AN ABSTRACT OF THE THESIS OF

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Title: A STUDY OF THE BIOSYNTHESIS OF STEROLS AND RELATED COMPOUNDS BY A BOVINE AORTA

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Dr. Wilbert Gamble

Previous investigations of the biosynthesis of sterols by the arterial wall have shown that the intact aorta is capable of low level incorporation of labeled precursor into a sterol digitonide. The biosynthesis of sterols and related lipids by a cell-free preparation of bovine aorta was investigated in the present study.

The cell-free system was the 60,000 x g supernatant of a Waring Blender homogenate of aorta and 0.1 M phosphate nicotinamide buffer. When the 60,000 x g supernatant was incubated in the presence of adenosine triphosphate and mevalonic acid-2-C\(^{14}\), the nonsaponifiable fraction (NSF) of the incubation mixture was radioactive. Acetate-2-C\(^{14}\) was not incorporated into the NSF. A sterol digitonide of low specific activity (60 to 237 counts per min per mg cholesterol) could be isolated from the NSF, but the major amount of
radioactivity in the NSF was not digitonin precipitable.

The 60,000 x g supernatant was capable of forming phospho-
mevalonic acid, mevalonic acid pyrophosphate, and isopentenyl pyro-
phosphate from MVA.

Chromatography of the NSF on silicic acid-celite columns
showed the labeled material to be slightly less polar than cholesterol.
Samples of the labeled material isolated (by preparative layer chrom-
atography) from large amounts of NSF did not give a positive Lieber-
mann-Burchard reaction and did not form a 2,4-dinitrophenylhydra-
ze. On silver nitrate impregnated layers of silicic acid, the la-
beled material appeared to be chromatographically similar to farnes-
ol and squalene.

Gas chromatography of a sample of labeled material showed all
the radioactivity present in one peak with a retention time intermed-
iate between the retention times of farnesol and squalene. The mass
spectrum of the labeled material indicated the presence of compounds
with m/e of 380, 384, and 386.

The results of these experiments indicated that the major bio-
synthetic product is not cholesterol or other similar sterol, but may
be a "prenol" of some kind.
A Study of the Biosynthesis of Sterols and Related Compounds by a Bovine Aorta Preparation

by

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A STUDY OF THE BIOSYNTHESIS OF STEROLS AND RELATED COMPOUNDS BY A BOVINE AORTA PREPARATION

INTRODUCTION AND LITERATURE REVIEW

The scientific interest in cholesterol is reflected in the extensive research that has been done on the structure, chemistry, and metabolism of this compound. The results of these investigations have been summarized and discussed in several texts (26, 50, 64). Of particular interest, has been the elucidation of the biosynthetic pathway leading to cholesterol (and other terpenoid compounds) because of the widespread occurrence, biological significance and relatively complexity of this compound. The biosynthesis of cholesterol has been reviewed comprehensively in several articles (9, 10, 11, 22, 28, 81, 82, 126) and texts (89, 124).

The biosynthesis of sterols is a complex procedure involving many transformations between precursor and product. That many mammalian tissues can perform this synthesis has been established, but most investigations have been limited to liver systems; ergosterol biosynthesis has been studied in yeast. The arterial wall is a functioning organ and as such, should contain the complex enzyme systems found in living cells. While much work has been done relating to the presence and activity of single enzymes in the arterial wall, the isolation and study of enzyme systems from the arterial wall
(including those enzymes responsible for sterol biosynthesis) have not been widely investigated.

However, there is sufficient evidence (46, 51, 118) to indicate that the arterial wall is capable of sterol synthesis, but these studies have been limited to intact systems. The demonstration that synthesis can take place in cell-free preparations of the tissue in question is desirable to clearly define the requirements, conditions, and course of such biosynthesis. For these reasons, this study was undertaken to determine the biosynthetic capacities of a cell-free system obtained from bovine aorta. In the brief survey presented below, the biosynthesis of sterols is outlined, and the investigations on the biosynthesis of sterols by the aorta are reviewed.

**Early Studies on the Biosynthesis of Cholesterol**

Prior to the introduction of isotope tracer methods, several groups of investigators (79, 88, 98) established, by the use of balance methods, that cholesterol could be either synthesized or destroyed by mammals. In 1937, Rittenberg and Schoenheimer (90) showed that deuterium from deuterium oxide was introduced into body cholesterol into stable, nonexchangeable positions. The maximum incorporation of deuterium into cholesterol was 50 percent of the concentration in the body fluids. The authors interpreted their results to mean that cholesterol biosynthesis involved the coupling together of a large
number of small molecules. In 1942 Bloch and Rittenberg (13) using both rats and mice, demonstrated that deuterium acetate was incorporated into cholesterol, and that the incorporation from deuterium acetate was 30 times greater than from deuterium oxide. In 1946, Bloch, Borek, and Rittenberg (12), using rat liver tissue slices, were able to demonstrate the incorporation of acetate-\(1^3\text{C}\) into cholesterol, but could not duplicate this incorporation using a Waring Blender homogenate of the same tissue. Little and Bloch (69), using methyl or carboxyl labeled acetate, demonstrated that acetate was the principle and possibly the only primary precursor to biosynthetic cholesterol. When doubly labeled acetate was used \((\text{C}^{14}\text{H}_3\text{C}'^{13}\text{OO}^-)\) as a precursor to cholesterol in rat liver slices, 15 carbons of the cholesterol molecule were derived from the methyl group of acetate and 12 from the carboxyl group.

The discovery of the singular role of acetate in cholesterol biosynthesis stimulated investigations into the origin of each of the individual carbon atoms within the molecule. A complete discussion and summary of these degradation studies can be found in the text Steroids (50, p. 404-409). Figures 1 and 2 show the numbering and labeling scheme in cholesterol.

Little and Bloch (19) showed that carbon atoms 18, 19, 26, 27 and presumably 17 in cholesterol arose from the methyl carbon of acetate. Wüersch, Huang and Bloch (130) established the origin of
Figure 1. Numbering of carbon atoms in cholesterol.

Figure 2. Source of individual carbon atoms in cholesterol biosynthesized from labeled acetate.
the carbon atoms at positions 20, 22, and 24 to also be the methyl carbon, and 21 and 23 to be the carboxyl carbon. Dauben and Take-mura (36) reported the methyl carbon of acetate to be the source of carbon atom 7. Cornforth, Hunter, and Popjak (30) reported the degradation of the A ring and later the C and D rings (29) and thus completed the elucidation of the source of the individual carbon atoms. The possibility of a terpene intermediate was postulated when the m-\text{c}-m-c \label{1} labeling pattern was observed in the side chain of the cholesterol molecule.

**Squalene as an Intermediate in Cholesterol Biosynthesis**

The possibility that squalene, a \( \text{C}_{30} \text{H}_{50} \) triterpene hydrocarbon (Figure 3) might be an intermediate in cholesterol biosynthesis was first suggested by Heilbron, Morris and Owens in 1926 (56). Channon (20), also in 1926, fed squalene to rats and observed that the liver cholesterol increased 100 percent as a result of such feeding.

In 1953 Langdon and Bloch (66) demonstrated that both carbons of acetate were also used in the biosynthesis of squalene. They were also able to isolate labeled squalene without the use of "cold" carrier squalene, and therefore concluded that squalene was a normal constituent of rat liver. Feeding such biosynthetically labeled squalene to mice resulted in a 4.1 to 6.1 percent incorporation of label into the sterol fraction. Tchen and Bloch (110) demonstrated the
conversion of squalene to lanosterol (Figure 4), a C\textsubscript{30} sterol closely related to cholesterol. Later, Clayton and Bloch (23) reported the conversion of lanosterol to cholesterol by rat liver.

Previous to this work, in 1953, Woodward and Bloch (125) proposed the now accepted hypothesis for the cyclization of squalene to lanosterol, in which proper folding of the squalene molecule and a nonstop series of transformations between squalene and lanosterol are the major features. Such a cyclization would account for the biosynthesis of both cholesterol and lanosterol from squalene.

Subsequent investigations showed the labeling pattern in biosynthetic cholesterol to be entirely consistent with the labeling pattern in the Woodward-Bloch postulate for cyclization. Further verification for the cyclization scheme came from the degradation of labeled squalene by Cornforth and Popják (32). The labeling pattern in squalene (Figure 3) was also in total agreement with the Woodward-Bloch scheme.

A mechanism for the series of concerted cyclization and rearrangement reactions leading to lanosterol (and other triterpenes as well) has been proposed by Ruzicka (93, 94). In this proposal, cyclization proceeds from all trans squalene, which is folded on the particular enzyme surface (squalene oxidocyclase for lanosterol biosynthesis) in a series of potential chair and boat cyclohexane rings. In the case of lanosterol, this is a chair-boat-chair-boat-unfolded form.
Figure 3. Labeling pattern in squalene biosynthesized from labeled acetate.

Figure 4. Lanosterol.
A cationic attack on a terminal double bond initiates a concerted cyclization reaction which proceeds by planar trans additions to the double bonds; this is immediately followed by a double hydride shift, two 1,2 methyl shifts, and the loss of a proton to produce lanosterol.

Experimental evidence for this mechanism came from the laboratories of Bloch and of Cornforth and Popják. Tchen and Bloch (111, 112, 113), using hog liver homogenate which would produce lanosterol (but not cholesterol) from squalene, studied the cyclization process in detail. No uptake of isotope, present as either D₂O or H₂O¹⁸ in the reaction medium, occurred when squalene was converted to lanosterol. However, incubating with O₂¹⁸ resulted in isotope incorporation into the 3ß-hydroxyl group. From these data, they concluded that there is no participation of protons from an external source; hydride shifts must be responsible for the transfer of methyl groups; and "activated oxygen" (112) was both the agent initiating the cyclization reaction and the source of the 3ß-hydroxyl group.

Additional work by Maudgal, Tchen, and Bloch (76), and Cornforth (33, 34) established that a double 1,2 methyl shift occurs during the reaction sequence, rather than a single 1,3 methyl shift. Such a shift was shown to occur only intramolecularly.
The Discovery of Mevalonic Acid

The knowledge that acetate $\rightarrow$ squalene $\rightarrow$ cholesterol prompted extensive research into the intermediate steps of this biosynthetic sequence. Of special interest was the search for an isoprenoid intermediate, a five- or six-carbon unit which would condense to form squalene, and, presumably, serve as a universal precursor to terpenes. A number of compounds were investigated, among them acetoacetic acid, isovaleric acid, $\beta,\beta$-dimethyl acrylic acid, and $\beta$-hydroxy-$\beta$-methylglutaric acid. The results of many experiments indicated that breakdown to acetate occurred, in all cases except acetoacetic acid, prior to incorporation into cholesterol (15). The search for the intermediate was thus largely unsuccessful until the discovery of mevalonic acid (MVA, $\beta,\gamma$-dihydroxy-$\beta$-methyl valeric acid) in 1956 by a Merck research group (127, p. 20-45).

Their work on an "acetate replacing factor" for Lactobacillus acidophilus led to the isolation (128) and characterization (122) of MVA. Later, chemical synthesis and demonstration of biological activity (123) established its identity with the proposed structure:

$$\text{OH}$$
$$\text{CH}_2 - \text{C} - \text{CH}_2 - \text{CH}_2 \text{OH}$$
$$\text{CH}_2 - \text{COOH}$$
The structure of MVA is closely related to β-hydroxy-β-methylglutaric acid which had been previously postulated as a precursor to cholesterol. Tavormina, Gibbs and Huff (108) using β-hydroxy-β-methylglutaric acid-3-\(^{14}\)C, β,β-dimethyl acrylic acid-4-\(^{14}\)C, and MVA-2-\(^{14}\)C in a rat liver homogenate obtained a 0.16, 0.8, and 43 percent incorporation, respectively, for each precursor into cholesterol. If only one enantiomer of the racemic MVA were biologically active, the incorporation of the labeled MVA would imply almost a total incorporation of the active isomer. This efficient utilization of MVA, in addition to results obtained in in vivo studies (81, p. 545) suggested strongly that, at least in these systems, MVA was used solely for sterol and terpene biosynthesis.

When MVA-1-\(^{14}\)C was incubated in a rat liver system, there was no incorporation of isotope into cholesterol (102) but the isotope could be recovered almost quantitatively (calculating on the basis of utilization of one half of the racemic mixture) as barium carbonate. Thus at some stage of the biosynthetic process the carboxyl carbon of MVA was lost as carbon dioxide. Dituri et al. (41) degraded squalene biosynthesized from MVA-2-\(^{14}\)C and found that six carbon atoms of the squalene molecule were labeled. The labeling pattern indicated that, during biosynthesis, there was little change in the MVA molecule and that three MVA or MVA-derived units were condensed in a head to tail linkage (carbon two of one molecule of MVA
linked to carbon five of a second molecule of MVA) to form a 15 carbon unit, two of which could be united in a head to head fashion to give, finally, squalene. Amdur, Rilling and Bloch (2) using MVA-2-C\(^{14}\)-5-di-H\(^3\) found no change in the C\(^{14}\)/H\(^3\) ratio in going from MVA to squalene in a soluble yeast system. Thus there is no oxidative change in the number five carbon of MVA during biosynthesis.

**Conversion of Acetate to Mevalonate**

The pathway from acetate to MVA is shown in Figure 5. This scheme has been generally accepted as the biosynthetic route in both mammalian and yeast systems (22). The overall conversion of acetate-1-C\(^{14}\) to MVA was reported in 1959 by Knauss, Porter and Wesson (61), who found that such biosynthesis required both a soluble fraction and a microsomal fraction isolated from rat liver.

The initial step in the formation of MVA involves the formation of acetyl coenzyme A from acetate and coenzyme A (CoASH), a reaction which requires ATP (adenosine triphosphate). This step is followed by the condensation of two acetyl coenzyme A units to form acetoacetyl coenzyme A. A third condensation step with another molecule of acetyl coenzyme A forms \(\beta\)-hydroxyl-\(\beta\)-methylglutaryl coenzyme A, accompanied by the release of free coenzyme A. The coenzyme A group on the carboxyl carbon of acetoacetyl
Figure 5. Acetate to mevalonic acid.
coenzyme A is retained (92) in this step.

Both yeast (48, 91) and rat liver (30) systems can form β-hydroxyl-β-methylglutaryl coenzyme A. The condensation of aceto-acetyl coenzyme A and acetyl coenzyme A does not appear to be readily reversible. However, β-hydroxyl-β-methylglutaryl coenzyme A is poorly utilized by in vitro systems as a precursor to squalene and and sterols, in contrast to the very efficient utilization of MVA. Two enzymes, both which catalyze reactions that appear to be irreversible, have been described which may account for the poor incorporation of β-hydroxyl-β-methylglutaryl coenzyme A into cholesterol. Bachawat, Robinson, and Coon (7) reported a β-hydroxyl-β-methylglutaryl coenzyme A cleavage enzyme, which splits β-hydroxy-β-methylglutaryl coenzyme A to acetoacetic acid and acetyl coenzyme A. Dekker, Schlesinger and Coon (39) have described another enzyme which converted β-hydroxy-β-methylglutaryl coenzyme A to free β-hydroxy-β-methylglutaric acid and free coenzyme A. These two enzymes may serve as controls for the formation of MVA and therefore, formation of cholesterol.

β-hydroxy-β-methylglutaryl coenzyme A can also arise from a completely different pathway, starting with amino acid leucine (89, p. 177-186). Leucine can be converted to branched five- and six-carbon acids, one of which is β-hydroxy-β-methylglutaryl coenzyme A.

Reduction of the bound carboxyl group of β-hydroxy-β-
methylglutaryl coenzyme A to MVA has been described by Lynen et al. (71) and Ferguson, Durr, and Rudney (47); both studies used a yeast enzyme system which required NADPH (reduced nicotinamide adenine dinucleotide phosphate). A similar enzyme system, which preferred NADH (reduced nicotinamide adenine dinucleotide) has been isolated from pig liver by Schlesinger and Coon (96). That the first reduction product, mevaldehyde (MVALD) can serve as an intermediate to mevalonate was demonstrated by Wright et al. (129). While it has been shown that MVALD is probably converted to MVA in in vitro systems, its occurrence in the biosynthetic pathway is not one of a free intermediate. MVALD probably does not exist as a free unit but rather in the form of a hemithioacetal with coenzyme A or the enzyme (18, p. 184). Neither of these postulated intermediates would readily convert to the free aldehyde, but presumably be converted to MVA. The isotope studies of Amdur, Rilling and Bloch (2) demonstrated that MVA lies further along the pathway to cholesterol than does MVALD. The reduction of MVALD was later shown to be stereospecific (43).

Malonyl coenzyme A in combination with acetyl coenzyme A has been implicated in the formation of MVA. Brodie, Wasson, and Porter (17, 18) using a pigeon liver preparation have postulated a biosynthetic pathway in which the intermediate acids are all enzyme bound until the final reduction steps to mevalonate. The free acids or their
coenzyme A esters are not involved in the pathway. This reaction sequence is similar to that of the fatty acid biosynthetic pathway.

**Mevalonate to Squalene**

The sequence of reactions for the transformation of MVA to the isoprenoid condensing units is shown in Figure 6. The pathway is generally accepted for the intermediate metabolism of MVA to terpenoid compounds and has been shown to operate in both plant and animal systems.

The first step in this reaction scheme is the phosphorylation of MVA to phosphomevalonic acid (MVA-5-P). This step requires ATP. The formation of a monophosphate ester of MVA by a yeast enzyme, mevalonic acid kinase, was reported by Tchen (109) in 1957. It was also noted in this step that only one enantiomer of racemic MVA was phosphorylated. With further incubation, MVA-5-P undergoes a second ATP dependent phosphorylation step to give pyrophosphomevalonic acid (MVA-5-PP) (14). This compound was characterized by Chaykin et al. (21); the enzyme responsible, phosphomevalonic kinase, has been purified from yeast (14) and hog liver (57). This enzyme is separate and distinct from the first phosphorylating enzyme, mevalonic kinase.

The formation of the pyrophosphate ester is followed by a concerted dehydration and decarboxylation step, which was also shown to
Figure 6. Formation of isoprenoid condensing units.
be ATP dependent (21). However, no new phosphate group was introduced into the product, isopentenyl pyrophosphate (IPP), the biological isoprene unit. When synthetic MVA-3-O\(^{18}\) was converted to IPP, the O\(^{18}\) was transferred to inorganic phosphate (68). This evidence supported a mechanism for the formation of IPP previously proposed by Bloch (14) in which a hypothetical intermediate is formed by phosphorylating the tertiary hydroxyl group of MVA-5-PP; this intermediate then undergoes simultaneous decarboxylation and loss of the C-3 hydroxyl function. Lynen et al. (73) have shown that MVA-2-C\(^{14}\) gives rise to IPP with all C\(^{14}\) activity in the methylene group.

Lynen et al. (72) described the chemical synthesis of IPP and its biosynthetic incorporation into squalene by a yeast system. Agranoff et al. (1) have isolated an enzyme from yeast termed isopentenyl pyrophosphate isomerase. This enzyme was shown to catalyze the almost irreversible formation of \(\beta,\beta\)-dimethylallyl pyrophosphate (DMA) from IPP. There was no randomization of the label in the molecule when isotopically labeled MVA was used as a precursor. Studies of terpene biosynthesis have shown that the methylene group of IPP becomes the trans methyl group of DMA (89, p. 200). A proton is acquired from the reaction medium and is incorporated into DMA. However, the irreversible nature of the reaction and the subsequent condensation reactions of DMA and IPP are such that the proton acquired in the isomerization reaction appears only in the
terminal methyl groups of squalene.

The condensation reaction between DMA and IPP, by which the ten and 15 carbon intermediates are formed, is shown in Figure 7. DMA, by the loss of its pyrophosphate group, provides a favorably stabilized cationic species, which then attacks the methylene group of IPP. Elimination of a proton then produces geranyl pyrophosphate, GPP. GPP can acquire another molecule of IPP by the same mechanism to form farnesyl pyrophosphate, FPP. While this mechanism is favorably accepted as a working model for the condensation of isoprene units, the enzymes required have not been isolated in purified form. Thus more extensive proof for this reaction sequence is not available, and alternate pathways may exist.

Lynen and co-workers in his laboratory identified FPP as a precursor to squalene, using a yeast system (72) and later (73) they were able to show that GPP preceded FPP in the biosynthetic pathway. Popják and Cornforth (31) obtained the same results using a rat liver system. Using MVA-2-C$^{14}$ they isolated labeled DMA, trans-trans-geraniol, and trans-trans-farnesol.

The final step, the head to head condensation of two FPP molecules to form squalene has been demonstrated in animal (55), plant (8), and yeast (72) systems, but has yet to be fully clarified. Several mechanisms have been proposed, but experimental evidence is lacking. Cornforth and Popják and their co-workers (84, 85, 86) have
Figure 7. Condensation of isoprenoid units.
elucidated some of the details of squalene formation. They have found (using a liver microsome system) that squalene biosynthesized from MVA-5-D$_2$ contains 11 atoms of deuterium per squalene molecule, not 12 as expected. The center of such a biosynthesized molecule had this pattern of labeling:

\[ -\text{CHD-CD}_2 - \]

This result was explained when it was found that one molecule of farnesyl pyrophosphate loses a proton (during squalene formation) which is later replaced by a proton from NADPH. This replacement was later shown to be stereospecific (35, 95). There was no incorporation of label from H$_3$HO into squalene during its synthesis from farnesyl pyrophosphate.

The conversion of farnesyl pyrophosphate to squalene by a pig liver enzyme, squalene synthetase, has been recently reported by Krishna et al. (63). The enzyme was solubilized (in contrast to earlier studies, which required a microsomal fraction) and partially purified. The enzyme reacts with farnesyl pyrophosphate to form a bound intermediate, with the release of pyrophosphate. Both a C$_{15}$ and a C$_{30}$ intermediate were isolated, but not identified; they were similar, but not identical, to farnesol and squalene. The results prompted the authors to propose a mechanism whereby farnesol is bound to the protein by a sulfhydryl linkage (via a methionine residue); the enzyme-bound intermediate then reacts with a second
molecule of farnesyl pyrophosphate to form a C\textsubscript{30} enzyme bound intermediate. Reduction of this intermediate with NADPH would produce free squalene, which would then be available for the cyclization reaction to produce lanosterol.

**Lanosterol to Cholesterol**

For the transformation of lanosterol to cholesterol, there remains only the removal of the three methyl groups, the saturation of the $\Delta^{24}$ bond, and the relocation of the $\Delta^{8(9)}$ bond to the $\Delta^{5(6)}$ position. Whether these reactions take place in a specific sequence in a given tissue, and if so, what that sequence is, has been difficult to establish. Many of the details of these final steps of the biosynthetic pathway remain to be clarified. However, it is known that the removal of the methyl groups occurs prior to the nuclear transformations.

The overall conversion of lanosterol to cholesterol was demonstrated by Clayton and Bloch (24). Oxygen was required for this conversion, which was carried out in a rat liver preparation. Olson, Lindberg and Bloch (78) used lanosterol labeled biosynthetically from acetate-2-$^{14}$C\textsubscript{14}; that is, such lanosterol was labeled in the methyl carbons on the sterol nucleus. Conversion of this lanosterol to cholesterol resulted in the release of $^{14}$CO\textsubscript{2}. The ratio of activity released to the activity in the cholesterol formed was one:five, which
would be anticipated for lanosterol labeled in 18 positions converted to cholesterol labeled in 15 positions and three labeled carbons released as $^{14}\text{C}O_2$. The authors further demonstrated that $^{14}\text{C}O_2$ does not arise from any part of the lanosterol molecule except from the three methyl groups.

The oxidation of the methyl groups probably takes place in a stepwise fashion. Bloch (10, p. 139) has reported the isolation of a lanosterol metabolite which had the characteristics of a diol, which he tentatively identified as a $C_{30}$ diol in which one of the methyl groups (presumably the C-14 substituent) was partially oxidized to a hydroxymethyl substituent. This product could be converted to cholesterol with the release of three moles of $^{14}\text{C}O_2$. Oxidation of the methyl groups therefore probably continues to the formation of a carboxyl group, which is then released as carbon dioxide.

Further evidence has been accumulated to show that the C-14 methyl group is removed prior to the C-4 methyl groups. Gautschi and Bloch (53, 54) isolated trace amounts of a material more polar than lanosterol, which was identified as $\Delta^8(9), \Delta^{24}_{-4, 4}$-dimethyl-cholestadienol. This compound could be converted to cholesterol by a rat liver homogenate, but produced only two moles of carbon dioxide for each mole of substrate in the transformation.

The loss of the two methyl groups at C-4 may be initiated by the oxidation of the C-3 hydroxyl group to a ketone. Bloch et al. (67)
have provided evidence for such a reaction sequence. When \( 3\alpha-H^3 \)
sterols with one or two methyl groups in the C-4 position were me-
tabolized to cholesterol, the tritium was lost, but the intermediate
oxidized sterols could not be isolated. However, lanosta-8,24-dien-
3-one and 4,4-dimethyl-cholest-8-ene-3-one could be converted to
cholesterol but the evidence did not show such ketones to be oblig-
atory intermediates. That the oxidation and elimination of one methyl
group is probably completed before the second one (at the C-4 posi-
tion) is oxidized was suggested by the isolation of 4-\( \alpha \)-methyl-\( \Delta^7 \)-
cholestenol from mammalian tissues (77, 117). This sterol has been
prepared biosynthetically from acetate-1-C\(^{14}\) in rat tissues (116).
When this sterol was labeled in the C-4 position and administered to
rats, it was efficiently converted to cholesterol (52). The order of
removal of the extra methyl groups need not be the same in all sys-
tems; Djerassi (42) has isolated from plant sources a 14-methyl
sterol, 14-\( \alpha \)-methyl-\( \Delta^8 \)-cholest-3\( \beta \), 6\( \alpha \)-diol (MacDougallin), but no
comparable sterol has been isolated from mammalian tissues.

The details of the reduction of the side chain double bond and
the relocation of the nuclear double bond are not known. Apparently
two pathways exist for these transformations, differing by the pre-
sence or absence of the side chain double bond. It may be that the
reductase responsible for the removal of the double bond is nonspe-
cific, or different substrate specificity may occur in different
tissues (61). The pathways, as suggested by Dempsey (38, p. 4177), are shown in Figure 8.

Zymosterol \([\Delta^8(9), \Delta^{24}\text{-cholestradien-3}\beta\text{-ol}]\) and 24, 25 dihydrozymosterol are both metabolized to cholesterol by a Bucher-type liver homogenate (58); oxygen was required for the conversion of both sterols to cholesterol. The requirement for oxygen was interpreted to mean that the \(\Delta^5(6)\) double bond was formed by hydroxylation and subsequent dehydration reactions. \(\Delta^5,7\text{-cholestadien-3}\beta\text{-ol} (7\text{-dehydrocholesterol})\) has been shown to be an essential intermediate in the relocation of the double bond (37, 104). It has been shown that \(\Delta^7\text{-cholesten-3}\beta\text{-ol} (\Delta^7\text{-cholestenol})\) can be converted to cholesterol (104) but this conversion also required oxygen. However, 7-dehydrocholesterol could be converted to cholesterol in the absence of oxygen, but NADPH was required (14). The sequence for the change in double bond position therefore appears to be \(\Delta^8(9) \rightarrow \Delta^7 \rightarrow \Delta^5,7 \rightarrow \Delta^5\).

The oxygen apparently is required for the introduction of the \(\Delta^5\) bond into \(\Delta^7\text{-cholestenol}\).

However, the nature of the intermediate biochemical mechanisms for these nuclear transformations remains to be clarified, but recently some experimental evidence has been presented. Slaytor and Bloch (104) prepared \(4\text{-C}^{14}\Delta^7\text{-cholesten-3}\beta,6\alpha\text{-diol}\) and \(4\text{-C}^{14}\Delta^7\text{-cholesten-3}\beta,6\alpha\text{-H}^3\text{-ol}\) and described some of the metabolic transformations of these compounds by a liver homogenate.
Figure 8. Final steps in the biosynthesis of cholesterol.
These two compounds and $6\alpha-H^3\Delta^7$-cholestene-3$\beta$, 6$\beta$-dial, could be converted to cholesterol only in the presence of oxygen. Under anaerobic conditions, these three sterols were converted to $\Delta^7$-cholestenol; 7-dehydrocholesterol was not an intermediate. The authors noted that the failure of anaerobic conversion of the diols to cholesteryl excluded these compounds as intermediates between $\Delta^7$-cholestenol and 7-dehydrocholesterol; the requirement for oxygen in these nuclear transformations has yet to be explained.

Dempsey (38) has shown that $\Delta^5, 7, 24$-cholestatrien-3$\beta$-ol is also an intermediate in cholesterol biosynthesis, again in a rat liver homogenate. She has demonstrated that this trienol is formed, under aerobic conditions, from $\Delta^7, 24$-cholestadien-3$\beta$-ol. In other experiments the conversion of labeled zymosterol to $\Delta^7, 24$-cholestadien-3$\beta$-ol (no oxygen was required) and to $\Delta^5, 7, 24$-cholestratrien-3$\beta$-ol (oxygen was required) was shown. She concluded that, in the liver enzyme system, a saturated side chain pathway for cholesterol biosynthesis operates when there is an excess of oxygen and NADPH. She also observed that enzymic conversion of C27 sterols to cholesterol can take place by several pathways, and that the steps in the pathways appear to be irreversible.
Biosynthesis of Sterols by the Aorta

The first report of the biosynthesis of sterols by arterial tissue came from Siperstein, Srere, and Chaikoff (103) in 1951. Using tissue slices from aortas of chickens and rabbits, the authors demonstrated that such a tissue preparation could convert acetate-1-C\textsuperscript{14} to cholesterol (isolated as the digitonide), but the amount of acetate incorporated was low. The specific activity of the isolated cholesterol ranged from 15 to 420 counts per min per mg of cholesterol.

In 1952 Schwenk and Werthessen (101) perfused intact pig aorta with acetate-1-C\textsuperscript{14} and obtained incorporation of a small amount of label into a pentane extract (1338 counts per min per mg) of the saponified tissue. No digitonide precipitation of the extract was attempted, but when a dibromide of the extract was prepared, 75 percent of the label was lost, resulting in the isolation of a dibromide with a specific activity of 329 counts per min per mg.

Werthessen et al. (118) reported cholesterol biosynthesis by the intact bovine aorta. Using a perfusion technique on calf aorta and acetate-1-C\textsuperscript{14} as a precursor, the authors isolated a labeled sterol digitonide from the aorta wall. Such perfusions were carried out for at least 72 hours and for as long as 239 hours. The specific activities of the isolated digitonides ranged from 32 to 8500 counts per min per mg cholesterol, a variation too extreme for the authors to analyze.
the ability of the bovine aorta to synthesize cholesterol. When the labeled digitonide isolated during the perfusion experiments was purified by the formation of cholesterol dibromide, 20 percent of the label was recovered in the cholesterol dibromide. It had been previously shown (101) by Schwenk and Werthessen that cholesterol can be isolated from a sterol digitonide by selective precipitation of the cholesterol dibromide. However, the presence of radioactivity (from a labeled precursor) in the cholesterol-like companions of cholesterol in the digitonide did indicate the utilization of precursor for sterol biosynthesis. Isolation of a labeled digitonide, therefore, in the perfusion investigation (118) was taken as adequate evidence for biosynthesis of sterols by the intact bovine aorta.

Azarnoff (6) in 1958 studied cholesterol biosynthesis from acetate-1-C\(^{14}\) in arterial tissue obtained from humans and several species of adult animals. Slices of the intimal layer of the human and dog aorta were used as the tissue preparation; with other species, the whole organ was used. Cholesterol synthesis was measured by the amount of radioactivity found in the dibromide prepared from the digitonide of the nonsaponifiable fraction of the incubated tissue, but specific activities of either the isolated digitonide or dibromide were not determined. The author concluded that aorta tissue from humans, dogs, cats, and rats could not synthesize cholesterol; while the isolated digitonide from the incubated aortas ranged from 100 to 608
counts per min, no label could be found in the dibromide prepared from these samples. The same tissue from chickens, rabbits and guinea pigs could convert acetate to cholesterol under the same conditions; the level of incorporation in the digitonide ranged from 450 to 2280 counts per min, and a labeled dibromide was isolated from the digitonide.

Whereat (120) reported lipid biosynthesis by aortic intima (instead of the whole organ) obtained from both normal and cholesterol-fed rabbits. Both intact and sliced intimal layer were used. When acetate-1-C\(^{14}\) was used as a precursor, no label was found in the digitonide isolated from the incubated tissue. When MVA-2-C\(^{14}\) was used as a precursor, the isolated digitonide was not labeled, even though mevalonate is a more efficient precursor to sterols.

Other investigators (46, 51, 75) have also reported sterol biosynthesis from labeled acetate by the intact or sliced aorta. Feller and Huff (46) reported an average incorporation of 0.15 percent (400 to 3800 counts per min from each aorta) of added label into the non-saponifiable fraction of rabbit aorta. Foster and Siperstein (51), using rat aorta, found acetate-1-C\(^{14}\) incorporated into a sterol digitonide. The radioactivity ranged from 51 to 1526 counts per min in the digitonide prepared from each aorta. Marcó and Van Bruggen (75), also using rat aorta, reported a total incorporation of 18 to 72 counts per min for the digitonide isolated from each aorta,
representing a total incorporation of 0.02 to 0.04 percent of the label added.

The preparation of cell-free systems from arteries has been complicated by the toughness of the tissue, due to a large amount of connective tissue in the organ. This toughness makes conventional homogenization techniques difficult, if not impossible. Sterol biosynthesis by an aorta homogenate preparation was reported in 1955 by Eisley and Pritham (45). The authors used a Latapie grinder to prepare minced turkey aorta, which was allowed to incubate for 24 hours in the presence of acetate-1-C^{14} and phosphate buffer. For comparison, tissue slices of turkey aorta were incubated under the same conditions. A labeled digitonide was isolated from the mince preparation; it contained from 0.12 to 7.07 percent of the labeled acetate. The authors found the tissue mince to be about two and one-half times as active as tissue slices in converting acetate to sterol; in the mince incubations, the average recovery of label in the digitonide was 3440 counts per min per mg digitonide. The authors also noted that they could not show the tissue mince to be free of intact cells.

Terner and Darey (114) reported the incorporation of labeled mevalonate and acetate into lipids by a homogenate of turtle aorta. The homogenate was prepared with a Latapie mill and a Potter homogenizer, then the homogenate was squeezed through muslin. The
resulting preparation could convert labeled acetate and mevalonate into an acid fraction. The acetate was converted to long chain fatty acids and mevalonate was converted to short chain acids, but neither acidic product was identified. However, the homogenate could not incorporate either labeled acetate or labeled mevalonate into a sterol digitonide. The authors did describe the incorporation of mevalonate into an "unsaturated hydrocarbon fraction". No further identification of this product was made. Mevalonate was also incorporated into both phosphatide and glyceride fractions. In the phosphatide fraction, radioactivity from mevalonate was found in polyglycerophosphatides, phosphatidylethanolamines, and phosphatidyl cholines, but the authors suggested that the radioactivity in these fractions was localized in short chain acids, as was found in the glyceride fraction. The authors did not investigate the coenzyme requirements for incorporation, but included in the incubation both phosphate and Tris buffers, ATP, NAD, NADP, nicotinamide, glucose, fumarate, coenzyme A, cytochrome C, and ethylenediamine tetraacetate. From their data, they concluded that in arterial tissue, mevalonate was a more active precursor to major classes of lipids than acetate.

Investigations of the biosynthesis of sterols by the aorta have shown a variety of results. Studies with intact systems have shown that the arterial wall is capable of low level incorporation (in most cases, less than 0.1 percent of the added label) of precursor into a digitonin
precipitable fraction. It is difficult to compare studies because of the manner in which the data have been reported and the variety of tissues used. However, attempts to purify the sterol digitonide (6, 118) to show the incorporation into cholesterol itself have shown that cholesterol is not the only sterol synthesized by the arterial wall. At least one investigator (105) reported that the intact arterial wall could not synthesize sterols. Studies with the bovine aorta (118, 119) have been limited to perfusion experiments utilizing the entire aorta. These investigations have shown that the intact arterial wall from calves could synthesize sterols, at a low rate, from acetate.
MATERIALS AND METHODS

The bovine aortas used in this investigation were obtained without cost from the D. E. Nebergall Meat Company, Albany, Oregon.

Preparation of the Crude Homogenate

Aortas, from cattle two to three years old, were obtained immediately after sacrifice of the animals and chilled on ice. The adventitia and excess fat were removed with scissors, and the aortas were then rinsed with cold distilled water. Only the portion (12 to 14 inches in length) below the bifurcation of the aorta was used. During the preparation of the aortas, rubber gloves were worn to prevent any contamination of the homogenate.

The aortas were then either ground in a meat grinder or cut into small strips with scissors. Either method of preparation produced the same results. The tissue was kept on ice during most of the preparation period. The ground (or cut) aortas were next homogenized in a Waring blender at high speed with an equal weight of cold 0.1 M phosphate nicotinamide buffer (0.067 M K₂HPO₄, 0.042 M KH₂PO₄, 0.03 M nicotinamide, and 0.006 M MgCl₂). Aortas were also homogenized by the same procedure with either cold distilled water or 0.25 M sucrose as the homogenizing medium, in place of the 0.1 M phosphate nicotinamide buffer. However, the phosphate
buffer preparation was selected for further investigation on the basis of its ability to incorporate radioactivity into the nonsaponifiable fraction (NSF).

The resulting thick homogenate was then either forced through several layers of cheesecloth or centrifuged at 5,000 rpm in a Servall refrigerated centrifuge for 40 minutes. The solid material removed by the cheesecloth or by centrifuging was discarded. Either method of separation produced a homogenate of the same activity. The liquid portion, referred to as the crude homogenate, could be kept frozen over a period of several months without loss of activity. However, if the crude homogenate was thawed and refrozen more than two times, the activity of the preparation declined. The activity of the preparation varied with different batches of aortas.

Preparation of the Cell-free System

The cell-free preparation employed in most of the incubations was prepared by centrifuging crude homogenate (both the distilled water and the 0.1 M phosphate nicotinamide buffer preparations) at 60,000 x g in a Beckman Model L Spinco centrifuge for 120 minutes. The resulting clear, cell-free extract was used within a few hours after centrifuging. The supernatant could be stored, frozen, for about two weeks with only slight loss of activity. However, to insure no variation of activity because of freezing, the supernatant was used
the same day it was centrifuged. The cell-free extract is referred to as the 60,000 x g supernatant.

The 0.25 M sucrose crude homogenate was prepared according to the sucrose centrifugation method of Schneider (97): the crude homogenate was centrifuged at 2000 rpm in a Servall refrigerated centrifuge for ten minutes to remove debris and whole cells. The supernatant was centrifuged again at 9,000 rpm for 30 minutes to remove the mitochondrial fraction; the supernatant from this step was centrifuged a third time at 60,000 x g for one hour to obtain the final supernatant used in the incubation mixtures.

**Preparation of DL-mevalonic Acid-2-C\(^{14}\) and Acetate-2-C\(^{14}\)**

The DL-mevalonic acid-2-C\(^{14}\) (MVA-2-C\(^{14}\)) was obtained from the New England Nuclear Corporation of Boston, Massachusetts, as the N,N'-dibenzylethlenediamine salt, (MVA-2-C\(^{14}\)\(_2\)-DBED. The MVA-2-C\(^{14}\) was prepared in its free form as follows: 100 mg of "cold" MVA, also as the DBED salt, (obtained from Mann Research Laboratories, Inc.) was added to 0.01 mc of (MVA-2-C\(^{14}\)\(_2\)-DBED and the mixture dissolved in 200 ml distilled water. The DBED salt was converted to the free acid on a column of Dowex-1X4 (chloride form). The solution of the free acid (0.05 µc per 1.9 µmoles per ml) was used for routine incubations in this study and could be stored, frozen, until needed. Paper chromatographic
analysis according to the method of Tchen (109) showed the MVA so prepared to be free of any labeled contaminant.

The sodium acetate-2-C\(^{14}\) used in the incubation procedures was also purchased from the New England Nuclear Corporation. The labeled acetate was diluted with "cold" sodium acetate in an aqueous solution to give a final specific activity of 0.25 µc/0.01 mmole/ml.

**Incubation Procedure**

Incubations were carried out in 125 ml Erlenmeyer flasks. Solutions of coenzymes, 0.1 M phosphate nicotinamide buffer, and labeled compounds were added to each flask, followed by the addition of 60,000 x g supernatant or crude homogenate. Each flask was swirled in a stream of oxygen (95 percent O\(_2\), 5 percent CO\(_2\)) for 15 seconds, stoppered, and water sealed before incubation. The flasks were incubated in a Research Specialties oscillating water bath at 37° for two hours and fifty minutes.

At the completion of the incubation period, ten ml of alcoholic potassium hydroxide (10 percent in 80 percent ethanol) were added to each flask to stop the reaction. The contents of each flask were saponified overnight (12 to 16 hours) on a steam bath. During saponification each flask was equipped with a "cold finger" which consisted of a water-filled 50 ml glass conical centrifuge tube.
Determination of Coenzyme Requirements

Part of the investigation dealt with the determination of coenzymes required by the crude homogenate and the 60,000 x g supernatant for the incorporation of radioactivity into the nonsaponifiable fraction (NSF).

The volume of the incubation mixture was kept constant at 10.5 ml; coenzymes were added to the incubation mixture as aqueous solutions and in combination with each other to maintain the constant volume of the incubation mixture. Only ATP and NAD were routinely added to the incubation mixture.

Antibiotics (Penicillin G, potassium salt, 1585 units per mg, and streptomycin sulfate, 960 units per mg, from Nutritional Biochemicals Corporation) were dissolved in 0.1 M phosphate nicotinamide buffer. These antibiotics were tested on their ability to change the level of radioactivity incorporated into the NSF to preclude any biosynthetic activity by microorganisms.

The amounts of coenzymes added are included in the experimental results section. The effect of the coenzyme on the system was judged on the stimulation of incorporation of radioactivity into the NSF. The coenzymes tested were selected on the basis of their requirement in other sterol synthesizing systems (83).

Reduced glutathione (GSH), and coenzyme A (CoA) were added
to the incubation mixture dissolved in either 0.1 M phosphate nicotinamide buffer or water. Glucose-6-phosphate was added with nicotinamide adenine dinucleotide phosphate (NADP). ATP and NAD were usually added in separate aqueous solutions. Pyridine nucleotides and ATP were obtained from P-L Biochemicals, Inc. Remaining coenzymes were purchased from the Sigma Biochemicals Corporation.

**Measurement of Glucose-6-phosphate Dehydrogenase Activity**

Other investigators have established that reduced NADP (NADPH) is required for sterol biosynthesis in cell-free systems, both prior to (44) and after (3, 60) mevalonate formation. Because NADPH is a relatively expensive coenzyme, an enzyme system which would generate NADPH within the incubation mixture was used instead.

NADP was reduced by glucose-6-phosphate dehydrogenase (GPD):

\[
\text{glucose-6-phosphate} + \text{NADP} + \text{H}_2\text{O} \xrightarrow{\text{GPD}} 6\text{-phosphogluconic acid} + \text{NADPH} + \text{H}^+ 
\]

To insure the availability of NADPH in the 60,000 x g supernatant glucose-6-phosphate dehydrogenase was assayed by the method of Kornberg and Horecker (62).

**Reagents:**

- Phosphate nicotinamide buffer, 0.1 M, pH 7.0
- Magnesium chloride, 0.1 M
NADP, sodium salt, $1.5 \times 10^{-3} \text{ M}$

Glucose-6-phosphate, disodium salt, $0.02 \text{ M}$

Procedure:

Glucose-6-phosphate dehydrogenase activity was measured by the increase in optical density at $340 \text{ m}\mu$; absorption was followed with a Beckman Model DB Spectrophotometer.

Each one cm quartz cuvette contained $1.6 \text{ ml}$ water, $0.2 \text{ ml}$ glucose-6-phosphate, $0.2 \text{ ml}$ NADP, $0.4 \text{ ml}$ magnesium chloride, and $0.1 \text{ ml}$ phosphate nicotinamide buffer. At zero time $0.6 \text{ ml}$ of $60,000 \times g$ supernatant was added to the sample cell and $0.6 \text{ ml}$ of buffer to the reference cell. Readings were taken at one minute intervals.

Protein content of the $60,000 \times g$ supernatant was determined by the method of Lowry (70), using crystalline bovine serum albumin as the standard. The supernatant was found to contain $10.1 \text{ mg}$ protein per $\text{ ml}$.

A unit of glucose-6-phosphate dehydrogenase is defined as the amount of enzyme needed to bring a change of one optical density unit per minute, under the above conditions. The $60,000 \times g$ supernatant contained glucose-6-phosphate dehydrogenase with a specific activity of $0.016 \text{ units per mg}$ protein. This is sufficient activity to reduce $0.5 \mu\text{ mole}$ of NADP with glucose-6-phosphate within several minutes.
Isolation of the Nonsaponifiable Fraction

The saponified incubation mixture was allowed to cool at room temperature before the extraction procedure. The contents of each flask were then extracted four times with 50 ml portions of 30-60° petroleum ether, according to the method of Wright and Cleland (127). Each extraction consisted of swirling the flask four separate times for a period of ten to 15 seconds, and then carefully decanting the petroleum ether layer into a 250 ml Erlenmeyer flask. The combined petroleum ether extracts were dried over 30 gm of anhydrous sodium sulfate for a period of not less than one hour.

The petroleum ether extract was then filtered through a fluted Whatman No. 1 filter paper into a second 250 ml Erlenmeyer flask. The sodium sulfate was washed two times with ten ml of petroleum ether, and the washings added to the original extract.

The filtered extract and washings were taken to dryness on a stream bath. The material remaining after evaporation of the petroleum ether is referred to as the nonsaponifiable fraction (NSF). Control experiments, which had no aorta preparation in the incubation mixture, showed that no radioactivity is transferred into the NSF when the usual incubation, saponification, and isolation procedures were preformed.
Assay of Radioactivity

The radioactivity of the NSF was assayed by liquid scintillation counting in a Packard "Tri-Carb" liquid scintillation spectrometer, Model 314-DC or Model 3003. Because of the solubility of the NSF in organic solvents, liquid scintillation counting proved to be the method of choice. Liquid scintillation counting also eliminates the problems of self-absorption, geometry, and sample preparation inherent in other systems. One disadvantage of this method is the fact that the material being assayed cannot be recovered for use in further investigations.

The scintillation fluid used for counting the radioactivity in the samples consisted of four gm of 2, 5-diphenyloxazole (PPO) and 30 mg of 1, 4-bis-(phenyloxazolyl)-benzene (POPOP) dissolved in one liter of reagent grade toluene. The samples (NSF) were dissolved in five ml of this fluid and the resulting solution was then carefully decanted into a scintillation vial. The flask was rinsed with five ml of scintillation fluid, and the rinse added to the original solution in the vial. The sample to be counted was always dissolved in ten ml of scintillation fluid.

The Model 314-X instrument was set at a voltage tap of 960 volts, using discriminator settings of 100 to 1000 and 1000 to $\infty$. The samples were usually cooled for ten minutes prior to counting. The
efficiency of the instrument was determined (prior to this study) to be about 66 percent with the toluene-PPO-POPOP system. On the Model 3365 instrument, the toluene-POP-POPOP samples were counted at an eight percent gain setting; the instrument has an 85 percent efficiency at this setting. The window setting was maintained at 50 to 1000. Aqueous samples were counted in Bray's solution (16) at an 18 percent gain setting on the Model 3003, or at a voltage tap of 1250 volts in the Model 314-X.

**Cholesterol Determination by the Liebermann-Burchard Reaction**

The Liebermann-Burchard reaction (27, 115) was used to determine the amount of cholesterol present in the NSF. The reaction is a fairly specific colorimetric reaction for sterols having the C-27 sterol skeleton and a C-3 hydroxyl group (27, p. 379-380). Therefore, it is not necessarily specific for cholesterol alone, but cholesterol is specifically analyzed by reading the optical density of the sample at a longer wavelength and at an exact time period after mixing the sample and reagent together. The blue-green color which develops reaches a maximum intensity between 27 and 37 minutes and is supposed (115) to remain stable during the ten minute time period. Because such color stability was not observed when the Liebermann-Burchard reaction was used for analysis, the samples were read 30 minutes after the addition of the color reagent, as part of the general
procedure.

Reagents:

- Glacial acetic acid
- Concentrated sulfuric acid
- Acetic anhydride
- Cholesterol standard, one mg cholesterol per ml glacial acetic acid

Procedure:

The color reagent was freshly prepared for each analysis:

20 ml of acetic anhydride were chilled in the ice bath for about 15 minutes, before the addition, with mixing, of one ml of concentrated sulfuric acid. In each analysis, a standard determination using one mg of cholesterol was performed. In some instances, standards containing 0.1 mg to 1.0 mg cholesterol were used to construct a standard calibration curve. The standards and the samples to be analyzed (NSF or digitonides) were dissolved in one ml of glacial acetic acid. Two ml of the Liebermann-Burchard reagent were added to the sample and the solution mixed vigorously. The samples were placed in the dark for the 30 minute development period. The color was read at 690 m\(\mu\) in a Bechman Model DB Spectrophotometer. For a reference sample four ml of glacial acetic acid were mixed with eight ml of the Liebermann-Burchard reagent.

The NSF of an incubation mixture of five ml of the 60,000 x g
supernatant contained 95 to 120 µgm of cholesterol, depending upon the preparation used, as determined by the Liebermann-Burchard reaction; or approximately 9.4 to 14.4 µgm cholesterol per mg protein.

For some analyses, especially those which did not involve solution of a sterol digitonide, the samples were dissolved in chloroform and Liebermann-Burchard reagent was added to the sample in the same volume ratio as described above. The color was read at 620 mµ.

**Cholesterol Determination by Digitonide Formation**

Digitonin, a saponin, has long (50, p. 29-31) been known as a specific precipitating agent for cholesterol; a very insoluble 1:1 complex of cholesterol and digitonin is formed when appropriate solutions of the two compounds are combined. Later (106) it was shown that digitonin precipitates not only cholesterol, but other sterols having a 3β-hydroxyl group similar to cholesterol.

The digitonide of the NSF was prepared according to the method of Sperry and Webb (105).

**Reagents:**

- Digitonin, 0.5 percent solution in 50 percent ethanol
- Acetone, 50 percent (v/v) in absolute ethanol
- Acetone, 50 percent (v/v) in anhydrous diethyl ether
Acetic acid, ten percent

Procedure:

The NSF, in a 250 ml Erlenmeyer flask, was transferred to a 15 ml plastic conical centrifuge tube with five ml of 30-60° petroleum ether. The flask was washed two times with five ml of petroleum ether and the washings added to the original. The solvent was removed with an air stream. "Cold" carrier cholesterol, when used, was added to the centrifuge tubes in the acetone-ethanol solution. The NSF, if no carrier was added, was directly dissolved in one ml of acetone-ethanol solution. The tubes were warmed slightly in a water bath to insure complete solution of the NSF. Sufficient digitonide solution was then added to triple the sample volume; several drops of ten percent acetic acid were then added with mixing. The mixture was allowed to stand, covered, overnight.

The precipitate which formed was removed by centrifuging the tube for 15 minutes at maximum speed (2000 rpm) in a clinical centrifuge and the supernatant was carefully decanted. The digitonide was washed with two ml of acetone-diethyl ether solution and was centrifuged for 15 minutes at the same speed. The supernatant was decanted and added to the original supernatant. The digitonide was then washed two more times with two ml diethyl ether, following the same procedure. The digitonide was dried by placing the centrifuge tube in a warm water bath for several minutes.
The digitonide could be used directly in the Liebermann-Burchard procedure by dissolving the precipitate in glacial acetic acid. Slight heating in a boiling water bath helped to bring all the digitonide into solution. For assay by scintillation counting, the method of Kritchevsky and Shapiro was used (65): two ml of methanol were added to the centrifuge tube containing the digitonide and the tubes immersed in a hot water bath. The methanol was allowed to boil gently for two minutes, and the solution was then decanted into a scintillation vial. The procedure was repeated using one ml of methanol, then each tube was rinsed with one ml of anhydrous diethyl ether and the ether rinse added to the vial. Ten ml of scintillation fluid was added and the vials counted as usual.

It was found that 0.1 mg of "cold" cholesterol could be precipitated and recovered by this procedure. Gravimetric analysis of such small amounts of cholesterol was not as convenient a method as analyzing by the Liebermann-Burchard method, as it was very difficult to remove the excess precipitant from such small amounts of sterol.

When the NSF of five ml of 60,000 x g supernatant was analyzed for cholesterol by the digitonin procedure, 90 to 110 µgm of cholesterol were found in the precipitate. The digitonide was analyzed by the Liebermann-Burchard method.

To demonstrate that cholesterol could be quantitatively precipitated by digitonin in the presence of the NSF of the 60,000 x g
supernatant, labeled cholesterol of known specific activity was used as a standard and as labeled carrier in combination with a "cold" NSF.

Cholesterol-4-C\text{14} with a specific activity of 1157 counts per min per mg was used as the standard. The cholesterol was purified prior to use via the dibromide using the method of Kabara and McLaughlin (59) and regenerated with zinc according to the method of Fieser (49), then crystallized to constant specific activity.

For the standard determination (no NSF was used) 11 mg of labeled cholesterol was dissolved in ten ml of acetone. Using one ml of this solution (1.1 mg cholesterol) the digitonide was isolated and counted as described above. Because methanol is a known quenching agent, a standard vial was prepared using one ml (the same amount used in the digitonide precipitation) of the above cholesterol solution. The solution was pipetted into a scintillation vial and the acetone removed by an air stream. Three ml of methanol, one ml of diethyl ether, and ten ml of scintillation fluid were added. The results are summarized in Table 1.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Counts per min</th>
<th>Percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Cholesterol</td>
<td>778</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>776</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>797</td>
<td>102</td>
</tr>
<tr>
<td>3</td>
<td>729</td>
<td>94</td>
</tr>
<tr>
<td>4</td>
<td>755</td>
<td>97</td>
</tr>
</tbody>
</table>
The data presented in Table 1 indicate that precipitation of one mg of cholesterol by the digitonin procedure could be considered a nearly quantitative procedure.

An unlabeled NSF was prepared by incubating a mixture of 3.0 ml water, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml 60,000 x g supernatant. The NSF was isolated as before, and was transferred to plastic centrifuge tubes with a total of 15 ml 30-60° petroleum ether. The petroleum ether was removed with an air stream. For a standard, ten mg cholesterol (specific activity of 1157 counts per min per mg) was dissolved in ten ml acetone. To each NSF in the plastic centrifuge tubes was added one ml (one mg cholesterol) of the standard cholesterol solution and the digitonide isolated and counted as above. A standard vial was again prepared using one ml of the cholesterol standard solution; the standard was prepared for counting by the same procedure as the standard in the previous digitonide precipitation. The results are summarized in Table 2.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Counts per min</th>
<th>Percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Cholesterol</td>
<td>680</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>654</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>663</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>666</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>668</td>
<td>98</td>
</tr>
</tbody>
</table>
The results in Table 2 indicate that digitonide precipitation of one mg of cholesterol is a nearly quantitative procedure for isolation of cholesterol; this procedure is equally effective by itself or in the presence of the NSF.

In later experiments, this method for isolating the digitonin precipitable sterols was modified by centrifuging the sample at higher speeds (10,000 rpm) and counting the digitonide according to the method of Avoy et al. (5). In this counting procedure, the digitonide was dissolved in anhydrous reagent grade pyridine (which splits the digitonin-cholesterol complex), and the solution transferred to a scintillation vial. The pyridine was removed by heating the vial in a stream of air until the solvent had been removed. Small amounts of toluene were added to the vial and removed in the same manner until the odor of pyridine could no longer be detected. Ten ml of scintillation fluid were then added and the radioactivity determined in a scintillation counter. Nearly quantitative recovery of standard samples (one mg) of cholesterol-4-C$^{14}$ converted to the digitonide was obtained by this method.

Silica Gel Chromatography of the NSF

Because of the small amount of sterol in the NSF, the micro method of column chromatography of Wycoff and Parsons (131) was selected and used with some modifications. The column was made of
8 mm. Pyrex glass tubing, constricted at one end. A small wad of cotton was used to plug the column, and silicic acid (from Bio-Rad Laboratories, minus 25 mesh, "Specially Prepared for the Chromatography of Lipids") was packed as a slurry in 60-70°C petroleum ether to a height of five cm.

The column was washed first with ten ml of 60-70°C petroleum ether, then with 15 ml of 30-60°C petroleum ether. The sample (NSF), dissolved in 30-60°C petroleum ether, was applied to the column. This was followed by two ml of petroleum ether which had been used to rinse the sample container. Fifteen ml graduated centrifuge tubes were used to collect the fractions, two ml each. The flow rate was maintained at about two ml per minute with the aid of air pressure.

Unless otherwise specified, the order and number of fractions eluted with each solvent were: fractions one through four, petroleum ether; fractions five through eight, 50 percent chloroform in petroleum ether (v/v); fractions nine through 12, 50 percent ethyl acetate in petroleum ether (v/v); fraction 13, anhydrous diethyl ether; and fraction 14, methanol. The fractions were evaporated on the steam bath or in a stream of air. Radioactivity was assayed by scintillation counting; or by a combination of thin layer chromatography and autoradiography.

When cholesterol -4-C<sup>14</sup> alone was chromatographed by this method, the radioactivity appeared in the last three chloroform-
petroleum ether fractions and in the first petroleum ether-ethyl acetate fraction. When squalene was used as a sample of chromatography, it was eluted completely in the first four fractions (petroleum ether); the presence of squalene in the fractions was determined by thin layer chromatography.

Silicic Acid-celite Chromatography of the NSF

A second method of column chromatography was used to characterize the labeled material in the NSF. The procedure used was that developed by Clayton, Nelson, and Frantz (25). This method will separate most of the naturally occurring sterols in mammalian tissues.

The column (1 cm by 100 cm) was prepared by packing (under air pressure) a slurry of silicic acid (Mallinkrodt, 100 mesh) and celite (Johns-Manville, Hyflo SuperCel), two:one (w/w), in distilled reagent grade benzene. The sample was applied as a solution in benzene, and benzene was used as the eluting solvent. The flow rate of such columns was about eight to ten ml per hour; fractions were collected for 15 min periods and were found to be of a constant volume throughout the elution process. Aliquots (ten percent of each fraction) were taken for counting; one half of the remainder of each fraction was used for Liebermann-Burchard analysis.
Thin Layer Chromatography

Thin layer chromatography (TLC) was employed for further analysis of the NSF and of the labeled material. Silica Gel G (from Brinkmann Instruments) was used as the adsorbent. Glass plates, 20 cm by 20 cm, were used and prepared according to Randerath (87, p. 21-25). Thirty grams of Silica Gel G were slurried in 60 ml of distilled water and the slurry immediately applied to the glass plates with a Research Specialties Company applicator. This method produced a silica gel layer about 275 µ thick. The plates were stored at room temperature until just prior to use; then heating the plate in a 110°C oven for 30 minutes activated the silica layer. The samples were applied as solutions in petroleum ether or other suitable solvents. The plates were then developed in a glass tank lined with Whatman No. 1 paper to insure an evenly saturated atmosphere. The solvent was allowed to migrate to a height of 11.5 to 13.5 cm from the origin before the plates were removed and allowed to air dry. The compounds were detected on the plates with one of the following sprays: 0.5 percent (w/v) Rhodamine B; 0.04 percent (w/v) 2,7-dichlorofluorescein in methanol; 50 percent (v/v) sulfuric acid in ethanol; or ten percent (w/v) phosphomolydic acid in ethanol. With Rhodamine B used as the detecting agent, spots appeared violet on a rose background; with 2,7-dichlorofluorescein, the plates were
viewed under ultraviolet light, when the spots appeared as a fluorescent yellow-green. With either the sulfuric acid or phosphomolybdic acid as the spray, the plates were heated in the oven for five to 30 minutes.

Preparative layer chromatography was used to isolate specific fractions in the NSF. Control experiments using Silica Gel G as the adsorbent demonstrated that this material is not pure enough to obtain clean fractions, especially for subsequent use in gas chromatography. Silica Gel H (Brinkmann Instruments) was purified for preparative chromatography by the method of Parker and Peterson (80): One hundred and twenty-five gram lots of Silica Gel H were purified at one time. All solvents were reagent grade, distilled prior to use. The silica gel was washed, on a Büchner funnel, with one liter of methanol: formic acid: water, two:one:one (v/v/v). This wash was followed by two liters of distilled water. The washed silica gel was oven dried at 110° for 48 hours, then stored in a covered container until needed.

The preparative plate, 40 cm by 20 cm, was prepared from a mixture of 50 gm of purified Silica Gel H and 85 to 90 ml of distilled water; the slurry was mixed in a Waring Blender for five minutes at low speed. The slurry was applied with a Desaga adjustable applicator (Brinkmann Instruments) and the thickness of the plates used varied from 500 µ to 750 µ. The plate was allowed to air dry for one
hour; just prior to use it was activated at 110° for one hour. This produced plates with a very smooth and stable surface. The sample (usually the combined NSF of large incubations) was applied as a single streak on the plate; this could be accomplished without disturbing the surface of the gel to any great extent. Labeled cholesterol (60 to 100 µgm, 250 counts per min per µgm) was sometimes applied as a marker when the plates were exposed to film.

A large incubation mixture contained 169 to 254 µmoles of ATP, 69 µmoles of NAD, 0.50 µc MVA-2-C14, and 50 ml 60,000 x g supernatant in a total volume of 80 ml. The reaction mixture was incubated for two hours and fifty minutes, then stopped with 80 ml alcoholic KOH and saponified overnight. Extraction was carried out as before, with a total of 800 ml distilled (30-60°) petroleum ether. Aliquots were taken from each NSF for counting.

To obtain enough (one mg) of labeled material for analysis for an infrared spectrum, the combined NSF of 28 large incubations was chromatographed on five 750 µ preparative plates in benzene:ethyl acetate, five:one (v/v) (4). Each plate was chromatographed in the solvent three times to facilitate better separation. Using autoradiographs for guides, (the labeled band migrated just above cholesterol) the labeled band from each plate was scraped off, and the silicic acid from the five plates was combined. The silicic acid was eluted immediately with 60 ml of distilled chloroform, and the chloroform
removed under reduced pressure. The combined labeled band sample was then rechromatographed on two 500 µ preparative plates in benzene:acetone, nine:one (v/v) (87, p. 12). The labeled bands were again located by autoradiography, combined and eluted from the silicic acid as before to give a sample containing approximately one mg of labeled material. A portion of this sample was used for gas chromatography. When the labeled bands were removed from the preparative plates, the remainder of the plate was sprayed with the phosphomolybdic acid spray to show that separation of cholesterol and the labeled band had taken place.

The procedure for obtaining enough material for a mass spectrum analysis was essentially the same. The NSF of 21 large incubations was chromatographed first on three 750 µ preparative plates in the benzene:ethyl acetate system as above. The area above the cholesterol band on each plate (located by spraying with 2, 7-dichloro-fluorescein) was removed, combined and eluted; the sample so obtained was rechromatographed on two 500 µ preparative plates in petroleum ether:diethyl ether:acetic acid, 70:30:1 (v/v/v) (74). The labeled band was located by autoradiography (the labeled band again migrated slightly above cholesterol) and eluted from the silicic acid. A second sample of the labeled band was isolated from the NSF of seven large incubations. This combined NSF was chromatographed on one 750 µ preparative plate three times in the petroleum ether:ethyl...
ether:acetic acid system. The autoradiograph of this plate showed the labeled band to be separate from cholesterol. The two samples of the labeled product from the NSF were combined and used as the sample for obtaining a mass spectrum.

**Autoradiography**

For detection of radioactive spots on thin layer and paper chromatograms, autoradiography was used (100). Kodak Blue Sensitive single coated X-ray film (14" by 17") was used. The exposure time was determined by the estimated radioactivity in the sample; usually this was calculated on the basis that the minimum exposure time required for visible darkening of the film is the time required to have \(10^6\) events occur (in the labeled sample) per square cm of film exposed (100, p. 8). Thus if a sample had an estimated activity of 100 dpm per square centimeter of the plate (or paper), then the minimum exposure time would be one week.

The film was developed in Kodak X-ray developer and fixer according to the manufacturer's directions. Until the development procedure was completed all operations were carried out in the darkroom with a Kodak safety light for illumination. All solutions used were kept at room temperature. The exposed film was placed in a holder and immersed in the developer solution for five minutes, then was briefly rinsed in a running water bath. The film was then immersed
in the fixer solution for five minutes; during this period, the film was agitated frequently to insure removal of the emulsion. Finally the film was placed in the water bath for 30 minutes, then air dried.
EXPERIMENTAL RESULTS

Selection of the Homogenizing Medium

Three different crude homogenates were prepared from bovine aortas, using distilled water, 0.1 M phosphate nicotinamide buffer, or 0.25 M sucrose as the homogenizing medium. None of these crude homogenates, when incubated with various coenzymes, has shown any incorporation of MVA-2-C$^{14}$ or acetate-2-C$^{14}$ into the NSF.

The incorporation of labeled MVA into the NSF by the 60,000 x g supernatants of the three crude homogenates is summarized in Table 3. The data (Table 3) show that only the 0.1 M phosphate nicotinamide buffer preparation incorporated the label significantly; this preparation was therefore used in subsequent experiments.

Table 3. Incorporation of MVA-2-C$^{14}$ into the NSF by three different aorta preparations.\(^1\)

<table>
<thead>
<tr>
<th>No. of flasks</th>
<th>0.25 M sucrose</th>
<th>Distilled water</th>
<th>0.1 M phosphate nicotinamide</th>
<th>Counts per min (NSF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M phosphate Supernatant</td>
<td>0.1 M phosphate Supernatant</td>
<td>0.1 M phosphate Supernatant</td>
<td>Counts per min</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10.0 ml</td>
<td>0.0 ml</td>
<td>0.0 ml</td>
<td>10-22</td>
</tr>
<tr>
<td>4</td>
<td>0.0 ml</td>
<td>10.0 ml</td>
<td>0.0 ml</td>
<td>6-15</td>
</tr>
<tr>
<td>4</td>
<td>0.0 ml</td>
<td>0.0 ml</td>
<td>10.0 ml</td>
<td>480-782</td>
</tr>
</tbody>
</table>

\(^1\) Each incubation mixture also contained 3.3 µmoles ATP, 27.8 µmoles NAD, 0.05 µc MVA-2-C$^{14}$, and 5.0 ml 0.1 M phosphate nicotinamide buffer in a total volume of 18 ml.
Figure 9 shows the incorporation, with time, of MVA-2-C\textsuperscript{14} into the NSF over a period of 240 minutes. Each incubation mixture contained 5.0 ml 60,000 x g supernatant, 2.5 ml 0.1 M phosphate nicotinamide buffer, 0.05 \(\mu\)c MVA-2-C\textsuperscript{14}, 3.3 \(\mu\)moles ATP, and 27.8 \(\mu\)moles NAD. Two flasks were removed from the incubation bath at the time interval indicated and the reactions were stopped with alcoholic potassium hydroxide. Each point in the figure represents the average counts per min of the two NSF's. At 200 minutes the amount of incorporated label apparently becomes constant; prior to this, incorporation increases with time.

**Effect of Antibiotics on the Incorporation of Radioactivity into the NSF**

Table 4 summarizes the results of several experiments in which antibiotics were added to the incubation mixture to preclude any biosynthetic activity by microorganisms. The results indicate that the antibiotics have no effect on the incorporation of MVA-2-C\textsuperscript{14} into the NSF; the same amount of labeled MVA is incorporated whether or not antibiotics are present in the incubation mixture. The amount of acetate-2-C\textsuperscript{14} incorporated, with or without antibiotics in the incubation mixture, was not significant.
Figure 9. Incorporation of MVA-2-\textsuperscript{14}C into the NSF.
Table 4. Effect of antibiotics on the incorporation of MVA-2-C\textsuperscript{14} and acetate-2-C\textsuperscript{14} into the NSF.

<table>
<thead>
<tr>
<th>No. of flasks</th>
<th>MVA-2-C\textsuperscript{14}</th>
<th>Acetate-2-C\textsuperscript{14}</th>
<th>Antibiotics\textsuperscript{2}</th>
<th>Counts per min in NSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Average</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.25 μc</td>
<td>+</td>
<td>12-34</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>0.25 μc</td>
<td>-</td>
<td>13-51</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>0.05 μc</td>
<td>+</td>
<td>676-978</td>
<td>878</td>
</tr>
<tr>
<td>4</td>
<td>0.05 μc</td>
<td>-</td>
<td>689-999</td>
<td>872</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Each flask also contained 3.3 μmoles ATP, 27.8 μmoles NAD, 5.0 ml 60,000 x g supernatant, and 0.1 M phosphate nicotinamide buffer in a total volume of 10.5 ml.

\textsuperscript{2} Each ml of antibiotics solution contained Penicillin G, 1585 units per mg, 0.5 mg; and streptomycin sulfate, 960 units per mg, 1.0 mg. The antibiotics were dissolved in phosphate nictoinamide buffer.

**Effect of Coenzymes on the Incorporation of Acetate-2-C\textsuperscript{14} into the NSF**

Table 5 shows the effect of various coenzymes on the incorporation of acetate-2-C\textsuperscript{14} into the NSF. In general, no significant incorporation of labeled acetate has been demonstrated; while several coenzymes were included in the incubation mixture, their presence did not stimulate any biosynthetic activity.

Reduced glutathione (GSH) was included to protect any -SH containing enzymes from oxidation (61). Coenzyme A was added because of its participation in the biosynthetic pathway from acetate to mevalonate. Glucose-6-phosphate and NADP were added to provide a source of NADPH.
Table 5. Incorporation of acetate-2-C\textsuperscript{14} into the NSF.\textsuperscript{1}

<table>
<thead>
<tr>
<th>No. of flasks</th>
<th>Glucose-6-phosphate and NAD\textsuperscript{2}</th>
<th>Coenzyme A (\mu)moles</th>
<th>Reduced glutathione (\mu)moles</th>
<th>Counts per min NSF</th>
<th>Range</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>+</td>
<td>1.0</td>
<td>0</td>
<td>0-9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>1-15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>1.0</td>
<td>150</td>
<td>1-6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>1.0</td>
<td>150</td>
<td>0-1</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} Each flask also contained 3.3 \(\mu\)moles ATP, 27.8 \(\mu\)moles NAD, 0.25 \(\mu\)c acetate-2-C\textsuperscript{14}, 5.0 ml 60,000 x g supernatant, and 0.1 M phosphate nicotinamide buffer in a total volume of 10.5 ml.

\textsuperscript{2} One ml added which contained 3.3 \(\mu\)moles glucose-6-phosphate and 1.9 \(\mu\)moles NADP.

Effect of Coenzymes on the Incorporation of MVA-2-C\textsuperscript{14} into the NSF

Tables 6 to 11 summarize the effect of various coenzymes on the incorporation of MVA-2-C\textsuperscript{14} into the NSF. Table 6 shows that, for the incorporation of MVA-2-C\textsuperscript{14} into the NSF, there is a requirement for ATP. The requirement for NAD was not so definite, some incorporation taking place when NAD alone was added, but the amount of incorporation was not nearly as high as that obtained with ATP alone. ATP by itself appeared to be just as effective as the combination of ATP and NAD in the incubation mixture. No significant incorporation took place when both coenzymes were omitted from the incubation mixture.

The effect of adding increasing amounts of NAD to the incubation mixture with a constant amount of ATP is shown in Table 7.
Table 6. Effect of NAD and ATP on the incorporation of MVA-2-C\textsuperscript{14} into the NSF.\textsuperscript{1}

<table>
<thead>
<tr>
<th>No. of flasks</th>
<th>ATP µmoles</th>
<th>NAD µmoles</th>
<th>Counts per min NSF Range</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0-4</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>27.9</td>
<td>185-408</td>
<td>280</td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>0</td>
<td>1465-1697</td>
<td>1581</td>
</tr>
<tr>
<td>3</td>
<td>3.3</td>
<td>27.9</td>
<td>1535-1729</td>
<td>1627</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Each flask also contained 0.05 µc MVA-2-C\textsuperscript{14}, 2.5 ml 0.1 M phosphate nicotinamide buffer, 5.0 ml 60,000 x g supernatant, and water in a total volume of 10.5 ml.

Table 7. Effect of NAD on the incorporation of MVA-2-C\textsuperscript{14} into the NSF.\textsuperscript{1}

<table>
<thead>
<tr>
<th>No. of flasks</th>
<th>NAD µmoles</th>
<th>Counts per min NSF Range</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6.9</td>
<td>143-663</td>
<td>383</td>
</tr>
<tr>
<td>4</td>
<td>13.8</td>
<td>451-872</td>
<td>613</td>
</tr>
<tr>
<td>4</td>
<td>20.7</td>
<td>172-564</td>
<td>442</td>
</tr>
<tr>
<td>4</td>
<td>27.8</td>
<td>169-607</td>
<td>500</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Each flask also contained 3.3 µmoles ATP, 2.5 ml 0.1 M phosphate nicotinamide buffer, 0.05 µc MVA-2-C\textsuperscript{14}, and 5.0 ml 60,000 x g supernatant, in a total volume of 10.5 ml.

Table 8. Effect of NADPH on the incorporation of MVA-2-C\textsuperscript{14} into the NSF.\textsuperscript{1}

<table>
<thead>
<tr>
<th>No. of flasks</th>
<th>NAD µmoles</th>
<th>NADPH µmoles</th>
<th>ATP µmoles</th>
<th>Counts per min NSF Range</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>2346-2735</td>
<td>2601</td>
</tr>
<tr>
<td>4</td>
<td>6.9</td>
<td>0</td>
<td>25</td>
<td>2392-2897</td>
<td>2577</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>1.0</td>
<td>25</td>
<td>2577-2897</td>
<td>2744</td>
</tr>
<tr>
<td>4</td>
<td>6.9</td>
<td>1.0</td>
<td>25</td>
<td>2290-2801</td>
<td>2518</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1.0</td>
<td></td>
<td>9-13</td>
<td>11</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Each flask also contained 0.05 µc MVA-2-C\textsuperscript{14}, 5.0 ml 60,000 x g supernatant and 0.1 M phosphate nicotinamide buffer in a total volume of 10.5 ml.
### Table 9. Effect of NAD and NADH on the incorporation of MVA-2-C\textsuperscript{14} into the NSF.

<table>
<thead>
<tr>
<th>No. of flasks</th>
<th>NAD µmoles</th>
<th>NADH µmoles</th>
<th>Counts per min NSF Range</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>27.8</td>
<td>0</td>
<td>1731-2002</td>
<td>1881</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>27.8</td>
<td>371-998</td>
<td>793</td>
</tr>
</tbody>
</table>

1. Each flask also contained 3.3 µmoles ATP, 0.05 µc MVA-2-C\textsuperscript{14}, 5.0 ml 60,000 x g supernatant and 0.1 M phosphate nicotinamide buffer in a total volume of 10.5 ml.

### Table 10. Effect of NAD, NADPH, and NADH on the incorporation of MVA-2-C\textsuperscript{14} into the NSF.

<table>
<thead>
<tr>
<th>No. of flasks</th>
<th>NAD µmoles</th>
<th>NADH µmoles</th>
<th>Glucose-6-phosphate and NADP\textsuperscript{2}</th>
<th>Counts per min NSF Range</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>13.9</td>
<td>0</td>
<td>-</td>
<td>782-836</td>
<td>779</td>
</tr>
<tr>
<td>3</td>
<td>13.9</td>
<td>0</td>
<td>+</td>
<td>745-778</td>
<td>751</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>773-902</td>
<td>862</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>13.9</td>
<td>+</td>
<td>319-408</td>
<td>368</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>13.9</td>
<td>-</td>
<td>399-443</td>
<td>421</td>
</tr>
<tr>
<td>3</td>
<td>13.9</td>
<td>13.9</td>
<td>+</td>
<td>640-712</td>
<td>659</td>
</tr>
</tbody>
</table>

1. Each flask also contained 16.5 µmoles ATP, 0.05 µc MVA-2-C\textsuperscript{14}, 5.0 ml 60,000 x g supernatant, and 0.1 M phosphate nicotinamide buffer to make a final volume of 10.5 ml.

2. To each flask indicated was added 59 µmoles glucose-6-phosphate and 2.2 µmoles NADP.
Table 11. Effect of ATP on the incorporation of MVA-2-C$^{14}$ into the NSF.  

<table>
<thead>
<tr>
<th>No. of flasks</th>
<th>ATP µmoles</th>
<th>Counts per min NSF</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>16-23</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>3.3</td>
<td>1910-2020</td>
<td>1967</td>
</tr>
<tr>
<td>3</td>
<td>8.2</td>
<td>2361-2490</td>
<td>2412</td>
</tr>
<tr>
<td>3</td>
<td>16.5</td>
<td>2625-2826</td>
<td>2694</td>
</tr>
<tr>
<td>2</td>
<td>24.6</td>
<td>1792-2675</td>
<td>234</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>2437-2588</td>
<td>2225</td>
</tr>
</tbody>
</table>

1 Each flask also contained 13.9 µmoles NAD, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml 60,000 x g supernatant, in a total volume of 10.5 ml.

While this experiment did not show whether ATP alone was required for the incorporation of MVA-2-C$^{14}$ into the NSF, the results do indicate that the amount of NAD employed did not significantly alter the level of incorporation of radioactivity into the NSF.

The results of adding NADPH to the incubation mixture is shown in Table 8. NADPH was added directly to the incubation mixture (instead of using the glucose-6-phosphate dehydrogenase-NADP system) to see if adding this coenzyme directly would affect the level of incorporation. When NADPH is added alone, there is no incorporation of MVA-2-C$^{14}$. When ATP was present in the incubation mixture, NAD, or NADPH, or the combination of the two coenzymes did not stimulate the level of incorporation. ATP appeared to be just as effective alone as in combination with the pyridine nucleotides.

The effect of adding NADH directly to the incubation mixture is
summarized in Table 9. While NADH is required by a rat liver preparation (44) for the incorporation of MVA or acetate into the sterol fraction, NADH in this preparation of bovine aorta depressed the amount of MVA-2-C\textsuperscript{14} incorporated into the NSF to about 42 percent of the amount of MVA incorporated into the NSF when NAD instead of NADH was included in the incubation mixture.

This effect is also shown in Table 10, which compared the effect of NAD, NADP, and NADH on the incorporation of MVA-2-C\textsuperscript{14} into the NSF. Either NAD, or the combination of NADP and glucose-6-phosphate appeared to be equally effective in incorporating radioactivity into the NSF. Approximately the same level of incorporation was obtained when both NAD and NADP were added together. Adding NADH to the incubation mixture decreased the amount of label in the NSF to about 50 percent of that obtained with the other incubations, both when NADP was also added to the incubation mixture, and when NADH alone was added. However, when NAD, NADH and NADP were all included in the incubation mixture, the amount of incorporation was about 75 percent of that when no NADH was present in the incubation mixture.

Table 11 and Figure 10 show the effect of adding increased amounts of ATP to the incubation mixture; with NAD alone in the incubation mixture, incorporation of MVA-2-C\textsuperscript{14} was very low. With increasing amounts of ATP, there was a steady rise in the
Figure 10. Effect of ATP on the incorporation of MVA-2-\textsuperscript{14}C into the NSF.
incorporation of radioactivity. Maximum incorporation took place when 16.5 µmoles of ATP were added to the incubation mixture; larger amounts of ATP neither stimulated nor depressed this level of incorporation.

**Formation of Phosphorylated Intermediates by the 60,000 x g Supernatant**

The first step in the incorporation of MVA into sterols is the phosphorylation of MVA to phosphomevalonic acid; this compound is subsequently phosphorylated to mevalonic acid pyrophosphate, which is converted to isopentenyl pyrophosphate by a simultaneous decarboxylation and dehydration step. Each of these steps is catalyzed by a different enzyme, and each requires ATP:

\[
\begin{align*}
\text{MVA} + \text{ATP} & \quad \xrightarrow{\text{Mevalonic kinase}} \quad \text{MVA-5-P} + \text{ADP} \\
\text{MVA-5-P} + \text{ATP} & \quad \xrightarrow{\text{Phosphomevalonic acid kinase}} \quad \text{MVA-5-PP} + \text{ADP} \\
\text{MVA-5-PP} + \text{ATP} & \quad \xrightarrow{\text{Anhydrodecarboxylase}} \quad \text{IPP} + \text{CO}_2 + \text{ADP} + \text{Pi}
\end{align*}
\]

Because of the requirement for ATP for the incorporation of MVA into the NSF of the 60,000 x g supernatant, it was of interest to determine the presence of these three enzymes in the 60,000 x g supernatant. The presence of the enzymes which catalyze these reactions can be shown by using labeled mevalonate and detecting the products by paper and ion exchange chromatography (14, 21). These
methods were therefore used to demonstrate the preliminary utilization of MVA by the 60,000 x g supernatant.

MVA-2-C\textsuperscript{14} (0.37 µc, 0.19 µmole) was incubated with 15 µmoles of Mg\textsuperscript{++}, 10 µmoles ATP, and 1.0 ml of the 60,000 x g supernatant, in a total volume of 2.6 ml. The mixture was incubated for one hour in a 37°C oscillating water bath. The reaction was stopped by immersing the tubes in a 100°C water bath for three minutes. To remove the precipitated protein the tubes were centrifuged for 40 minutes at 10,000 rpm in a Servall refrigerated centrifuge. The pellet was washed with three ml distilled water and centrifuged again under the same conditions. The second supernatant was pooled with the original supernatant; this solution, containing the MVA and any phosphorylated derivatives, was dried in an air stream.

Paper chromatography and autoradiography were used initially to identify the products of the incubation. The reaction mixture was applied as a single spot to Whatman No. 1 paper and developed in t-butanol:formic acid: water, 40:10:16 (v/v) (21) for 24 hours using an ascending solvent technique.

The autoradiograph of this paper showed extensive darkening from slightly above the origin to just below the solvent front. MVA has a reported $R_f = 0.80$ to $0.85$ in this solvent system using a descending technique. MVA had an $R_f = 0.85$ to $0.95$ using an ascending technique; this was determined using known samples of MVA-2-C\textsuperscript{14}.
Using the autoradiograph as a guide, the radioactive area on
the paper was cut out and the radioactivity eluted with water. The
eluate was dried in an air stream and chromatographed again, using
two dimensional paper chromatography. The sample was applied on
Whatman No. 1 paper and developed in the first direction in n-butanol:
formic acid:water, 77:10:13 (v/v) (109), then in t-butanol:formic acid:
water in the second direction.

The autoradiograph of this paper is reproduced in Figure 11.
On the basis of their migration in both solvent systems the compounds
on the chromatogram could be tentatively identified. The \( R_f \) values
in the second solvent system are summarized in Table 12. Compound
III, isopentenyl pyrophosphate, was identified on the basis of its be-
havior in the first solvent system, to differentiate it from phospho-
mevalonic acid. In the first solvent system, compound III had an
\( R_f = 0.17 \); the \( R_f \) of isopentenyl pyrophosphate in this solvent sys-
tem has been reported as 0.18 (121). In addition, a small amount of
labeled material was found near the solvent front (compound V in
Figure 11), \( R_f = 0.98 \), which was not identified.

To further characterize the labeled products of the incubation,
ion exchange chromatography (14) was used. A 1 cm by 2.5 cm col-
umn of Dowex-1 (formate) was prepared by packing the column with
the resin (chloride form) and then washing it with 3 N sodium formate
until there was no chloride in the eluent. The column was then washed
Figure 11. Autoradiography of two-dimensional paper chromatography of phosphorylated derivatives of MVA.
with 25 ml water, 20 ml 1 N sodium formate in 6 N formic acid, 20 ml 90 percent formic acid, and water until the eluent was neutral to litmus.

Table 12. Identification of phosphorylated derivatives of MVA.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_f$ Reported</th>
<th>$R_f$ Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mevalonic Acid (IV)</td>
<td>0.80-0.85</td>
<td>0.93</td>
</tr>
<tr>
<td>Phosphomevalonic Acid (I)</td>
<td>0.53-0.61</td>
<td>0.59</td>
</tr>
<tr>
<td>Mevalonic Acid Pyrophosphate (II)</td>
<td>0.29-0.35</td>
<td>0.29</td>
</tr>
<tr>
<td>Isopentenyl Pyrophosphate (III)</td>
<td>0.53-0.61</td>
<td>0.65</td>
</tr>
</tbody>
</table>

1 Numerals refer to Figure 11.
2 The values are those of Chaykin et al. (21).
3 This value has also been reported by Wittig et al. (121).

The sample, the dried supernatant of an incubation mixture described above, was dissolved in two ml water and transferred to the column. Elution was then carried out with 62.5 ml 0.2 N formic acid; 125 ml 4 N formic acid; and 125 ml 0.8 N ammonium formate in 4 N formic acid. Two and one-half ml fractions were collected with a Technicon fraction collector. For assay of radioactivity 0.5 ml aliquots were removed from each fraction. A series of radioactive peaks were obtained (Figure 12). Bloch has reported (14) that MVA is eluted with 0.2 N formic acid; phosphomevalonic acid is eluted with 4.0 N formic acid; mevalonic acid pyrophosphate and isopentenyl pyrophosphate are eluted together with 0.8 N ammonium formate in
Figure 12. Ion exchange chromatography of mevalonic acid derivatives.
4 N formic acid.

The fractions in each peak were combined and chromatographed on paper in the t-butanol:formic acid:water system. The autoradiogram of this paper showed the material in peak I to be MVA, $R_f = 0.86$. The second peak (II) was not identified but Bloch (14) has reported an acid hydrolysis product of IPP to be eluted in this region of the chromatogram. The labeled material in peak III had an $R_f = 0.71$, and can be tentatively identified as phosphomevalonic acid. However, the $R_f$ of this material on this chromatogram was higher than that reported (Table 12). The labeled material in peak IV appeared as two main spots on the autoradiograph, $R_f = 0.32$ and $R_f = 0.71$. The more polar compound was tentatively identified as pyrophosphomevalonic acid; and the other compound as isopentenyl pyrophosphate. The $R_f$ of isopentenyl pyrophosphate was identical to the $R_f$ of phosphomevalonic acid on this chromatogram; it has been reported that the two compounds cannot be separated in this solvent system (14). The two compounds could therefore be identified by their similar behavior on the paper chromatogram and by their elution on the ion exchange column. Also detected, but not identified, in the labeled material in peak IV was a faint spot with a $R_f = 0.57$.

The results of this series of experiments showed that mevalonic kinase, phosphomevalonic kinase, and anhydrodecarboxylase were present in the 60,000 x g supernatant. While these experiments have
been only a qualitative demonstration of the activity of these three enzymes, it is important to note that the 60,000 x g supernatant is utilizing MVA by pathways previously established in mammalian and yeast systems. The fact that phosphomevalonic acid, pyrophosphomevalonic acid, and isopentenyl pyrophosphate were all formed by the 60,000 x g supernatant in the presence of ATP indicated that MVA is being used for terpene biosynthesis.

**Isolation of the Sterol Digitonide from the NSF**

The sterol fraction of the NSF was isolated as the digitonide in a number of experiments, using the procedure described in the methods section. In control experiments it was shown that even when no "cold" carrier cholesterol was added to the NSF, the digitonin precipitable sterol present in the NSF could be isolated by the same procedure. This fraction contained 18 to 22 gms of cholesterol per ml of the 60,000 x g supernatant, (as analyzed by the Liebermann-Burchard reaction) depending upon the preparation of aortas used.

In one experiment to determine if MVA-2-C\(^{14}\) was being incorporated into the sterol fraction during the incubation period, the digitonide of the NSF of an incubation mixture (containing 5.0 ml 60,000 x g supernatant, 0.05 µc MVA-2-C\(^{14}\), 2.5 ml 0.1 M phosphate nicotinamide buffer, 16.5 µmoles ATP, and 6.9 µmoles NAD) was isolated and counted. The digitonide contained 25 counts per min: the
60,000 x g supernatant used in the incubation contained 21 µgm cholesterol per ml. The specific activity of the digitonide was 237 counts per min per mg of cholesterol. The washings and supernatant of the digitonide were not counted, but the NSF of a similar incubation mixture contained 2302 counts per min.

Similar results were obtained when "cold" carrier cholesterol (one mg) was added to the NSF of an incubation mixture (similar to that described above but from another incubation study) and the digitonide of carrier plus NSF isolated. The NSF of the 60,000 x g supernatant used contained 20 µgm cholesterol per ml of supernatant. The washings and supernatant from the digitonide were combined and taken to dryness on a steam bath, dissolved in methanol and ether, and counted in scintillation fluid. The digitonide was counted by the same procedure; for comparison, the NSF of an identically incubated sample was also counted. The results are summarized in Table 13. The results show that adding a ten-fold molar excess of "cold" carrier cholesterol did not increase the amount of label in the digitonide. From the results cited above and the results in Table 13, it would appear that the major portion of the radioactivity in the NSF (when MVA-2-C¹⁴ is used as a precursor in the incubation mixture) was not precipitated with digitonin under the conditions used. The range in the specific activities of the two digitonides (237 counts per min per mg cholesterol and 60 counts per min per mg cholesterol) may be due
to the different activities of the 60,000 x g supernatants used in the
two incubations; the two incubations used supernatants obtained from
two batches of aortas. However, during the course of this investiga-
tion, digitonides of low or below background activity has been isolat-
ed from nearly every batch of aortas prepared and used in similar
incubation mixtures, and at no time was the greater amount of radio-
activity incorporated into the NSF found in the sterol digitonide frac-
tion.

Table 13. Assay of radioactivity in the
digonide and the NSF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Counts per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digitonide of the NSF</td>
<td>61</td>
</tr>
<tr>
<td>Washings and supernatant</td>
<td>662</td>
</tr>
<tr>
<td>NSF (Identical sample)</td>
<td>668</td>
</tr>
</tbody>
</table>

1 Calculated on the basis of endogenous choles-
terol in the NSF, the specific activity of this
digonide is 60 counts per min per mg cho-
lesterol.

In another experiment the sterol digitonide was isolated from
the NSF of incubation mixtures incubated for different time periods.
The results are summarized in Table 14. Duplicate determinations
were carried out for each time period, and the isolated digitonide
counted by the pyridine method (5). The data in the table shows there
is little variation in the amount of label in the digitonide over the
three hour incubation period, even though there is an increase in the
amount of label in the NSF. The results also show that during the usual time period (two hours and fifty minutes) allowed for incubating the 60,000 x g supernatant in incubation mixtures, no digitonin precipitable sterol is formed.

Table 14. Isolation of the sterol digitonide from the NSF of incubation mixtures incubated for different time periods.

<table>
<thead>
<tr>
<th>Time incubated minutes</th>
<th>Counts per min in digitonide Flask 1</th>
<th>Counts per min in digitonide Flask 2</th>
<th>Counts per min in digitonide supernatant Flask 1</th>
<th>Counts per min in digitonide supernatant Flask 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>3</td>
<td>56</td>
<td>39</td>
</tr>
<tr>
<td>80</td>
<td>5</td>
<td>4</td>
<td>120</td>
<td>108</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>2</td>
<td>248</td>
<td>219</td>
</tr>
<tr>
<td>120</td>
<td>4</td>
<td>5</td>
<td>208</td>
<td>219</td>
</tr>
<tr>
<td>140</td>
<td>6</td>
<td>3</td>
<td>775</td>
<td>607</td>
</tr>
<tr>
<td>160</td>
<td>3</td>
<td>5</td>
<td>156</td>
<td>264</td>
</tr>
<tr>
<td>180</td>
<td>18</td>
<td>13</td>
<td>1050</td>
<td>1142</td>
</tr>
</tbody>
</table>

1 Each incubation mixture contained 24.7 µmoles ATP, 6.9 µmoles NAD, 0.05 µc MVA-2-C\textsuperscript{14}, 5.0 ml 60,000 x g supernatant, and 2.5 ml 0.1 phosphate nicotinamide buffer in a total volume of 10.5 ml. Duplicates were run for each time period.

2 Only one supernatant was counted.

In other experiments, various coenzymes were added to the incubation mixture to determine if such addition would be responsible for the incorporation of MVA-2-C\textsuperscript{14} into the sterol digitonide. When NADPH (1.0 µmole) or NADH (6.5 µmole) or the combination of these two coenzymes were included in the incubation mixture (which also contained 5.0 ml 60,000 x g supernatant, 16.5 µmoles ATP, 6.9 µmoles NAD, and 2.5 ml 0.1 M phosphate nicotinamide buffer) the
isolated sterol digitonide of the NSF's of all the incubation mixtures were below background. The same results occurred when only ATP and ATP plus NAD were the only coenzymes in the incubation mixture. Thus it appears that at the concentrations tested the coenzymes added to the incubation mixture did not influence the incorporation of MVA into the sterol digitonide.

**Silica Gel Chromatography of the 60,000 x g Supernatant**

To further characterize the labeled material in the NSF, silica gel column chromatography was used as described in the methods section, except only three petroleum ether fractions were collected. The combined NSF of two incubation mixtures (each containing 5.0 ml 60,000 x g supernatant, 16.5 µmoles ATP, 13.9 µmoles NAD, 0.05 µc MVA-2-C\(^{14}\), and 2.5 ml 0.1 M phosphate nicotinamide buffer in a total volume of 10.5 ml) was dissolved in petroleum ether and used as the sample for chromatography; the combined NSF contained approximately 4500 counts per min. The fractions were counted in the scintillation counter; three activity peaks were obtained, as shown in Table 15 and Figure 13. The radioactivity in the first peak may be due to squalene; the major portion of the radioactivity was eluted in the same fractions as cholesterol. The label in the third peak was not identified. Compared to chromatograms with standard cholesterol as the sample, the results of this chromatogram indicate that the
Figure 13. Silicic acid chromatography of the NSF.
labeled material in the NSF behaves chromatographically like cholesterol.

Table 15. Silica gel chromatography of the NSF isolated from the 60,000 x g supernatant.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Ml of eluent</th>
<th>Total counts per min</th>
<th>Percent of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>6</td>
<td>101</td>
<td>2.2</td>
</tr>
<tr>
<td>50 percent chloroform</td>
<td>8</td>
<td>4036</td>
<td>89.7</td>
</tr>
<tr>
<td>50 percent ethyl acetate</td>
<td>8</td>
<td>296</td>
<td>6.6</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>2</td>
<td>3</td>
<td>0.07</td>
</tr>
<tr>
<td>Methanol</td>
<td>2</td>
<td>6</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Thin layer chromatography and autoradiography were used to further characterize the nature of the radioactivity in the NSF. The fractions from a micro column assay were used as samples for thin layer chromatography. Column chromatography was carried out as before; the labeled material used as the sample consisted of the combined NSF (about 4500 counts per min) of three incubation mixtures (similar to those used in the previous chromatogram). The fractions were spotted on thin layer plates.

The thin layer plates were developed in benzene:ethyl acetate, two:one. After the solvent was removed by air drying, the plates were exposed to X-ray film for two weeks. Several radioactive spots were detected; $R_f$ values are given as calculated from the thin layer plates. The film results are summarized in Table 16.
Table 16. Results of autoradiography of fractions from micro-column chromatography of the NSF.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Solvent</th>
<th>Autoradiograph R_f</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>petroleum ether</td>
<td>0.84</td>
</tr>
<tr>
<td>5</td>
<td>petroleum ether:chloroform</td>
<td>0.82</td>
</tr>
<tr>
<td>6</td>
<td>petroleum ether:chloroform</td>
<td>0.82</td>
</tr>
<tr>
<td>7</td>
<td>petroleum ether:chloroform</td>
<td>0.82, 0.62</td>
</tr>
<tr>
<td>8</td>
<td>petroleum ether:chloroform</td>
<td>origin</td>
</tr>
<tr>
<td>9</td>
<td>petroleum ether:ethyl acetate</td>
<td>streak from origin</td>
</tr>
</tbody>
</table>

The labeled material in fraction four through seven ($R_f = 0.84$ to 0.82) may be squalene, which has an $R_f = 0.83$ in this solvent system. However, squalene would be expected to also appear in the first petroleum ether fraction, which, on this chromatogram, did not contain any labeled material. These nonpolar labeled spots were faint; the second spot in fraction seven ($R_f = 0.62$) appeared to contain most of the radioactivity on the film, as estimated from the density of the darkened film.

The plates were sprayed with the sulfuric acid spray; cholesterol ($R_f = 0.55$) appeared in fractions seven, eight and nine. Comparison of the spots on the plate and the spots on the film showed no label coincident with cholesterol, and that no spot was detected that coincides with the highly labeled spot in fraction seven. The labeled material in fractions eight and nine was probably due to decomposition of the labeled material.

For comparison of the labeled NSF with a sample of labeled
cholesterol, micro column chromatography of cholesterol-4-C\textsuperscript{14} followed by thin layer chromatography of the fractions was performed. The labeled cholesterol, 0.05 µc, was diluted with 100 µgm of "cold" cholesterol before applying it to the column. Autoradiography of the thin layer plates showed that cholesterol (R\textsubscript{f} = 0.57) appeared in the last three petroleum ether:chloroform fractions and the first petroleum ether:ethyl acetate fraction. When the plates were sprayed with the sulfuric acid reagent and charred, cholesterol was detected in the last two petroleum ether:chloroform fractions and the first petroleum ether:ethyl acetate fraction, and were coincident with the cholesterol spots on the film in those fractions.

Comparison of the two autoradiographs and the thin layer chromatograms from both column assays showed that cholesterol was eluted with the petroleum ether:chloroform solvent. The labeled material in the NSF was eluted with cholesterol, but cholesterol migrates below the labeled material on the thin layer plate. These results indicate that the labeled material in the NSF was not cholesterol, but it is very similar to cholesterol in its chromatographic behavior; the labeled material also appears in amounts below the level of detection when sulfuric acid is used as the detecting reagent on the thin layer plates.
Silicic Acid-celite Chromatography of NSF

Figure 14 shows the results of silicic acid-celite chromatography of labeled cholesterol added to a "cold" NSF. The sample was an unlabeled NSF of four large incubation mixtures (no MVA-2-C$^{14}$ was added). After the incubation period, 0.2 µc of cholesterol-4-C$^{14}$ (58 mc per mmole) was added to each incubation mixture, followed by 100 ml of alcoholic KOH. Saponification and extraction were carried out as usual, and the NSF's combined. The recovery of the labeled cholesterol from the unlabeled incubation mixture was 98 to 100 percent.

The figure shows the total counts per min and total Liebermann-Burchard color (in optical density units) for each fraction. The recovery of labeled cholesterol from the column was 96 percent; the elution volume of the cholesterol peak was 450 ml. In addition to the cholesterol peak, a large amount of "cold" material was eluted in fractions 40 to 80 which did not give any color in the Liebermann-Burchard reaction. The material appeared to be in excess of the cholesterol present, but was not identified. Initial studies with this method showed that a small amount of material giving a yellow color with the Liebermann-Burchard reagent is eluted early in the chromatogram (see below). Otherwise, cholesterol appears to be the only Liebermann-Burchard positive sterol in the NSF.
Figure 14. Silicic acid-celite chromatography of unlabeled NSF and cholesterol-4-C14.
Figure 15 shows the results of silicic acid-celite chromatography of the labeled NSF. The sample was the combined NSF of four large incubation mixtures similar to those above, except 0.50 µc MVA-2-C$^{14}$ was added to each flask, and no labeled cholesterol was added at the end of the incubation period. Chromatography was carried out as before, and the fractions analyzed and plotted in the same manner. Eighty one percent of the counts per min applied to the column were recovered in the fractions.

Two labeled peaks were obtained, the first peak (fractions 29 to 35) small in comparison to the second peak. In the Liebermann-Burchard reaction the fractions in this peak gave a yellow color; lanosterol also gives a yellow color with the Liebermann-Burchard reagent. The elution volume of this peak is comparable to the elution volume for lanosterol (25). However, no further identification of this material was made but the peak probably does represent a second biosynthetic product. Only these two sections of the chromatogram gave positive results with the Liebermann-Burchard reagent.

The second peak is only slightly less polar than cholesterol. The elution of the radioactivity and the elution of cholesterol did not give a "coincident" peak, as was obtained in the chromatogram in Figure 14. Instead, the peaks overlapped each other, the labeled peak traveling slightly ahead of the cholesterol peak. This behavior of the labeled material again indicated that the labeled material was not
Figure 15. Silicic acid-celite chromatography of labeled NSF.
cholesterol. The elution volume of cholesterol on this column was 420 ml.

In another experiment, labeled NSF from four large incubation mixtures was combined with 0.8 µc cholesterol-4-C\textsuperscript{14} \footnote{This is likely a reference to a specific isotope.} and the sample chromatographed on a similar silicic acid-celite column. The first small peak was again detected, but no separation (similar to Figure 15) of the biosynthetically labeled product and the labeled cholesterol occurred; only the single large cholesterol peak was obtained. The fractions in the "cholesterol" peak were pooled and further investigated on thin layer plates and autoradiography.

Three portions of the pooled labeled fractions were spotted on a thin layer plate. The three peak samples contained approximately 40, 80, and 200 µgm cholesterol. For comparison, on the same plate, labeled cholesterol standards were also run, containing 80 to 640 µgm of labeled cholesterol (150 counts per min per µgm cholesterol). A sample (0.01 µc) of the labeled cholesterol added to the NSF for this column was also run to check the purity of the sample. The plate was developed in benzene:ethyl acetate, two:one. The autoradiograph of the thin layer plate (Figure 16) showed two labeled spots present in the first two peak samples; the size of the sample increases from left to right. In the 40 and 80 µgm samples the \( R_f \)'s of the two spots were 0.48 and 0.57; in the 200 µgm sample, separation was not complete. The slower moving spot in each peak sample
was identified as cholesterol by comparison with the standard cholesterol sample. The second, less polar spot in each sample was identified as the labeled biosynthetic product of the incubation. Figure 17 shows that the labeled cholesterol used in these studies appeared to be nearly chromatographically pure, $R_f = 0.48$.

After the film was developed, the plate was sprayed with Rhodamine B; only the cholesterol in the peak samples and in the standard samples could be detected. The cholesterol spots on the plate were coincident with the cholesterol spots on the film. The second, biosynthetically labeled spot could not be detected with the spray.

The results of the silicic acid-celite chromatography of the NSF indicated that the labeled product in the NSF is very similar to cholesterol in its chromatographic behavior. It also may be concluded that this material occurs in very small quantities in the NSF. However, separation of the labeled product from cholesterol did occur on the thin layer chromatograms.

Silver Nitrate Chromatography of the NSF

The combined NSF from four large incubations was chromatographed on a 375 μ layer of Silica Gel H which had been impregnated with silver nitrate. The adsorbent layer was prepared from 60 gm of silica Gel H and 120 ml of 12.5 percent silver nitrate solution, according to the method of Ditullio et al. (40). The sample was applied
Figure 16. Autoradiograph of thin layer chromatography of sample of pooled labeled peak from silicic acid-celite chromatography.

Figure 17. Autoradiograph of thin layer chromatography of labeled cholesterol.
to the plate, along with cholesterol-4-C\textsuperscript{14} (60 \, \mu gm, 253 counts per min per \mu gm), farnesol (100 \, \mu gm), and squalene (100 \, \mu gm) as standards. The plate was developed in chloroform:acetone, 95:5. The autoradiograph of the plate showed that all the labeled material in the NSF remained at the origin. The cholesterol band was located about 10 cm above the origin. When the plate was sprayed with Rhodamine B, a third band was located near the solvent front, but was not labeled. Squalene and farnesol remained at the origin.

The results of this experiment indicated that the labeled product is an unsaturated material, similar in chromatographic behavior in this system to farnesol with three double bonds and squalene with six double bonds. In a previous experiment with a small silver nitrate plate prepared with the same adsorbent as the preparative plate showed that cholesterol could be separated from desmosterol, 7-dehydrocholesterol, cholestanol, dihydrolanosterol and lanosterol. None of these sterols remained at the origin when developed in the chloroform:acetone system. The result indicated that the labeled material did not contain any of these naturally occurring sterols, unless they were of such low activity not to be detected by the film. However, since the labeled material did not migrate at all on the plate, it was difficult to conclude that the labeled material is either radiochemically or chemically homogeneous.
Liebermann-Burchard Analysis of the Labeled Material

To obtain enough of the labeled product for analysis by the Liebermann-Burchard reaction, the combined NSF from six large incubations was chromatographed on a 750 µ preparative plate in benzene:ethyl acetate, five:one.

The labeled material was located by autoradiography. The labeled band was scraped off, eluted, and the solvent removed on a Büchi evaporator to leave a faintly yellow solid residue on the bottom of the flask. For comparison, an equivalent area of silica gel was scraped off a "blank" plate; this plate had no sample, but was developed in the same solvent system and was prepared at the same time as the "sample" plate. The blank silica gel was also eluted with chloroform and the solvent removed as above.

Two-thirds of each sample (blank and labeled band) were used separately for Liebermann-Burchard analysis. The labeled band sample (in 1.6 ml chloroform) contained approximately 24,000 counts per min; this solution was mixed with 0.8 ml of Liebermann-Burchard reagent and readings were taken at 620 mµ over a 30 minute time period. The reference cell contained 1.6 ml chloroform and 0.8 ml Liebermann-Burchard reagent. There was no development of the characteristic blue-green color; the sample turned pale yellow in the first five minutes and remained that color for the rest of the time.
period. The blank band was analyzed by the same procedure and
gave readings (over the 30 minute time period) that were similar to
those from the sample band. If only the MVA added to the incubation
mixture were used for biosynthesis (no endogenous MVA was avail-
able) then the specific activity of the product would be the same as
the precursor, or about 400 counts per min per µgm. Thus the 24,000
counts per min in the labeled band used for Liebermann-Burchard
analysis represents at least 60 µgm of material. Under the same
conditions that the Liebermann-Burchard analysis was performed,
60 µgm of cholesterol would have an absorbance of 0.12 at 620 mµ.

Gas Chromatography of the Labeled Material

Prior to running the infrared spectrum (see below) of the la-
beled band, an aliquot of the sample was used for gas chromato-
graphy. Previous chromatography of the sample on a 3.8 percent SE-30
column on Diatoport S (the prepacked columns were obtained from the
F & M Scientific Corporation), in a Model 402 F & M Gas Chromato-
graph, showed all the label in the sample was eluted with the solvent
front, at a column temperature of 210°.

Chromatography of the labeled band was then performed on a
column (4' by 1/4") of 1.25 percent diethylene glycol adipate polyester
(Wilkens Instrument Company) on Chromosorb G (80/100 mesh,
High Performance Grade, from Johns-Manville Corporation). At a
column temperature of 180°, farnesol has a retention time of less than four minutes (sample impure); squalene, 24.8 minutes. At 190° the retention time of farnesol was not determined but would be expected to be eluted almost with the solvent front; the retention time for squalene was 15.6 minutes, cholestane 13.5 minutes. The samples were dissolved in hexane and 5 µgm of each standard was used.

When a sample of the labeled band (containing 550 counts per min) was chromatographed at 190°, two main peaks were eluted, with retention times of 8.5 minutes and 21 minutes. To assay for radioactivity in the same sample, the stream splitter on the instrument was opened. The split ratio had been previously determined to be 4.8:1, the ratio of the effluent split between the auxiliary port and the detector. After injection of the labeled sample (2750 counts per min), collections of the effluent were made for three minute periods with Kontes glass traps. The traps were rinsed with toluene into scintillation vials and the excess solvent removed with an air stream before adding scintillation fluid. The chromatogram obtained was similar to the one run above, except retention times for the peaks were about two minutes longer, and a group of peaks was eluted almost with the solvent front. The chromatogram is reproduced in Figure 18; Table 17 shows the radioactivity eluted in each fraction.

In the fourth and fifth fractions of the chromatogram, corresponding to the elution of the first main peak, 57 percent of the
Figure 18. Gas chromatography of the labeled material.
injected radioactivity, calculated from the split ratio, was recovered.

Table 17. Radioactivity in fractions from gas chromatography of the labeled material.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Counts per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>878</td>
</tr>
<tr>
<td>5</td>
<td>494</td>
</tr>
<tr>
<td>6</td>
<td>72</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
</tr>
<tr>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
</tr>
</tbody>
</table>

With labeled cholesterol as a sample, the recovery of radioactivity by this method was about 62 percent of the calculated value. The results of this chromatogram indicated that the labeled material in the NSF is neither farnesol nor squalene, and was present in one peak eluted from the column. The labeled peak was accompanied by other nonradioactive compounds, which were not identified.

The fact that the labeled material was eluted off the SE-30 column at $210^\circ$ with the solvent front excluded the possibility that the radioactivity was present in sterols such as cholesterol, desomsterol, lanosterol, $\Delta^7$-cholesteno1, and 7-dehydrocholesterol; or in cholest-4-en-3-one, and 4,6-cholestadien-3-one. All these compounds were found to have retention times greater than 15 minutes on
Infrared Spectrum of the Labeled Material

The labeled material remaining after gas chromatographic analysis was used as a sample for an infrared spectrum. The spectra were obtained on a Perkin-Elmer Model 21 Infrared Spectrophotometer, using a micro cell. The sample was dissolved in 15 μl of spectrograde solvent; both carbon disulfide and carbon tetrachloride were used. Figure 19 shows the spectrum of the labeled band in carbon tetrachloride. Strong absorption bands were present at 2940-2820 cm\(^{-1}\), 1695 cm\(^{-1}\), and 1510 cm\(^{-1}\). Less intense bands were present at 1450-1425 cm\(^{-1}\), 1375 cm\(^{-1}\), 1275 cm\(^{-1}\) and 936 cm\(^{-1}\). Figure 20 shows the spectrum in carbon disulfide; two strong bands are seen at 2895 cm\(^{-1}\) and 2825 cm\(^{-1}\). There is also a strong absorption band at 1700 cm\(^{-1}\), and less intense bands are present in at 1275 cm\(^{-1}\) and 930 cm\(^{-1}\).

A blank sample was obtained by treating an equivalent amount of purified silicic acid from a blank plate in the same manner as the labeled band sample in the last chromatographic step. The spectrum of this sample in carbon tetrachloride showed adsorption bands at 2880 cm\(^{-1}\) and 2820 cm\(^{-1}\) of medium intensity and less intense bands at 1710 cm\(^{-1}\) and 1450 cm\(^{-1}\). The absorption bands of the labeled sample at coincident points, however, were more intense and appeared
Figure 19. Infrared spectrum of the labeled material. Solvent: carbon tetrachloride.
Figure 20. Infrared spectrum of the labeled material. Solvent: carbon disulfide.
to be significant.

The absorption in the 2940-2820 cm\(^{-1}\) region in both spectra is probably due to C-H stretching vibrations. The absorption is very strong and broad in the carbon tetrachloride spectrum, while the carbon disulfide spectrum shows two bands in this region. The difference in the two spectra is probably due to loss of compound while handling the sample. However, the absorption bands in both spectra in this region was indicative that -CH\(_3\), -CH\(_2\), and C-H groups are present in the sample. This is reflected in the absorption in the 1450-1420 cm\(^{-1}\) region, which also could have been due to a =CH\(_2\) group. The presence of a -CH\(_3\) group was indicated by the absorption at 1375 cm\(^{-1}\) in the carbon tetrachloride spectrum.

The strong absorption at 1700 cm\(^{-1}\) indicated the presence of a carbonyl group in the sample. Absorption in this region may also be due (although this frequency is high) to the presence of nonconjugated double bonds, or the presence of a C = C-O-function.

The results of the gas chromatography of the labeled band showed that this sample is not chemically homogeneous, and further interpretation of the infrared spectrum of the biosynthetic product should be performed when purer samples are available.
Mass Spectrum of the Labeled Material

The mass spectrum of the labeled band was obtained from the Morgan Schaffer Corporation. The results and interpretation of the spectrum of the labeled band indicated that the sample contained more than one compound. The main component observed had a m/e at 386 and gave a spectrum almost identical to cholesterol. However, in addition to cholesterol, compounds were present at m/e = 396, 398, 400, and 402, accompanied by m/e = 384, 382, and 380, which could indicate a loss of water and these latter peaks are fragment ions.

Another spectrum of the sample indicated that the 380 to 384 group of peaks was unaccompanied by the higher m/e values, suggesting that the 380 to 384 group of peaks may be due to separate compounds.

The presence of cholesterol-like compounds in the labeled band was also indicative that the chromatographic procedures used for isolation were not adequate, since there appeared to be some cross-contamination by the cholesterol band and the labeled band. However, this contamination was not significant enough to invalidate the results of the Liebermann-Burchard analysis, since no Liebermann-Burchard color was obtained at all in the labeled band sample.

Digitonide and 2, 4-dinitrophenylhydrazone Formation

Another sample of the labeled material was isolated from the
combined NSF of six large incubations by preparative layer chromatography in petroleum ether:diethyl ether:acetic acid. Twenty-five percent of this material was used in digitonide precipitation. The material was transferred to a centrifuge tube and the digitonide precipitation procedure performed as described. Only a very small amount of precipitate appeared in the tube, apparently due to inadequate separation of the cholesterol band from the labeled band. As standards, six tubes, each containing one mg of labeled cholesterol, were also included in the experiment. The pyridine method of counting was used (5); recovery of the labeled cholesterol was about 94 percent. The digitonide of the labeled band contained six counts per min. The supernatant and washings solution from this sample was taken to dryness and used for a sample in the next experiment.

To test for the presence of a carbonyl group (as indicated in the infrared spectrum) in the labeled material, the formation of a 2,4-dinitrophenylhydrazone by this sample was attempted. Because such 2,4-dinitrophenylhydrazones are yellow in color, and therefore quench counts in the scintillation counter, a quench correction curve was prepared (99) using the 2,4-dinitrophenylhydrazone of cholest-4-en-3-one as the color additive. By using the automatic external standard on the Packard 3003 scintillation counter, the counting efficiency for C\textsuperscript{14} could be determined when the colored samples were counted.
For a ketone standard, 0.045 µc of cholestenone-4-C\textsuperscript{14} (from New England Nuclear Corporation) was diluted with 250 µgm of "cold" cholestenone to a final specific activity of 0.0045 µc per 25 gm; a 25 µgm sample contained 9400 counts per min. Twenty-five µgm samples of this materials were used to check the recovery in counts per min in the formation of the 2,4-dinitrophenylhydrazones. Aliquots of the labeled band (corresponding to eight and 12 µgm of material) were used, as well as the supernatant from the digitonide precipitation (containing about 25 µgm of material).

The method of Shriner et al. (102, p. 219) for the formation of 2,4-dinitrophenylhydrazone derivatives of aldehydes and ketones was used, reduced to a micro scale. Each sample (six 25 µgm samples of the cholestenone-4-C\textsuperscript{14} standard and the three samples of the labeled material) was dissolved in 30 µl of 95 percent ethanol and mixed with ten µl of 2,4-dinitrophenylhydrazine solution (0.4 gm of 2,4-dinitrophenylhydrazine in a mixture of two ml sulfuric acid, three ml water, and ten ml 95 percent ethanol). An orange precipitate immediately formed in the cholestenone tubes, but no change occurred in the labeled material tubes. After standing overnight, the tubes were centrifuged at 10,000 rpm for ten minutes. The supernatant from each tube was removed with a micro syringe and transferred to scintillation vials. In the labeled material tubes this meant removing the entire contents of each tube. All of these samples were the
"supernatant" samples (Table 18). The residue in each tube was then dissolved in scintillation fluid; these were the "residue" samples. The results are summarized in Table 18. The values for counts per min have been corrected for color quench. The data show that the labeled material does not appear to form a 2,4-dinitrophenylhydrazone derivative under the conditions that will quantitatively form this derivative of cholestenone.

Table 18. 2,4-dinitrophenylhydrazone formation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Supernatant Counts per min</th>
<th>Residue Counts per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 µgm labeled material</td>
<td>5100</td>
<td>147</td>
</tr>
<tr>
<td>8 µgm labeled material</td>
<td>3200</td>
<td>196</td>
</tr>
<tr>
<td>Digitonide supernatant (25 µgm)</td>
<td>10800</td>
<td>230</td>
</tr>
<tr>
<td>Cholestenone-4-C¹⁴ (25 µgm)¹</td>
<td>206</td>
<td>9300</td>
</tr>
</tbody>
</table>

¹ Average of six determinations.
DISCUSSION

The biosynthesis of sterols is a complex process involving many steps. The presence of sterol-synthesizing systems in the arterial wall has been demonstrated by Siperstein, Srere and Chaikoff (103) and Eisley and Pritham (45), but the synthesis of sterols by this organ takes place at a low rate compared to the mammalian tissues. Terner and Darey (114) investigated lipid biosynthesis by a cell-free preparation obtained from turtle aorta, but they were not able to demonstrate either acetate or mevalonate incorporation into sterols.

The present investigation was undertaken to study the biosynthetic capacity of a cell-free system prepared from the bovine aorta. The aortas were obtained from mature cattle; this fact may be of some importance since tissue obtained from mature animals is metabolically less active than that obtained from immature animals. Werthesseen et al. (118, 119) demonstrated that the intact calf aorta was capable of sterol biosynthesis.

The cell-free preparation was obtained from a Waring Blender homogenate of aorta and 0.1 M phosphate nicotinamide buffer. The use of a Waring Blender in the procedure was rather severe treatment of the tissue, but gentler methods of homogenization were not feasible. The crude homogenate, obtained from the blender preparation, was centrifuged at 60,000 x g to obtain the cell-free system. This supernatant incorporated MVA-2-C^{14} into the NSF. Similar 60,000 x g supernatants, which were prepared by the same procedure
with either distilled H₂O or 0.25 M sucrose as the homogenizing medium, in place of the 0.1 M phosphate nicotinamide buffer, were inactive. The 0.1 M phosphate nicotinamide buffer used as the homogenizing medium has also been employed by other investigators (19) to prepare cell-free, sterol-synthesizing systems from other tissues.

The coenzyme requirements of the 60,000 x g supernatant for incorporation of MVA into the NSF were determined by the amount of radioactivity incorporated into the NSF when the coenzyme was present in the incubation mixture. The data show that in the presence of ATP, NAD, coenzyme A, GSH, glucose-6-phosphate plus NADP, and antibiotics, acetate-2-C₁⁴ was not incorporated into the NSF. This failure may be due to inadequate amounts of coenzyme added to the incubation mixture, but further experiments are needed to verify this possibility. It is more likely that the lack of incorporation is due to the absence or inactivity of proper enzymes for the formation of MVA, since MVA was utilized by the same supernatant in the presence of ATP and NAD. This defect was not investigated further, but it would be of interest to determine where the blockage of synthesis is located.

The coenzyme requirements for incorporation of MVA into the NSF were also investigated; of all the coenzymes tested, only ATP was absolutely required for incorporation. This requirement for ATP was not unexpected, since other cell-free sterol-synthesizing systems (21, 121) also require ATP. When NAD, NADP, NADH, or NADPH were added alone or in combination with each other to the
incubation mixture, there was little or no incorporation into the NSF. These compounds did not permit the system to incorporate MVA into the NSF without ATP in the incubation mixture; the amount of label incorporated with ATP and the pyridine nucleotides was not increased over the amount incorporated when ATP alone was in the incubation mixture. However, NADH in combination with ATP decreased the amount of MVA incorporated into the NSF. The depressed incorporation due to NADH is difficult to interpret, since a reduced pyridine nucleotide (preferably NADPH) is required for squalene formation (63) and the conversion of lanosterol to cholesterol (37) in rat liver homogenates.

ATP was utilized for the formation of phosphorylated derivatives of MVA. The 60,000 x g supernatant formed phosphomevalonic acid, mevalonic acid pyrophosphate, and isopentenyl pyrophosphate.

The isolation of a labeled sterol digitonide from the NSF was attempted in a number of experiments; whether carrier was added or not the amount of label in the isolated digitonide was less than five percent of the total label in the NSF. The specific activities of the labeled digitonides (60 to 237 counts per min per mg cholesterol) were comparable to those values obtained by other investigators. Siperstein, Srere and Chaikoff (103) reported a specific activity of 15 to 420 counts per min per mg cholesterol in the digitonides isolated from aortas of chickens and rabbits. Werthessen et al. (118) isolated sterol digitonides from perfused calf aorta with a specific activity of 32 to 8500 counts per min per mg cholesterol. The 60,000 x g
supernatant appears to be capable of a very low amount of incorporation of MVA into the sterol digitonide, but the major amount of the incorporated label in the NSF was not digitonin precipitable. The presence of oxidized and reduced pyridine nucleotides in the indubation mixture did not stimulate incorporation of MVA into the sterol fraction. The defect responsible for the low incorporation into the sterol digitonide was apparently not due to the lack of a coenzyme usually required for sterol synthesis. A sample of the labeled material purified by preparative layer chromatography did not form a labeled digitonide.

Both silica gel and silicic acid-celite chromatography were used to characterize the labeled product in the NSF. The labeled material is chromatographically very similar to cholesterol. Claytin, Frantz, and Nelson (25) have shown that most naturally occurring sterols in mammalian systems can be separated on silicic acid-celite columns; lanosterol and partially methylated sterols are eluted prior to cholesterol; sterols differing from cholesterol only in the placement of the nuclear double bond and the presence of a side chain double bond are eluted after cholesterol. This method showed cholesterol to be the major Liebermann-Burchard positive compound present in the NSF. On this type of column, two labeled peaks were obtained from the NSF; the first, minor peak was not identified but preliminary evidence indicated that this peak could have been due to labeled lanosterol. The elution of the second peak showed that the labeled component in this peak overlapped the cholesterol peak. Thin layer chromatography of the same peak from a similar column did
separate the biosynthetic product from cholesterol. The behavior of the labeled material on silica gel plates in three solvent systems indicated that there is one major biosynthetic product.

On silver nitrate impregnated preparative plates the labeled product(s) in the NSF behaved as an unsaturated material, similar to squalene and farnesol. In this method, the greater the number of double bonds a sample has, the greater is the attraction between the compound and the Ag⁺ in the adsorbent layer. This method has been widely used to separate compounds differing in only the number and/or placement of double bonds. Since separation of cholesterol and the labeled product was complete in this experiment, the labeled material appeared to be quite different from cholesterol in the amount of unsaturations present.

A sample of labeled material (about 60 µgm) did not form a color when reacted with the Liebermann-Burchard reagent, under the same conditions that 60 µgm of cholesterol would react to give a readable color. Since cholesterol is specifically identified at the wavelength used and is one of the least sensitive sterols in this reaction (26) the behavior of the labeled material indicated that this sample is insensitive to the Liebermann-Burchard reagent.

Gas chromatography of the labeled band demonstrated that the radioactivity is eluted in a single peak of the chromatogram; but the labeled peak was accompanied by other, nonradioactive peaks. The retention time of the labeled peak was intermediate between farnesol
and squalene; no comparable retention time was obtained for other sterols (they are not eluted off the column at the temperature used). The results of silver nitrate chromatography, the Liebermann-Burchard reaction, and gas chromatography of the labeled band indicated the labeled product could have been a "prenol" of some kind; but the results of these studies showed that the labeled product was not squalene, farnesol, or cholesterol. The gas chromatographic analysis also indicated that methods employed for isolation of the labeled material did not separate the material from other, nonradioactive compounds. These data are similar to those obtained by Terner and Darey (114), who reported the biosynthesis of an "unsaturated hydrocarbon fraction" by a cell-free system of turtle aorta.

The results of the infrared spectrum showed that the labeled material contained compound(s) with -CH\(_3\), -CH\(_2\), -CH, and possibly =CH\(_2\) or other carbon-carbon double bond functions. Other than the strong absorption of the carbonyl region, there was no indication of other oxygenated functional groups. The use of these spectra to characterize the nature of the labeled material is limited, owing to the possible impurity of the sample, and the small quantity of material that was used in obtaining the spectra.

Because the infrared spectrum indicated the presence of a carbonyl group, the formation of a labeled derivative, a 2,4-dinitrophenylhydrazone, from the labeled band was attempted; but no insoluble
derivative was formed.

The mass spectrum also showed that the labeled band was not pure, so a m/e value cannot be assigned to the labeled material. However, the components of the sample had m/e values comparable to cholesterol, thus excluding the possibility of farnesol being present in the material.
SUMMARY

A cell-free system (60,000 x g supernatant) was prepared from a Waring Blendor homogenate of bovine aorta in 0.1 M phosphate ni-
cotinamide buffer. The cell-free preparation incorporated MVA-2-
C$^{14}$ into the NSF.

ATP was required for incorporation of MVA in the NSF. No incorporation was obtained when NAD, NADP, NADPH, or NADH were added alone or in combination with each other, in the incubation mixture. These compounds, except NADH, neither stimulated nor depressed incorporation obtained when ATP alone was in the incubation mixture. When NADH was added in combination with ATP, the amount of label incorporation decreased, compared to the amount of incorporation obtained when ATP alone was present.

The 60,000 x g supernatant was capable of forming phosphoryl-
ated derivatives of MVA-2-C$^{14}$. The results of experiments demon-
strated that MVA was being metabolized by a route usually described for the formation of isoprenoid compounds.

The sterol digitonide isolated from the NSF contained only a small amount of the total label in the NSF.

Column chromatography showed that the major biosynthetic product was chromatographically very similar to cholesterol. On silicic acid-celite columns, a second biosynthetic product was
detected, but appeared in small quantity.

Since the labeled material, isolated from large samples of NSF, did not give a positive Liebermann-Burchard reaction and did not form a labeled digitonide, the product(s) does not have a cholesterol-like structure. The results of gas chromatographic analysis indicated that the labeled material was neither farnesol nor squalene, but all the radioactivity appeared in a single peak. Silver nitrate layer chromatography indicated the presence of unsaturated material in the labeled fraction of the NSF.


17. Brodie, Jonathon D., Gertrude W. Wasson and John W. Porter. The formation of malonyl-enzyme and its conversion to fatty acids and \( \beta \)-hydroxy-\( \beta \)-methylglutaryl coenzyme A. Biochemical and Biophysical Research Communications 12:27-33. 1963.


86. Popják, G. et al. Synthesis of \(1-\text{T}_2-2\text{-C}^{14}\) and of \(1-\text{D}_2-2\text{-C}^{14}\)-\text{trans-trans}-farnesyl pyrophosphate and their utilization in squalene synthesis. Biochemical and Biophysical Research Communications 4: 204-207. 1961.


114. Terner, Charles and Frances R. Darey. The incorporation of (2-C14) mevalonate and (1-C14) acetate into lipids of aorta and heart homogenates of the turtle (Pseudymis Sp.) Biochimica et Biophysica Acta 98:194-203. 1965.


