest-7-en-3-one (IX)	$[4\text{-}^3\text{H}_2]\text{NADH}$	6900
	$[4\text{-}^3\text{H}_2]\text{NADPH}$	7120
4 α -Methylcholest-7-en-3-one (V)	$[4\text{-}^3\text{H}_2]\text{NADH}$	13400
	$[4\text{-}^3\text{H}_2]\text{NADPH}$	13620
5 α -Cholest-7-en-3-one (VI)	$[4\text{-}^3\text{H}_2]\text{NADH}$	9840
	$[4\text{-}^3\text{H}_2]\text{NADPH}$	11540

of the nucleotides fairly accurately. Specific radioactivity based on the c.p.m./ E_{340} ratio is inaccurate because the nucleotides are not radiochemically pure. An attempt was made to overcome this problem by preparing $[4\text{-}^3\text{H}_2]\text{NADH}$ and $[4\text{-}^3\text{H}_2]\text{NADPH}$ from $^3\text{H}_2\text{O}$ samples that had the same specific radioactivity. If the transfer of ^3H from the medium to NAD^+ and NADP^+ shows the same isotope effect then the 4-position in the nucleotides must have identical specific radioactivities. By this criterion it appears that either NADH or NADPH may be used equally well to reduce the ketone. Kandutsch (1967), by measuring the reduction of model 3-ketones by spectrophotometric techniques, has shown that there is a preference for NADPH .

The alcohol derivative, compound (VIII), was subjected to oxidation with Jones's reagent (Bowden, Heilbron, Jones & Weedon, 1946) to regenerate the ketone (compound VI). This caused a loss of 90% of the $^3\text{H}_2$ showing that radioactivity was located at C-3 of the steroid.

Stereochemistry of the reduction of 5 α -cholest-7-en-3-one (VI) with NADPH . Microsomal preparations (5ml) were incubated with compound (VI) (500 μg) and stereospecifically ^3H -labelled NADPH (2 μmol) under anaerobic conditions for 1 h at 38°C . The use of $[4\text{A-}^3\text{H}_1]\text{NADPH}$ or $[4\text{B-}^3\text{H}_1]\text{NADPH}$ resulted in the synthesis of 5 α -cholest-7-en-3 β -ol (VIII) containing 0.65×10^3 and 37×10^3 c.p.m. respectively. The radioactivity was shown to be incorporated at C-3 since the chemical oxidation of compound

(VIII) to compound (VI) resulted in the loss of 95% of the ^3H . This result clearly establishes that in the NADPH-linked reduction of compound (VI) to compound (VIII) the hydride ion originates from the 4B side in NADPH.

All NADPH-linked reductases and dehydrogenases from mammalian sources show a correlation between the H atom transferred from the nicotinamide nucleotide and the stereochemistry of the new C-H bond. Enzymes catalysing the addition of a hydride ion to the α face of the steroid use the 4B-H atom of NADPH whereas it is the 4A-H atom of the coenzyme that is transferred to the β side of the steroid (Wilton, Watkinson & Akhtar, 1970; Akhtar, Watkinson, Rahimtula & Wilton, 1971).

Effect of cyclic AMP on demethylation as measured by the release of ^3H from 3α - ^3H -labelled compound (II). When cholesterol biosynthesis in rat liver homogenates is inhibited by cyclic AMP a steroid carboxylic acid similar to compound (I) accumulates (Bloxham & Akhtar, 1971). The enzyme responsible for the metabolism of this substrate is the 3β -hydroxy dehydrogenase enzyme in the demethylase complex. As we have now developed an assay based on this dehydrogenation it is possible to examine the effect of cyclic AMP on this step.

The effect of cyclic AMP on the release of tritium from 3α - ^3H -labelled compound (II) was measured in the presence of various concentrations of NAD^+ and a fixed concentration of NADPH in a microsomal preparation. Fig. 6 shows the results of this experiment presented as Lineweaver-Burk plot. The result approximates reasonably well to an inhibition by cyclic AMP of dehydrogenation at C-3 that is competitive with NAD^+ . Thus a V_{max} of 3.24 nmol/30 min per mg of protein was obtained in the presence or absence of cyclic AMP. However, cyclic AMP caused a very marked increase in the K_m for NAD^+ , which varies from $7.4\ \mu\text{M}$ in the absence of cyclic AMP to $59\ \mu\text{M}$ in the presence of cyclic AMP (5 mM).

When demethylation was assayed by the conventional method of measuring $^{14}\text{CO}_2$ evolution from $[4\alpha\text{-}^{14}\text{C}]$ methylcholest-7-en- 3β -ol (IIa) then an identical result was obtained. Therefore it appears that cyclic AMP inhibits C-4-decarboxylation by decreasing the activity of the 3β -hydroxy dehydrogenase. The observation that cyclic AMP inhibits the 3β -hydroxy dehydrogenase enzyme in the dealkylation at C-4 would explain the accumulation of the carboxylic acid derivative in cholesterol biosynthesis.

A. D. Rahimtula & J. L. Gaylor (personal communication) obtained an 18.1-fold purification of the 3β -hydroxy- 4β -methyl- 5α -cholest-7-en- 4α -carboxy decarboxylating enzyme and have shown

that cyclic AMP inhibited this partially purified enzyme.

Conclusion

The studies of Miller & Gaylor (1970a,b), Hornby & Boyd (1970) and Bloxham & Akhtar (1971) on the demethylation of 4-monomethyl and 4,4-dimethyl steroids have conclusively established the presence of a 4α -carboxylic acid intermediate in this process. In the present work we have investigated the subsequent reactions, which involve the oxidation of a 3β -ol to a 3 -ketone (reaction 2) with concomitant decarboxylation (reaction 3), before regeneration of the alcohol (reaction 4). The dehydrogenation reaction involves the participation of NAD^+ and results in the transfer of the 3α -H atom from the steroid to the 4-position of NAD^+ . The steroid-derived H atom appears to occupy the B-position in the resultant NADH. The reduction of several 3-oxo steroids appears to use NADH or NADPH with equal efficiency. For NADPH-linked reductions the 4B-H atom is transferred to the 3α -position on the steroid.

Fig. 6. Effect of cyclic AMP on the release of ^3H from 3α - ^3H -labelled compound (II). Microsomal fractions (0.5 ml) were incubated with 3α - ^3H -labelled compound (II) (0.17 μmol) in the presence of GSH (5 μmol), NADP^+ (1.25 μmol), glucose 6-phosphate (5 μmol), glucose 6-phosphate dehydrogenase (7.5 μg) and various concentrations of NAD^+ in a final volume of 1 ml. Incubations were for 30 min at 38°C in the presence of the following concentrations of cyclic AMP: $\circ$, 5 mM; $\triangle$, 2.5 mM; $\square$, 1.25 mM; $\bullet$, 0 mM.

Cyclic AMP has been clearly shown to act as a competitive inhibitor of the dehydrogenation reaction. This observation would account for the inhibition of cholesterol biosynthesis and the accumulation of the C-4 carboxylic acid steroid derivative that occurs in the presence of cyclic AMP. It is noteworthy that cyclic AMP has been shown to act as an inhibitor of Δ^5 - β -hydroxy steroid dehydrogenase in adrenal cortex (Koritz, Yun & Ferguson, 1968; McCune, Roberts & Young, 1970) and ovarian tissue (Sulimovici & Boyd, 1969) and of a mitochondrial dehydrogenase from adrenal cortex (Akhtar, Bloxham & Poat, 1970).

MATERIALS AND METHODS

Tween 80 was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., U.K. Cyclic AMP, NADP, glucose 6-phosphate dehydrogenase, lactate dehydrogenase and glutamate dehydrogenase were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. For analytical (0.4 mm) t.l.c., silica-gel preparation G_{F254} (nach Stahl) (E. Merck A.-G., Darmstadt, Germany) was used. Butyl-PBD [5-(4-biphenyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole] was obtained from CIBA (A.R.L.) Ltd., Duxford, Cambridge, U.K. NE 250 scintillation fluid was obtained from Nuclear Enterprises Ltd., Edinburgh, U.K. NaB³H₂ and [¹⁴C]methyl iodide were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were obtained from either BDH Chemicals Ltd., Poole, Dorset, U.K., or May and Baker Ltd., Dagenham, Essex, U.K.

Measurement of radioactivity. Radioactive samples in organic solvents were counted in 10–15 ml of butyl-PBD in toluene (8 g/l). Aqueous radioactive samples were counted in 10–15 ml of NE 250 scintillation fluid. All counting operations were performed in an Intertechnique ABAC SL40 liquid-scintillation counter at approx. 50% efficiency for ³H and 90% efficiency for ¹⁴C.

Preparation of radioactive compounds. [4-³H]-, [4A-³H]- and [4B-³H]-NADPH and [4-³H]₂NADH were prepared by the method of Wilton, Munday, Skinner & Akhtar (1968). [4 α -¹⁴C]Methylcholest-7-en-3 β -ol (3.8 $\times$ 10⁴ c.p.m./ μ mol) was prepared from 5 α -cholest-7-en-3-one by the method of Neiderhiser & Wells (1959) by using [¹⁴C]methyl iodide. 4,4-Dimethyl[3 α -³H]cholest-7-en-3 β -ol (1.6 $\times$ 10⁶ c.p.m./ μ mol) was prepared by the reduction of 4,4-dimethylcholesta-5,7-dien-3-one with NaB³H₄, before hydrogenation over Raney nickel. The conditions used in the hydrogenation of the 5,7-diene were the same as those used in the hydrogenation of the 14,15-double bond of 4,4-dimethylcholesta-8,14-dien-3 β -ol (Watkinson, Wilton, Munday & Akhtar, 1971), except that dioxan was used as a solvent.

Preparation and incubation of microsomal fractions. Male Wistar albino rats were killed by cervical dislocation and their livers were removed quickly. The liver (10 g) was homogenized in 0.154 M-KCl (10 ml) with a manually operated piston barrel homogenizer (clearance, 1 mm). Cell debris and mitochondria were removed by centrifugation at 10000g for 10 min. The supernatant was collected and the microsomal fraction obtained by centri-

fugation at 100000g for 45 min. The pellet was rinsed and resuspended in 0.154 M-KCl (10 ml) with a Jencon's hand-operated homogenizer. The microsomal fraction was re-collected by centrifugation at 100000g for 45 min. The pellet was washed and excess of medium was removed by inverting the tube over tissue paper. The microsomal fraction was finally suspended in 0.1 M-sodium phosphate buffer, pH 7.4, containing 4 mM-MgCl₂ (2.5 ml; 10–15 mg of protein/ml). The preparation was kept at 4°C throughout.

Incubations were performed in round-bottomed centrifuge tubes at 38°C with gentle shaking. Steroids were dissolved in Tween 80 (1 mg/100 μ g of steroid per ml of medium). Anaerobic incubations were performed under an atmosphere of N₂, which was purified by passage through Fieser's (1924) solution. The exact incubation conditions are described in the text.

Analysis of ³H released from 3 α -³H-labelled compound (II). (a) Acid-terminated reaction. To 1.0–1.5 ml of incubation medium 1.0 ml of 6% (w/v) HClO₄ was added to terminate the reaction. Protein was allowed to precipitate for 30 min on ice and was then removed by centrifugation. The supernatant was collected and neutralized by the addition of 20% (w/v) KOH (0.2 ml) followed by 20% (w/v) KHCO₃ (0.1 ml). After being kept for 30 min on ice the KClO₄ precipitate was removed by centrifugation. The volume of the supernatant (acid supernatant) was determined and a sample was counted for radioactivity to determine the total ³H released. The remainder was freeze-dried. The water from the medium was collected in a freezing trap: after thawing its radioactivity was counted directly. The freeze-dried residue was dissolved in water and counted for radioactivity. This fraction may be analysed further by paper chromatography.

Care must be taken in choosing the appropriate radioactive blank. It was found that if incubations to determine ³H release from 3 α -³H-labelled compound (II) were performed under CO₂ or with heat-treated microsomal protein there was a high incorporation of ³H into the acid supernatant. This radioactivity was completely extracted into diethyl ether and corresponded to the original steroid. Probably both of the treatments cause protein denaturation, which decreases the steroid-binding capacity. Incubations at 4°C in the presence of all co-factors gave very low blank values for release of ³H (0.1 nmol/30 min).

This technique is very rapid and allows the analysis of a large number of samples simultaneously. It could find general application in analysing the stability of C–H bonds in water-insoluble compounds.

(b) Base-terminated reaction. At the end of the incubation carrier NADH (1 μ mol) was added. The pH was adjusted to 10 with 0.1 M-NaOH (1.0 ml) and the sample was heated for 5 min in a boiling-water bath. After cooling the mixture the steroid was extracted with diethyl ether (5 $\times$ 3 ml). Traces of diethyl ether remaining with the aqueous phase were removed under a stream of N₂. The pH was adjusted to 8 with 1 M-HCl (45 μ l) with rapid stirring. After being kept at room temperature for 15 min the denatured protein was removed by centrifugation. The supernatant was applied to a Sephadex G-25 column (25 cm $\times$ 1 cm) previously equilibrated with 0.025 M-tris-HCl buffer, pH 8.0, and eluted with the

same buffer. Protein was eluted between 8 and 13 ml and the nicotinamide nucleotide fraction was eluted between 18 and 24 ml. Solvent was removed under reduced pressure from the nicotinamide nucleotide fraction, which was then analysed by paper chromatography.

Paper chromatography. Samples were analysed by descending paper chromatography on Whatman no. 4 paper (36 cm × 5 cm) with ethanol-1 M-ammonium acetate, pH 7.5 (7:3, v/v), as developing solvent. The following R_F values were obtained: NADP⁺, 0.015; NADPH, 0.03; NAD⁺, 0.10; NADH, 0.33; nicotinamide, 0.82; glutamate, 0.40; lactate, 0.72. NADPH and NADH were located by u.v. fluorescence. NAD⁺, NADP⁺ and nicotinamide were detected by the method of Kodicek & Reddi (1951).

For scanning radioactive compounds the paper was cut into strips (5 cm × 1 cm) and placed in a scintillation vial. Water (0.4 ml) was added and allowed to soak into the paper for 5 min. NE 250 scintillation fluid was then added before counting the radioactivity.

Extraction of carboxylic acid formed from 3 α -³H-labelled compound (II). The incubation was terminated by the addition of acetic acid (0.2 ml) and acetone (5 ml). Protein was allowed to precipitate and removed by centrifugation. The supernatant was collected and the solvent was removed under reduced pressure. The residue was dissolved in dichloromethane-methanol (1:1, v/v; 1 ml) and a 0.2 ml portion was analysed by t.l.c. with light petroleum (b.p. 60–80°C)-acetone (14:3, v/v) as developing solvent. The carboxylic acid (R_F 0) was eluted with methanol and counted for radioactivity.

Measurement of ¹⁴CO₂ evolution. Incubations were performed in manometer vessels. The reaction was terminated by the addition of 0.5 M-H₂SO₄ (0.5 ml) from the side arm and ¹⁴CO₂ was collected in 20% (w/v) KOH (0.1 ml) for 1 h at 38°C. The KOH in the centre well was quantitatively transferred to a scintillation vial, methanol being used to wash out the well, and counted for radioactivity.

Extraction of sterols. Incubations were terminated by the addition of 10% (w/v) methanolic KOH (5 ml). After saponification the sterols were extracted with light petroleum (b.p. 60–80°C)-diethyl ether (1:1, v/v). 5 α -Cholest-7-en-3 β -ol (VIII) and 4,4-dimethylcholest-7-en-3 β -ol were purified by recrystallization to a constant specific radioactivity with 50 mg of the authentic alcohol. 4 α -Methylcholest-7-en-3 β -ol (VII) was purified by t.l.c. with light petroleum (b.p. 60–80°C)-acetone (14:3, v/v) as developing solvent (R_F 0.6).

Determination of protein content. The protein content of microsomal preparations was determined by the method of Cleland & Slater (1953), which involves the extinction of lipid from the protein followed by treatment with biuret reagent.

We thank Professor K. A. Munday for his kind interest and encouragement. D. P. B. gratefully acknowledges the Science Research Council for a studentship.

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