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Abstract approved: _____
Wilbert Gamble

Previous investigations of the biosynthesis of sterols by arterial tissue have been mainly carried out with intact aorta or aorta slices, but a few investigators have used an aorta homogenate. A cell-free aorta homogenate has been made in this laboratory which shows low but significant incorporation of mevalonic acid (MVA) into a nonsaponifiable fraction (NSF). Thus this study was undertaken to characterize further the cell-free preparation with respect to isoprenoid biosynthesis.

The 60,000 x g supernatant exhibited little acetyl-CoA-kinase activity. This activity appeared to be independent of exogenous acetate and CoA but did show some dependence upon added ATP. There is an inhibitor of acetyl-CoA-kinase in the 60,000 x g supernatant which is heat-stable and dialyzable.

At pH 7.0 there is nonspecific phosphomonoesterase activity

in the 60,000 x g supernatant which can be inhibited by BSA but not by NaF. In addition, at pH 7.0 there is inorganic pyrophosphatase activity and ATPase activity in the 60,000 x g supernatant which can be inhibited by NaF. Both NaF and BSA inhibit the incorporation of MVA-2-C^{14} into the NSF in the 60,000 x g supernatant.

L-phenylalanine at high concentrations was found to inhibit the incorporation of MVA-2-C^{14} into the NSF in the 60,000 x g supernatant. The inhibition by L-phenylalanine can be overcome by increasing the ATP concentration. It was found that at low concentrations L-phenylalanine and p-chlorophenoxyisobutyric acid stimulated the incorporation of MVA-2-C^{14} into the NSF in the 60,000 x g supernatant.

NaF, p-nitrophenylphosphate, and p-nitrophenol at low concentrations can stimulate the incorporation of MVA-2-C^{14} into the NSF in the 60,000 x g supernatant. Other anions such as sulfate, chloride, and acetate weakly stimulate the incorporation of MVA-2-C^{14} into the NSF. In addition, phosphate must be present during the homogenization in order to obtain an active 60,000 x g supernatant.

Rat liver homogenate converted the radioactive NSF (cholesterol-free) from the 60,000 x g supernatant from aorta to an acidic compound(s). An insignificant amount of aorta NSF was converted to other nonsaponifiable compounds by the rat liver homogenate, suggesting that the radioactive compounds of aorta NSF are not

precursors of cholesterol.

The results indicate that, while the bovine aorta is capable of forming phosphorylated derivatives of mevalonate as in the normal biosynthesis of cholesterol, the primary products formed are aliphatic, branched chain polyisoprenoid derivatives. The latter appear to be normal constituents of aorta, since it was possible to extract them from untreated aorta using standard methods.

Isoprenoids of Bovine Aorta: Isolation, Purification, and Biosynthesis

by

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DEDICATION

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ISOPRENOIDS OF BOVINE AORTA: ISOLATION, PURIFICATION, AND BIOSYNTHESIS

INTRODUCTION

The biosynthesis of sterols by arterial tissue has been the subject of much investigation (6, 46, 47, 51, 71, 77, 103, 113, 115, 120, 121, 122). Some investigations of arterial tissue have employed whole aorta (120, 121), others have used aorta slices (47, 51, 71, 77, 103, 122) while only a few have been performed with an aorta homogenate (113, 115). A cell-free preparation from bovine aorta has been made in this laboratory which shows low but significant incorporation of labeled mevalonic acid (MVA) into cholesterol (115) but the major product synthesized by the cell-free preparation appears to be a polar isoprenoid compound of at least 20 carbon atoms. Thus part of this study was concerned with the further characterization of the cell-free preparation with respect to isoprenoid biosynthesis.

In most of the investigations of sterol biosynthesis in arterial tissue, acetate-C^{14} was shown to be incorporated into sterols. Furthermore, a number of investigators (47, 51, 77, 103) have shown that acetate can be oxidized to CO_2 in arterial tissue. Both of these findings imply that the initial step in the incorporation of acetate into sterols must be its activation to acetyl-CoA. It has been observed (115) in an homogenate of bovine aorta that acetyl-CoA, but not

acetate, can be incorporated into nonsaponifiable material. Thus the question arises as to why acetate cannot be activated to acetyl-CoA in a cell-free preparation of bovine aorta. Therefore part of this study was an investigation to determine if the enzyme responsible for activating acetate to acetyl-CoA could be found in a cell-free preparation of bovine aorta.

The low level of incorporation of labeled MVA into cholesterol and the high level of incorporation of labeled MVA into the polar isoprenoid may be due in part to a high level of phosphatase or pyrophosphatase activity. Thus another part of this study was concerned with determining if there is any phosphatase activity in the cell-free preparation of bovine aorta.

Below is a survey of the work that has been done in establishing the pathway for the biosynthesis of cholesterol from acetate and of the investigations on the incorporation of acetate into sterols in arterial tissue, the purification and properties of acetyl-CoA-kinase, and the nature of phosphatase activity in arterial tissue.

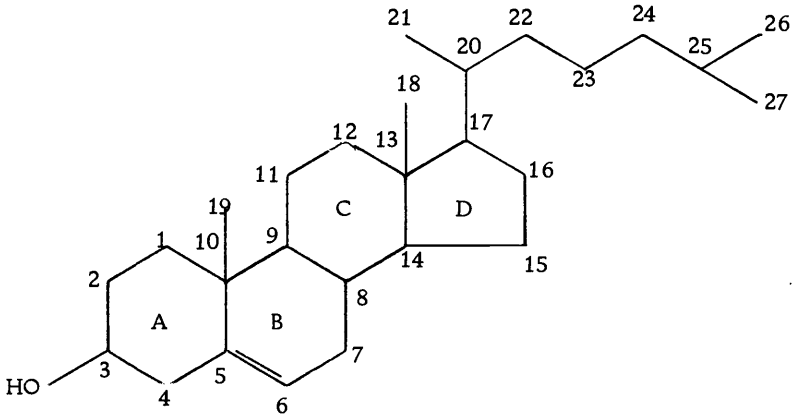
Early Studies on Cholesterol Biosynthesis

Several books (32, 50, 66) and many review articles (11, 12, 13, 29, 34, 85, 86, 127) have been written on the subject of cholesterol biosynthesis. It is now known that acetate is the sole carbon source of cholesterol. The number and origin of all the carbon atoms of

cholesterol are given in Figure 1. The entire pathway and most of the mechanisms of the individual reactions have been worked out in detail.

In 1925 (92) it was shown that rats were able to synthesize cholesterol. Rats were fed a diet free of cholesterol; yet it was found that there was a net increase in cholesterol in those rats. Therefore cholesterol had to be synthesized in vivo. Then in 1932 (83) it was shown that cholesterol was metabolized in a mammalian system. Using rabbits Page and Menschick found that large amounts of cholesterol were absorbed by the animals. The deposition of cholesterol occurred in all organs of the body with the exception of the brain. There is an equilibrium reached between the amount of cholesterol excreted and that which is stored. Schoenheimer and Breusch (99) confirmed these findings. Schoenheimer and Breusch found that mice could synthesize cholesterol. Up to this point it had been thought that fats contributed to cholesterol synthesis, but Schoenheimer and Breusch found that this was not true. They also found that cholesterol will only be metabolized when there are large amounts of cholesterol in the diet. Mice were found to synthesize large amounts of cholesterol only when it was not provided by the diet.

In 1937 Rittenberg and Schoenheimer (94) showed, using deuterium oxide (D_2O), that the hydrogens in cholesterol cannot be exchanged, but if mice were injected with D_2O, it was found that after



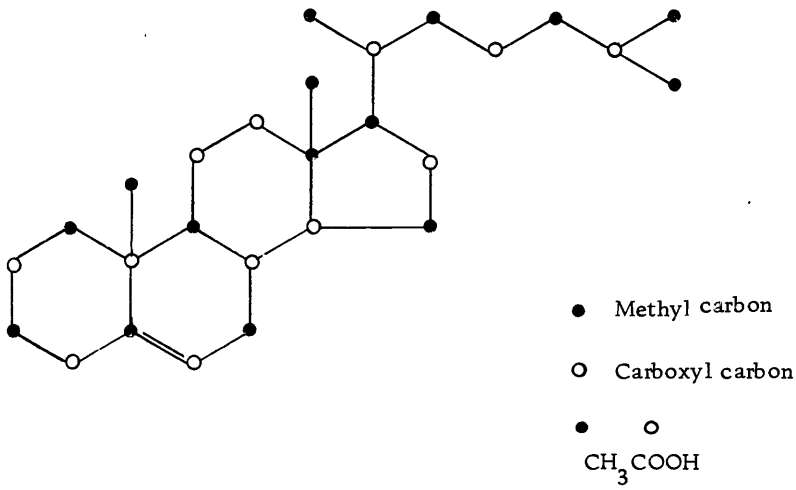


Figure 1. Numbering and origin of the carbon atoms in cholesterol.

98 days the cholesterol in the mice contained deuterium. Half of the hydrogens in the cholesterol were derived from the D_2O. The fact that the cholesterol contained deuterium shows that cholesterol is made up of smaller molecules. Then Bloch and Rittenberg (15, 16) demonstrated that if mice and rats were fed deuterio acetate, deuterio cholesterol was formed with deuterium in both the rings and side chain of cholesterol. A minimum of 13% of the hydrogen atoms of cholesterol were derived from acetate. In 1946 Bloch, Borek and Rittenberg (14), using slices of rat liver incubated aerobically in phosphate buffer in the presence of acetate containing deuterium in the methyl group and carbon-13 (C^{13}) in the carboxyl group (CH_2D-$C^{13}OOH$), showed that cholesterol contained both deuterium and C^{13}. Thus it was shown that the carbon source of cholesterol is acetate.

In 1950 Little and Bloch (70), using acetate-1-C^{14} and acetate-2-C^{14}, showed that the methyl group was the source of carbon atom 26 and carbon atom 27 and that carbon atom 25 was derived from the carboxyl carbon. Then Wüersch, Huang and Bloch (131) found that carbon atom 20 and carbon atom 23 were derived from the carboxyl carbon of acetate while carbon atom 21, carbon atom 22, and carbon atom 24 were derived from the methyl carbon of acetate. Carbon atom 7 was found by Dauben and Takemura (41) to be derived from the methyl group. Cornforth, Hunter and Popják (36) in 1953,

using acetate-1-C^{14} and acetate-2-C^{14}, partially degraded the ring structure of cholesterol whereby carbon atoms 1, 2, 3, 4, 5, 6, 10, and 19 were ultimately obtained as CO_2. From this study it was shown that carbon atom 2, carbon atom 4, carbon atom 6, and carbon atom 10 were derived from the carboxyl carbon while carbon atom 1, carbon atom 3, carbon atom 5, and carbon atom 19 were derived from the methyl carbon of acetate. Following a similar procedure for rings D and C, Cornforth, Gore and Popják (35) were able to establish that carbon atom 8, carbon atom 11, carbon atom 12, carbon atom 14, and carbon atom 16 were derived from the methyl carbon of acetate. With these findings, the origin of all carbon atoms in cholesterol was then known.

In 1956 Bucher and McGarrahan (22), using a rat liver homogenate, demonstrated that, for the biosynthesis of cholesterol from acetate, both the microsomes and soluble cell constituents of rat liver are required. It was also demonstrated that 90% of the cholesterol, made at short time intervals, was located in the microsomes and that the *in vitro* synthesis required oxygen, ATP, and NADH.

Thus it has been established that the sole carbon source of cholesterol is acetate. Fifteen carbon atoms of cholesterol are derived from the methyl group of acetate while the remaining 12 carbon atoms are derived from the carboxyl group of acetate. The site of synthesis appears to be the microsomes. ATP, NADH, and oxygen are required for synthesis *in vitro*.

Acetate to Mevalonate

The first major step in the breakdown of the pathway by which cholesterol is synthesized was the discovery that acetate (14) could be incorporated into cholesterol. The second major step in the breakdown of the pathway was the discovery of mevalonic acid (124, 125, 129). The pathway from acetate to mevalonate is given in Figure 2.

In 1956 Wright *et al.* (129) found a factor in *Lactobacillus acidophillus* which was identified (124) through structural degradation and chemical synthesis as β-hydroxy-β-methyl-δ-valerolactone. Investigation of its chemical nature by Wolf *et al.* (125) resulted in the determination of its structure as β-δ-dihydroxy-β-methyl valeric acid which is known as mevalonic acid. The structure was confirmed by synthesis. Tavormina, Gibbs and Huff (107) using a cell-free rat liver homogenate and β-hydroxy-β-methyl-δ-valerolactone labeled in the 2-position with C^{14}, demonstrated that mevalonic acid (MVA) could be incorporated into cholesterol. Only one optical isomer was utilized. Wright and Cleland (128) also developed a rat liver homogenate which would incorporate MVA into cholesterol if ATP, NAD, coenzyme A, and oxygen were present. In 1959 Porter, Knauss and Wasson (64) showed that mevalonic acid was synthesized from acetate-1-C^{14} in a rat liver homogenate in the presence of ATP,

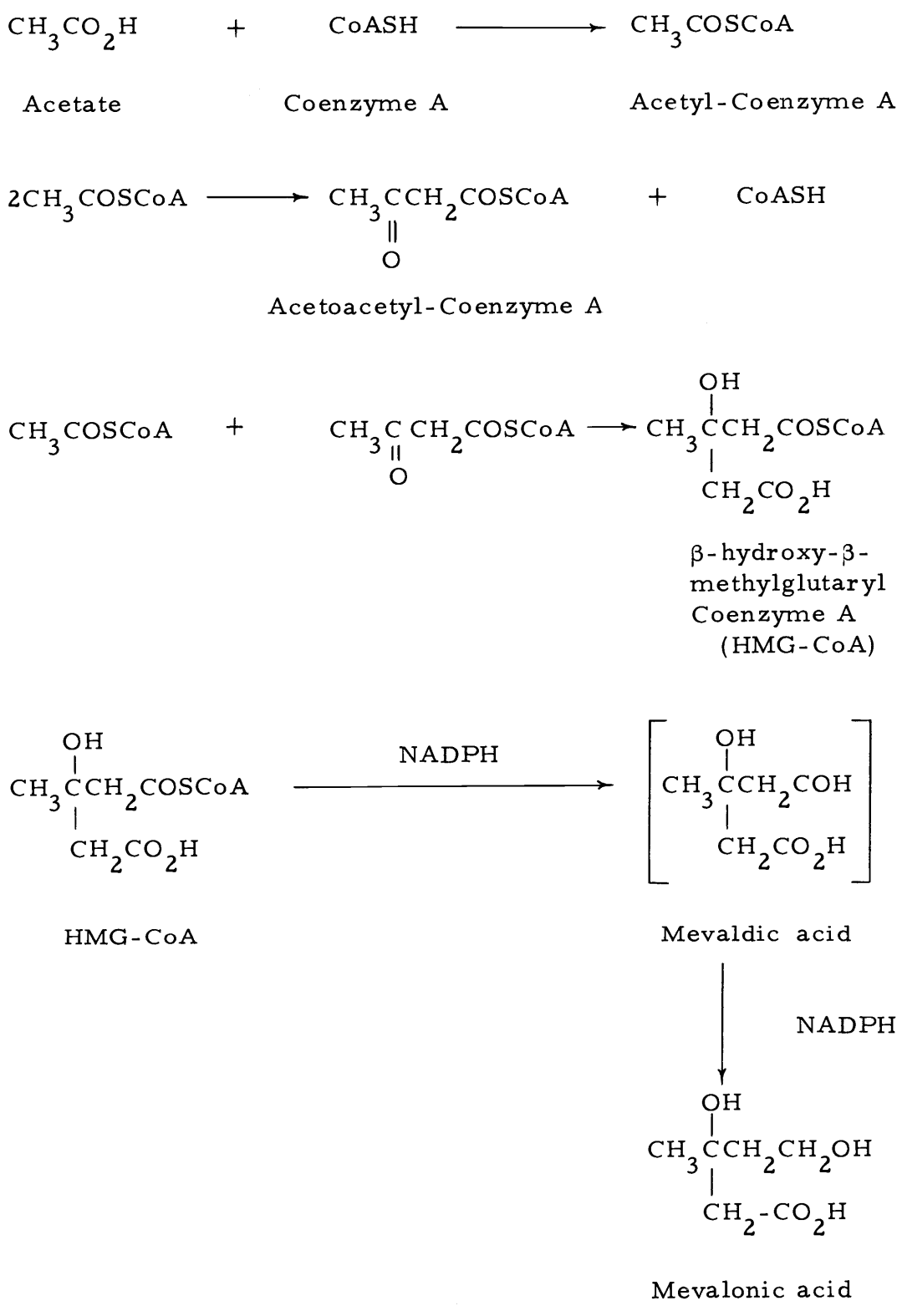


Figure 2. Acetate to mevalonic acid.

coenzyme A, glutathione, Mg^{+2}, and NAD. Thus the link between cholesterol, mevalonic acid, and acetate was established.

In 1959 Ferguson and Rudney (49) purified an enzyme (HMG-CoA condensing enzyme) from yeast which catalyzed the condensation of acetyl coenzyme A and acetoacetyl-coenzyme A to yield β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA). Rudney and Ferguson (95) have also shown that the coenzyme A released is from acetyl-CoA. HMG-CoA can also arise from the amino acid leucine (93, p. 177-186). In 1963 Brodie, Wasson and Porter (20, 21) reported that if acetyl-CoA and malonyl-CoA were incubated in the absence of TPNH in a pigeon liver homogenate, HMG-CoA was formed. Thus acetyl-CoA will condense with malonyl-CoA, yielding acetoacetyl-CoA and CO_2. Acetoacetyl-CoA will then condense with another acetyl-CoA molecule, yielding HMG-CoA.

It has been shown (48, 73) in yeast that HMG-CoA is readily reduced to MVA with the oxidation of two equivalents of TPNH. Mevaldic acid, which can arise from HMG-CoA with the oxidation of one equivalent of TPNH, was shown (130) in a rat liver homogenate to be converted into MVA. The enzyme, mevaldic reductase, has been partially purified (98) from pig liver; it has also been found in pig heart and kidney. The reaction is not reversible and either DPNH or TPNH is required. Donninger and Popják (45) have shown that mevaldic reductase belongs to the 'A' specific group of

dehydrogenases. The occurrence of free mevaldic acid as an obligatory intermediate in the conversion of HMG-CoA to mevalonate has not been shown. It may either be enzyme-bound or present as a hemithioacetal with coenzyme A (93, p. 184).

Thus it has been well-established that acetyl-CoA condenses with malonyl-CoA to yield acetoacetyl-coenzyme A, CO_2, and free coenzyme A. Then another molecule of acetyl-CoA condenses with acetoacetyl-coenzyme A to form β-hydroxy-β-methylglutaryl CoA and free coenzyme A. In the presence of one mole of NADPH, HMG-CoA is converted to mevaldic acid (which is not a free intermediate) and free coenzyme A. Finally mevaldic acid is converted to MVA in the presence of another mole of NADPH.

Mevalonate to Squalene

In 1926 (57) it was demonstrated that if squalene was given to rats their cholesterol content increased; thus evidence was provided that squalene was a precursor to cholesterol. Channon (26) in 1926 showed that the non-saponifiable fraction material increased by 2.6 times the normal amount in the liver of rats when squalene was added to their diet. The amount of cholesterol present increased by 100%. These results were confirmed by Bloch in 1953 (67). Cornforth and Popják (38) demonstrated that the carbon source of squalene was acetate. In 1955 Tchen and Bloch (109), using a rat liver homogenate

(microsomes and supernatant) and squalene-C^{14} made from acetate-C^{14}, showed that squalene could be converted to lanosterol and cholesterol. Both the cyclization of squalene and the demethylation to cholesterol took place when the microsomes were intact. The cell-free supernatant had only the ability to cyclize squalene.

Amdur, Rilling and Bloch (3), using a particle-free extract of dried baker's yeast, demonstrated that MVA-2-C^{14} in the presence of Mg^{+2}, ATP, and NAD could be incorporated into squalene. Dituri et al. (44) confirmed these findings with a rat liver homogenate. Degradation of the squalene revealed that carbon atom five of one molecule of MVA was attached to carbon atom two of the second molecule of MVA. Dituri et al. then speculated that decarboxylation of MVA took place during or after polymerization. The pathway for the conversion of MVA to squalene (as accepted today) is given in Figures 3 and 4. Tchen (108) isolated and partially purified from an extract of yeast an enzyme which catalyzed the formation of a monophosphate ester of mevalonate (MVA-P). There is a requirement for ATP, GTP, CTP, or UTP, but not ADP can replace ATP. Also, a divalent metal ion such as Mg^{+2} or Mn^{+2} is required. The active site may be a sulfhydryl group.

Mevalonic kinase has been partially purified from rabbit liver, hog liver and some plants (93, p. 193). Then Chaykin et al. (27), using MVA-P as the substrate in a partially purified yeast fraction

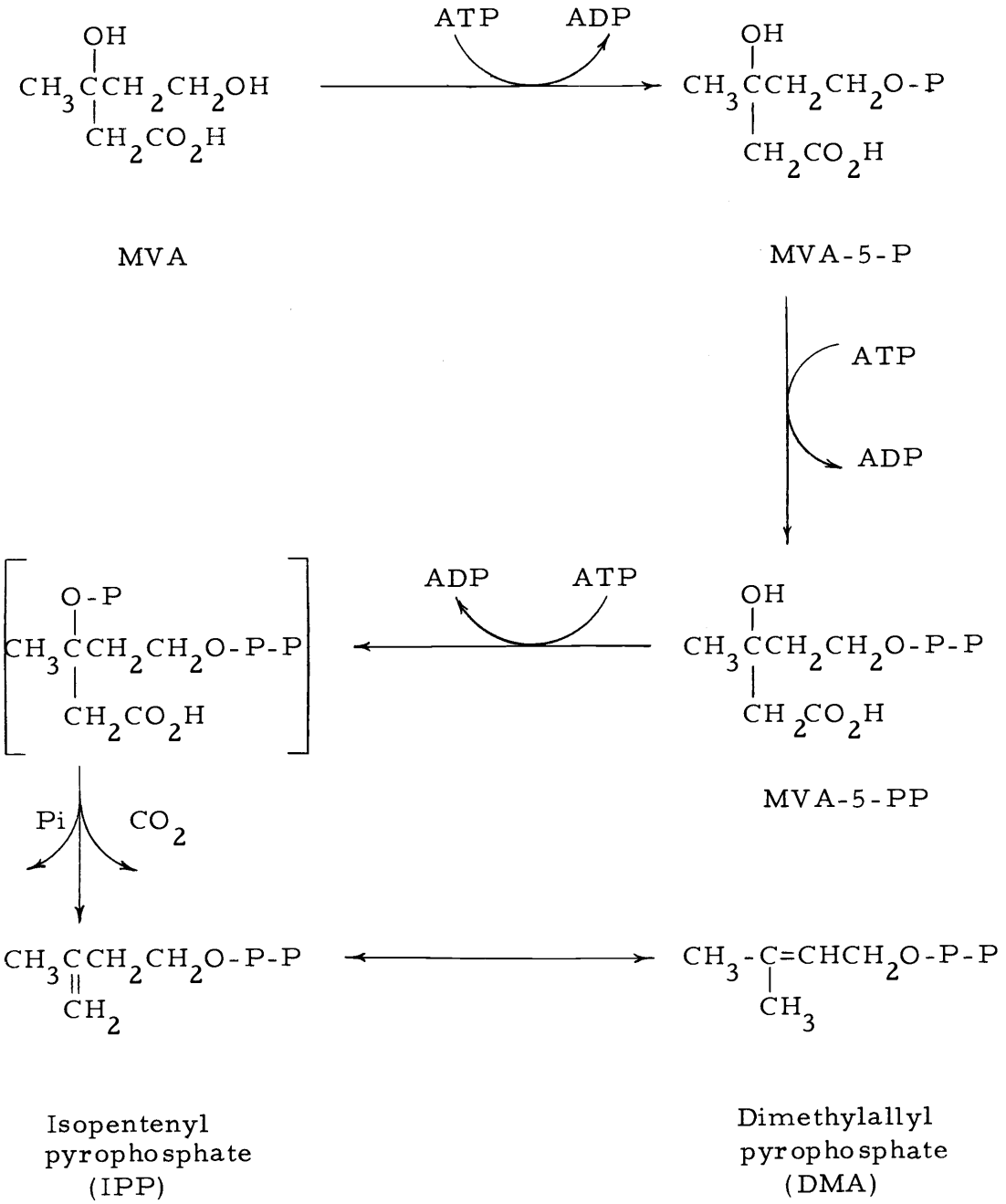


Figure 3. Formation of isopentenyl pyrophosphate from mevalonic acid.

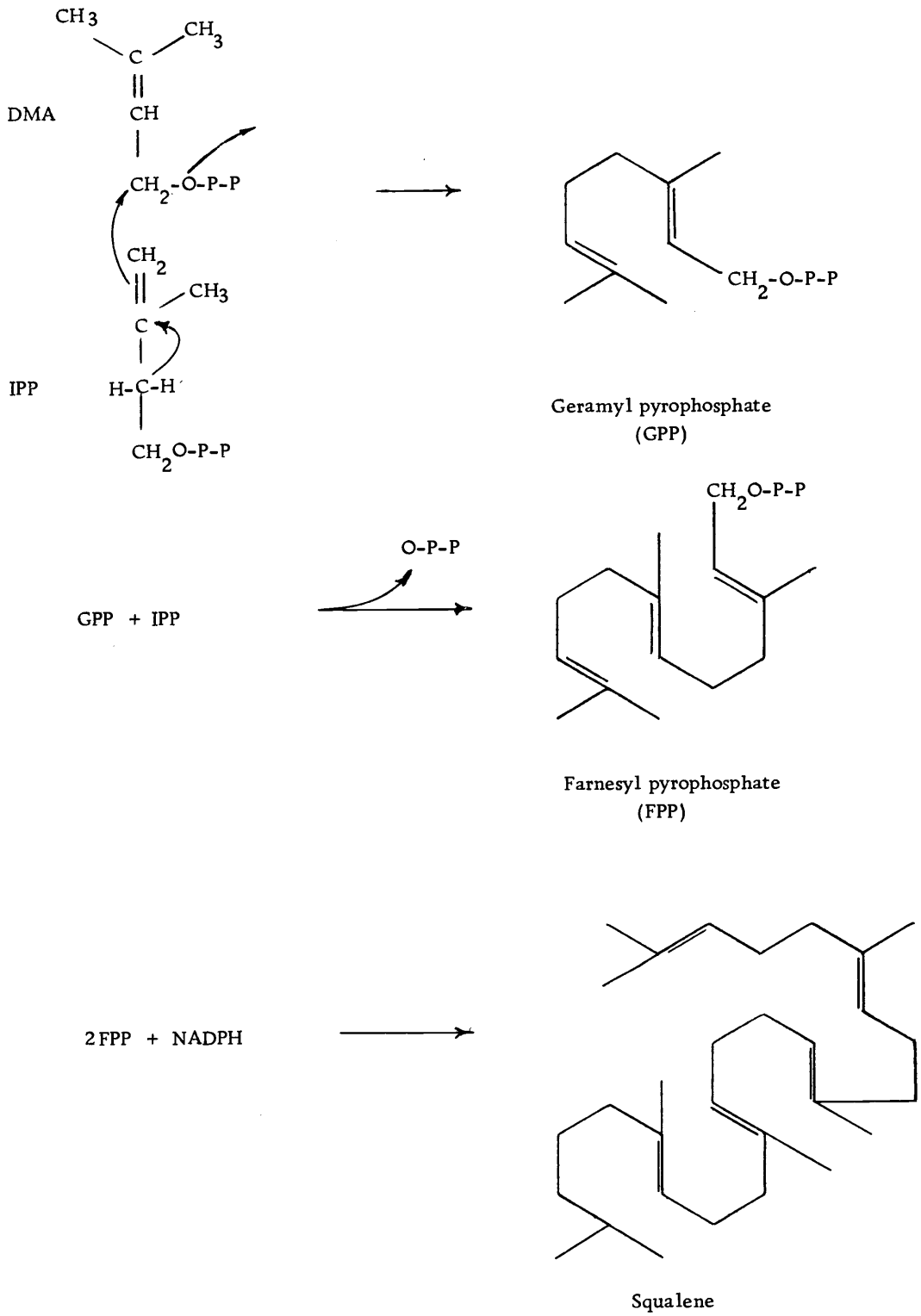


Figure 4. Condensation of isoprenoid units.

found two compounds which when used as substrates could be converted to squalene. They tentatively designated the structures as mevalonic acid-5-pyrophosphate (MVA-5-PP) and Δ^3-isopentenyl pyrophosphate (IPP). Popják (58) isolated phosphomevalonic kinase from pig liver and found that the reaction is reversible. ATP is required to convert MVA-P to MVA-5-PP. Mg^{+2} is also required for activation. No other nucleotide can act as a phosphate donor with this enzyme.

MVA-5-PP in the presence of ATP (27) will yield CO_2, ADP, PO_4^{-2}, and IPP. Lindberg et al. (69) have worked out the mechanism of the formation of IPP from MVA-5-PP. Bloch synthesized 3-O^{18}-mevalonalactone and converted it enzymatically to 3-O^{18}-mevalonate-5-pyrophosphate which was decarboxylated to IPP. The inorganic phosphate isolated from the reaction mixture contained O^{18} which showed that the oxygen had been transferred from the tertiary hydroxyl group of mevalonate-5-pyrophosphate to the terminal phosphate residue of ATP. This finding confirms that the 3-hydroxyl group was activated for expulsion as its phosphate ester. Lynen et al. (74) have found that carbon dioxide evolution and ADP production occur at identical rates. Thus the mechanism is as shown in Figure 3. 3-phospho-5-pyrophosphomevalonate has never been detected.

Lynen et al. (73) isolated IPP and converted it to dimethylallyl

pyrophosphate (DMA) by a soluble yeast enzyme fraction. The enzyme, isopentenyl pyrophosphate isomerase, has been isolated and purified from yeast (2, 75). This enzyme will catalyze the conversion of IPP to DMA. The reaction is reversible.

In 1957 Dituri et al. (44) found that C^{14}-farnesenic acid was incorporated into cholesterol; from these results they suggested that an intermediate containing the farnesyl structure should be considered. Lynen et al. (74) isolated IPP and farnesyl pyrophosphate in a cell-free yeast extract. They also showed that IPP is converted into farnesyl pyrophosphate and squalene with NADPH required. Lynen et al. (75) also detected geranyl pyrophosphate and found an enzyme in yeast, farnesyl pyrophosphate synthetase, which effected the connection of 5-carbon units. Goodman and Popják (54) have found DMA, geranyl pyrophosphate, and farnesyl pyrophosphate in rat liver. Cornforth and Popják (37) have proposed a mechanism for the condensation of DMA and IPP to form geranyl pyrophosphate with the expulsion of pyrophosphate; geranyl pyrophosphate is then attacked in turn by another molecule of IPP to form trans-trans-farnesyl pyrophosphate. Cornforth et al. (40) have worked out the stereochemistry of the condensation of DMA and IPP to geranyl pyrophosphate and of IPP and geranyl pyrophosphate to farnesyl pyrophosphate.

Squalene is formed by the dimerization of farnesyl

pyrophosphate (FPP). Lynen et al. (74) found that this conversion required NADH. Popják et al. (88, 89, 90, 91) have done a definitive set of experiments on the mechanism of the conversion of FPP to squalene. They found that (1) mevalonate-5-D_2-2-C^{14} incorporated only 11 atoms of deuterium into squalene (12 atoms would be theoretically possible), (2) mass spectrometric analysis showed that the labeling in the center of the chain was asymmetrical (-CHD-CD_2-), and (3) 1-T_2-2-C^{14} trans-trans-farnesyl pyrophosphate incorporated into squalene gave a H^3/C^{14} ratio of 0.76 (in theory, the ratio should be 1.00). From these findings it was concluded that one labeled hydrogen atom is lost from carbon atom one of one of the farnesyl pyrophosphate molecules.

Cornforth and Goodman (39, 97) found that during the dimerization of FPP, tritium is incorporated from TPNT (the hydrogen atom is removed from the B side of the reduced nicotinamide coenzyme). As the symmetry of squalene requires, there is an equal distribution of tritium between carbon atom 11 and carbon atom 12 of cholesterol.

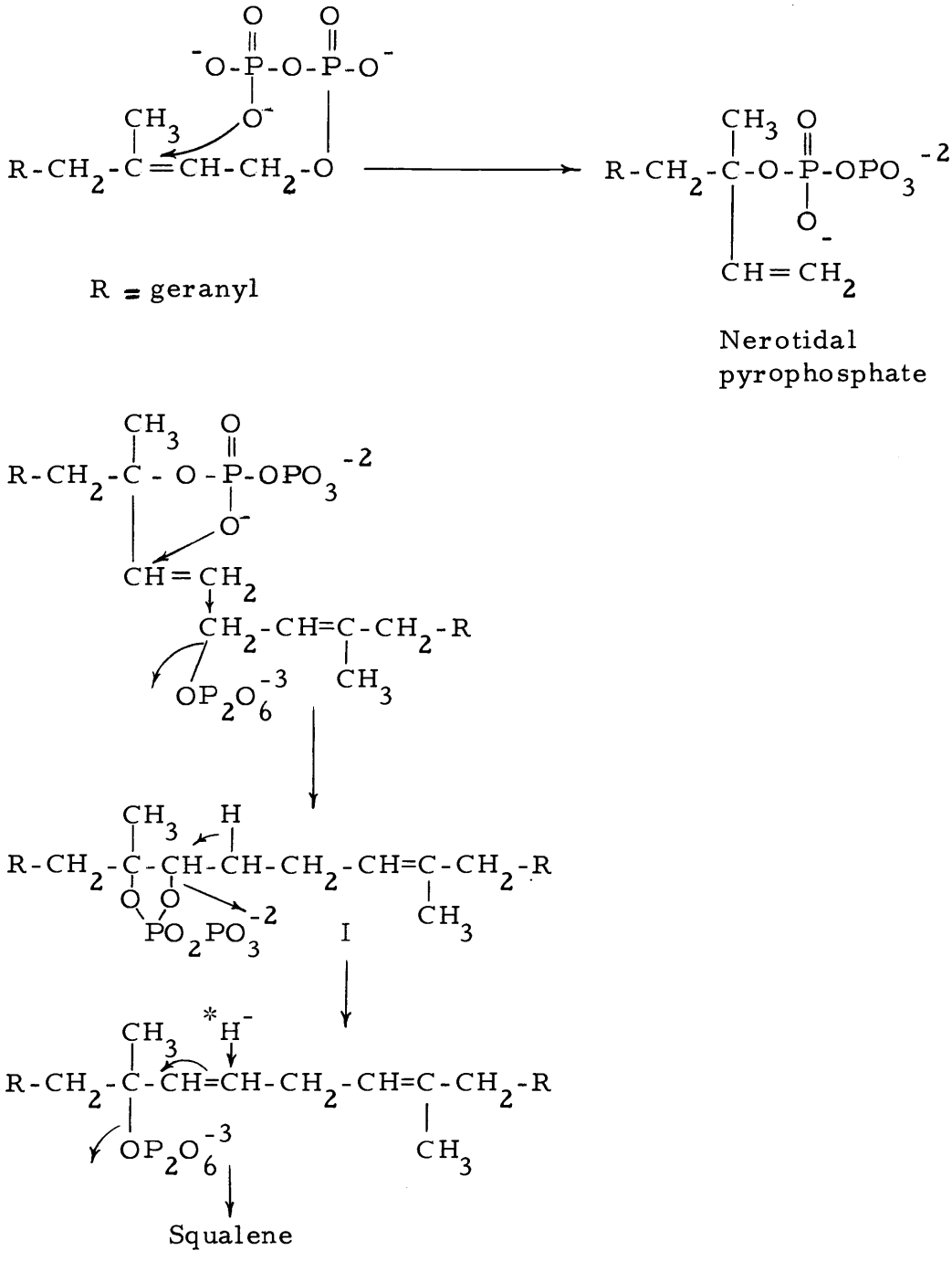
Thus, if squalene is formed from the dimerization of FPP, then one hydrogen atom of carbon atom one of one farnesyl residue is lost and is stereospecifically replaced by an hydride ion from NADPH. In addition, there must be inversion of configuration around carbon atom one of the second farnesyl residue which is not involved in the hydrogen exchange (89). The mechanism for

the condensation of two FPP molecules has been proposed as shown in Figure 5. This mechanism has led to the proposal of the intermediary formation of nerolidyl pyrophosphate and an intermediate such as I in Figure 5 (89). However, evidence has also been brought forth (65, 105) that only enzyme-bound intermediates exist between farnesyl pyrophosphate and squalene. Therefore, the mechanism for the condensation of farnesyl pyrophosphate to form squalene has not been conclusively proved.

Squalene to Lanosterol

In 1953 Woodward and Bloch (126) proposed the cyclization of squalene to lanosterol as shown in Figure 6 (scheme a). This distribution of acetate carbons in the cholesterol skeleton was clearly in accord with the proposed mechanism of cyclization. Then in 1955 Tchen and Bloch (109) demonstrated the conversion of squalene-C^{14} into lanosterol and cholesterol in a rat liver homogenate. This study confirmed squalene as a precursor to lanosterol. In 1957 Tchen and Bloch (110) isolated a partially purified enzyme fraction from rat liver that would convert squalene to lanosterol. The process required aerobic conditions and NADPH or NADH.

Tchen and Bloch showed (111, 112), using D_2O, H_2O^{18} and O_2^{18}, that no protons from the medium are incorporated into lanosterol and that molecular oxygen is incorporated into lanosterol. This result supported a concerted mechanism of cyclization.



*From NADPH

Figure 5. Mechanism of squalene synthesis from farnesyl pyrophosphate.

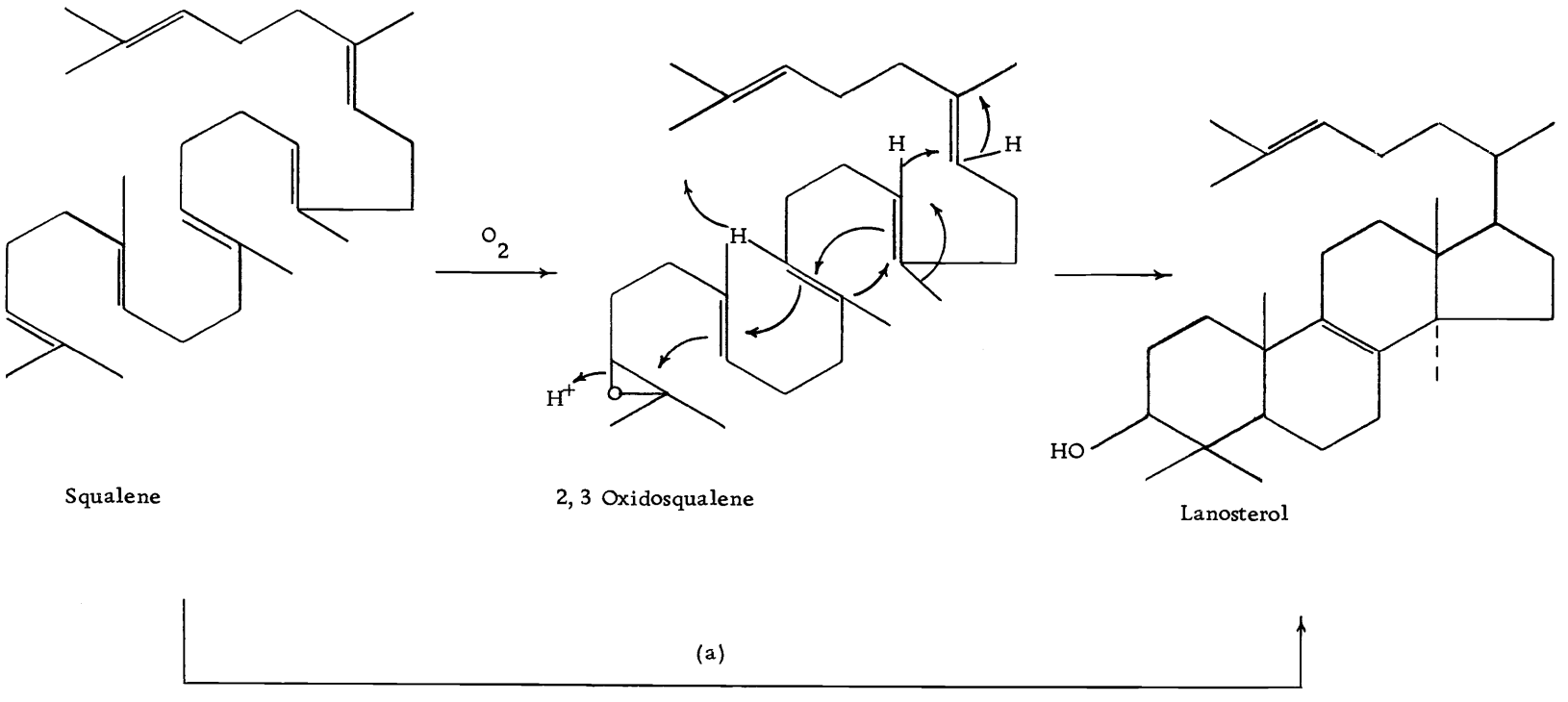


Figure 6. Pathway of squalene to lanosterol.

Another aspect of the cyclization mechanism proposed by Bloch involved the two methyl groups in the region of the C-D ring junction. There exist two possibilities: a single 1, 3 methyl shift from carbon atom 8 to carbon atom 14 or two 1, 2 methyl migrations. Maudgal, Tchen and Bloch (78) in an elegant series of experiments showed that the methyl migration was in fact two 1, 2 methyl migrations. Clayton and Bloch (30, 31) proved conclusively, using a liver homogenate, that lanosterol is synthesized from acetate and that labeled lanosterol can in turn be converted into cholesterol.

The origin of the hydroxyl group had been established as molecular oxygen but the exact mechanism of its insertion into squalene was not elucidated until 1966 when Corey, Russey and Ortiz de Montellano (33) made 2, 3-oxidosqualene-C^{14} (Figure 5) which was converted into cholesterol in a rat liver homogenate. At the same time Van Tamelen et al. (106) showed that 2, 3-oxidosqualene-C^{14} can be converted into lanosterol. In 1967 Willett et al. (123) demonstrated that the oxide is cyclized to lanosterol by a cyclizing system independently of the oxidative step and requires neither oxygen nor NADPH. The pathway is shown in Figure 6. The cyclase has been isolated from hog liver (42) and rat liver (123). In both cases, the cyclase was found in the microsomes.

Lanosterol to Cholesterol

For the final transformation of lanosterol to cholesterol there remains only the removal of the two methyl groups at carbon atom 4 and the methyl group at carbon atom 14, the reduction of the Δ^{24} sidechain double bond, and the relocation of the $\Delta^{8,9}$ ring junction double bond to $\Delta^{5,6}$.

In 1957 Olson, Lindberg and Bloch (82) showed that the metabolism of labeled lanosterol in rat liver homogenate yielded cholesterol and $C^{14}O_2$. They found no formation of formaldehyde during the demethylation reaction; therefore Olson and Bloch (81) proposed that the 4, 4'-methyl and the 14-methyl of lanosterol are oxidized first to an alcohol and then to a carboxyl group which is then eliminated as CO_2. Gautschi and Bloch then found (52) a compound which had dimethyl substituents at carbon atom 4, double bond at carbon atom 24, 25, and was enzymatically converted to cholesterol, yielding two moles of CO_2. Gautschi and Bloch later (53) definitely identified this intermediate as 4, 4'-dimethyl-cholestadienol (14-desmethylanosterol). Thus it has been established that the methyl group at carbon atom 14 is the first of the three methyl groups to be lost.

The loss of two methyl groups at carbon atom 4 appears to occur after the transformation of the hydroxyl group at carbon atom 3 to a ketone. Lindberg, Gautschi and Bloch (68) showed that

$\Delta^{8,24}$-lanostadien-3-one, 4-4'-dimethyl-Δ^{8}-cholestenol (14-des-methyl-24, 25-dihydrolanosterol) and 4, 4'-dimethyl-Δ^{8}-cholesten-3-one can be converted to cholesterol in a rat liver homogenate. Thus from these and other results, it appears that 4, 4'-dimethyl-cholestadienol is converted to $\Delta^{8,24}$-4, 4'-dimethyl cholestadien-3-one before the two methyl groups at carbon atom 4 are lost. The exact order of events resulting in loss of the carbon atom 4 methyls is not known with certainty but a carbon atom 4 mono methyl sterol, methosterol (4-α-methyl-Δ^{7}-cholesten-3-β-ol), has been isolated and identified (79, 119) in rat feces. When administered (118) to rats, methosterol was quickly absorbed and effectively converted to cholesterol.

The first carbon-27 intermediate appears to be zymosterol ($\Delta^{8,24}$-cholestadienol) (43). Johnston and Bloch prepared C^{14}-zymosterol from a yeast preparation and showed (59) that it could be converted to cholesterol by a homogenate of rat liver. All that remains is to reduce the Δ^{24} double bond and shift the Δ^{8} double bond to the carbon atom 5, 6 position. There are two pathways for converting zymosterol (43) to cholesterol. In one pathway, the reduction of the Δ^{24} double bond is the last step [$\Delta^{5,24}$-cholestadienol (desmosterol)$\rightarrow$cholesterol] and, in the second pathway, the Δ^{5} double bond is formed in the presence of the double bond at carbon atom 7 and thus the final step is the reduction of the Δ^{7}

double bond [$\Delta^{5,7}$-cholestadienol (7-dehydrocholesterol)→cholesterol]. The scheme for the conversion of lanosterol to cholesterol is shown in Figure 7.

Incorporation of Acetate into Sterols in Arterial Tissue

The first demonstration that acetate could be converted into cholesterol in arterial tissue was by Siperstein, Chaikoff and Chernick (103) in 1951. Using slices of aorta from rabbits and chickens, they found that acetate-C^{14} could be incorporated into cholesterol after three hours at 37° C. Rabbit and chicken aortas also had the capacity to oxidize significant amounts of acetate to CO_2.

Since 1951 various *in vitro* methods have been used to show that acetate-C^{14} can be incorporated into sterols, fatty acids, and CO_2 in arterial tissue. Werthessen *et al.* (120, 121) have shown that the whole aorta of calves can incorporate acetate into cholesterol. The authors found (120) that, if the whole aorta was incubated for long periods (72 to 239 hours) in the presence of glucose and acetate-C^{14}, acetate could be incorporated into cholesterol. Even with the inherent difficulties in a prolonged perfusion experiment of this type, there was enough activity, although varied, to establish definitely that cholesterol is synthesized in the calf aorta.

Key to Figure 7.

1. Lanosterol
2. 4, 4'-dimethyl-cholestadienol(14-desmethylamosterol)
3. $\Delta^{8,24}$-cholestadienol (zymosterol)
4. $\Delta^{8,24}$-4, 4' dimethyl cholestadien-3-one
5. $\Delta^{5,7}$-cholestadienol (7-dehydrocholesterol)
6. $\Delta^{5,24}$-cholestadienol (desmosterol)

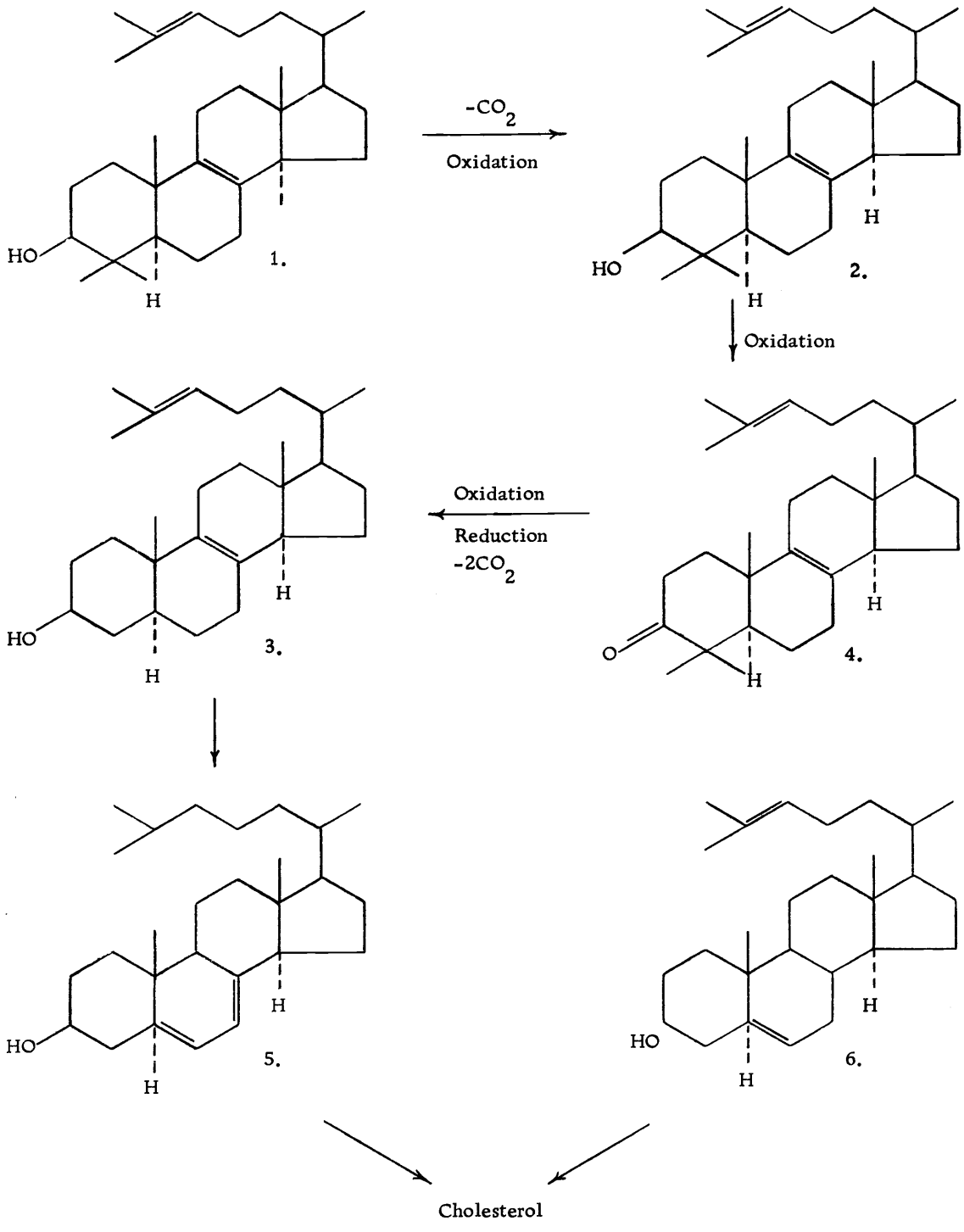


Figure 7. Formation of cholesterol from lanosterol.

Instead of using the whole aorta to demonstrate that acetate is a suitable substrate for cholesterol biosynthesis, some investigators have utilized only part of the aorta. Whereat (122) has studied the incorporation of acetate-C^{14} and mevalonic-2-C^{14} acid into the aortic intima (inner lining of the aorta) of normal and cholesterol-fed rabbits. The author did not observe any significant incorporation of either substance into cholesterol, but acetate was incorporated significantly into fatty acids.

Many of the investigations on arterial tissue have been carried out with slices of the tissue. Feller and Huff (47), using strips of rabbit aorta, found that acetate-2-C^{14} could be incorporated into nonsaponifiable lipids, fatty acids and CO_2. Foster and Siperstein (51) have also found that if acetate-C^{14} was incubated for three hours at 37° C in the presence of aorta slices from rats, it was converted into cholesterol and fatty acids. In addition, acetate was observed to be oxidized to CO_2. Marcó and Van Bruggen (77) have also shown, using rat aorta slices, that the tissue is metabolically active with respect to acetate-C^{14} uptake into sterols and fatty acids. In addition Loomeijer and Van Der Veen (71) have shown, using aorta slices from rats, that various lipids such as phospholipids, sterol esters, free fatty acids, triglycerides and sterols are manufactured <u>in vitro</u> using acetate-C^{14}. Finally Azarnoff (6), using slices from human aortas, has shown that acetate is incorporated into cholesterol-like compounds.

Some investigators have attempted to make a cell-free preparation of aorta in order to observe if acetate can be incorporated into cholesterol. Eisley and Pritham (46), using a Micro Model Latapie Grinder, have obtained incorporation of acetate-C^{14} into cholesterol from tissue minces of turkey and hog aorta. In order to obtain incorporation, an incubation period of 24 hours at 37° C was required. Instead of minced tissue, Terner and Darey (113) have used an aorta homogenate. After mincing the aortas of turtles in a Latapie Grinder and homogenizing them in a Potter homogenizer, Terner and Darey squeezed the homogenate through muslin. Then the homogenate was centrifuged at 500 x g for five minutes. After the homogenate was incubated for 60 minutes at 25° C, acetate was incorporated into both a nonsaponifiable fraction and a fatty acid fraction.

The incorporation of acetate into sterols and fatty acids has been observed in vitro in whole aorta as well as in homogenates of aorta. Human, chicken, cow, rat, turkey, dog, cat, and turtle aorta have been shown to incorporate acetate into sterols and fatty acids. Sterol and fatty acid synthesis in the aorta is low compared to their synthesis in the liver (103). In addition, a number of investigators (47, 51, 77, 103) have shown that acetate can be oxidized to CO_2 in arterial tissue. These investigations imply that in the incorporation of acetate to sterols acetate must be activated to acetyl-CoA. If

this is true, then acetyl-CoA-kinase must be present in arterial tissue. A cell-free preparation of bovine aorta made in this laboratory (115) was capable of incorporating acetyl-CoA but not acetate into a nonsaponifiable fraction. Thus it was of interest to know why acetate could not be activated to acetyl-CoA in a cell-free preparation of bovine aorta.

Acetyl-CoA-Kinase

$$\text{Acetate} + \text{ATP} + \text{Enzyme} \xrightleftharpoons{Mg^{+2}} \text{Acetyl-AMP}\cdot\text{Enzyme} + \text{PP}$$

$$\text{Acetyl-AMP-Enzyme} + \text{CoA} \rightleftharpoons \text{Acetyl-CoA} + \text{Enzyme} + \text{AMP}$$

The above mechanism for the acetylation of coenzyme A (CoA) by free acetate has been shown by Berg (10) and Webster (116).

One of the first attempts at purification of acetyl-CoA-kinase was with yeast (61). An approximately 10-fold purification of the enzyme was achieved with ammonium sulfate precipitation. The yeast enzyme will acetylate both CoA and dephosphoCoA. Propionate as well as acrylate can replace acetate, but the former substrates are not really as effective as acetate. There is a requirement for Mg^{+2} which may be replaced by Mn^{+2}. The enzyme has a maximal rate at pH 7.2, but the optimum is rather broad. The partially purified enzyme is stable when frozen in the presence of ammonium sulfate, but, upon further purification by dialysis, the enzyme will lose activity slowly even if frozen. At this point in the purification

scheme all the activity will be gone within a month.

The enzyme has been partially purified from ox brain (4). Using a calcium phosphate gel treatment, a 20-fold increase in purity was obtained. There is a requirement for Mg^{+2}, but it is not an absolute requirement; Zn^{+2}, Co^{+2} and Ni^{+2} will inhibit the enzyme activity. In contrast to the yeast enzyme, this enzyme has a sharp maximal rate at pH 7.0. The purified enzyme is found to be stable if the protein concentration is 5 mg/ml or greater, but upon dialysis the enzyme will lose activity.

Acetyl-CoA-kinase has been isolated from a bacteria. This enzyme exhibits some properties that are quite different from the yeast and ox brain enzymes. Using a calcium phosphate gel treatment, Abraham and Bachhawat(1) isolated the enzyme from Euglena Gracilis which can grow in a medium which contains acetate as the sole carbon source. A ten-fold increase in purity was obtained. The enzyme has a pH optimum of 5.0 in Tris-phosphate buffer which contrasts strikingly with the optimum for the yeast and ox brain enzymes. This difference may be related to the fact that Euglena grows maximally in a medium of pH 3.6 (1). There is an absolute requirement for Mg^{+2}. Ni^{+2} and Zn^{+2} have no effect on the enzyme activity. The purified enzyme is fairly stable until it is dialyzed.

Studies on lipid metabolism of pleuropneumonia-like organisms have demonstrated the incorporation of acetate-C^{14} into sterols (104).

Thus acetyl-CoA-kinase has been studied in several pleuropneumonia-like organisms (25). The pH optimum runs between 6.0 and 7.5. There is an absolute requirement for a divalent metal ion. Mg^{+2} or Mn^{+2} is required and both work equally well. Several acids besides acetate, namely propionate, butyrate, isobutyrate and several others, were tested and no activity was observed. The properties of this enzyme appear to be very similar to those of the yeast and ox brain enzymes.

In most of the attempts at isolation and purification of acetyl-CoA-kinase the enzyme has always exhibited some instability upon purification. Shah and Ramakrishnan (101) have partially purified (30-fold increase) the enzyme from Aspergillus niger. In this case even the crude preparation lost all its activity within one day. The enzyme was very similar to the enzyme from yeast in that it had an absolute requirement for Mg^{+2} and a pH optimum of 8.5.

The most extensive study on the acetyl-CoA-kinase reaction has been carried out on the enzyme isolated from bovine heart mitochondria (23). Webster claims also to have isolated the enzyme in crystalline form (117). This enzyme is very similar to those already described in that it has an absolute requirement for Mg^{+2} and a pH optimum of 8.0. In addition, the most purified fraction will lose its activity at 3°C. Repeated freezing and thawing of the enzyme causes substantial loss in activity. From sedimentation equilibrium data

Webster has estimated a molecular weight of approximately 32,000.

Sharkova (102) has attempted to determine what amino acid(s) is (are) involved in the active site. Using an acetone-dried powder and ammonium sulfate precipitation procedure, Sharkova isolated the enzyme from rabbit heart muscle. Titration of the enzyme with para-chloromercuribenzoate and amperometric titration with $AgNO_3$ showed approximately five sulfhydryl groups per mole of enzyme. Blockage of the sulfhydryl groups with para-chloromercuribenzoate caused no loss in enzymatic activity. Mild photooxidation inactivates the enzyme. The inactivity appears to be associated with the breakdown of the histidine imidazole ring. These facts would imply that histidine is required for enzymatic activity.

Phosphatase Activity in Arterial Tissue

In 1948 Baló, Banga and Josepovits (7) were not able to find myosin or actomyosin in the aorta of humans, in a water extract at pH 6.0, but they did find adenylpyrophosphatase activity. This enzyme will split off orthophosphate from ATP and ADP. In addition, using Weber's solution (0.6M KCl, 0.01M Na_2CO_3 and 0.04M $NaHCO_3$), they found no adenylpyrophosphatase activity in that part of the aorta which failed to go into solution. From this result it was concluded that the adenylpyrophosphatase was not bound to elastin but rather to collagen or other soluble proteins. The

adenylpyrophosphatase had a pH optimum of 9.0 and could not be activated with Mg^{+2} or Ca^{+2}. Other substrates such as sodium pyrophosphate, sodium hexosediphosphate, phosphoglyceric acid-sodium salt and α-glycerophosphate were tried but no orthophosphate was liberated. In 1959 Kirk (63) also found adenylpyrophosphatase activity which showed a pH optimum of 8.1 under his assay conditions.

In 1950 Banga and Nowotny (8) found in aorta of rabbits and humans a myosin-bound adenosine triphosphatase (ATPase) with a pH optimum of 7.0 which is activated by Mg^{+2}. This enzyme will remove only the terminal phosphate from ATP, but it will not work on ADP or AMP. Carr, Bell and Krantz (24) found that a Ca^{+2} activated ATPase with a pH optimum of 7.4 in male mongrel dog aorta. They also found a pronounced difference among species with respect to ATPase activity in rat, turtle, frog, chicken, guinea pig and rabbit aorta. Rat and dog aorta had the highest ATPase activity (26 mg and 16.5 mg inorganic phosphate liberated per mg of tissue in 15 minutes at 37°C, respectively). Frog and guinea pig aorta had half the activity of the dog aorta while the rabbit aorta had about one-fourth the activity of the dog aorta. Chicken and turtle aorta showed very little activity. Bonting, Simon and Hawkins (17) have found in smooth muscular tissue of the cat aorta a sodium-potassium activated adenosine triphosphatase. This enzyme presumably plays a role in active cation transport.

Kirk and Praetorius (62), using disodium phenylphosphate as the substrate, found inorganic pyrophosphatase activity in an homogenate of human arterial tissue. The enzyme had a pH optimum of 5.7-5.8. Kirk (63) has also found inorganic pyrophosphatase activity in human arterial tissue with a pH optimum of 7.1-7.2. This activity can only be seen if the $MgCl_2$ concentration is increased from 1.0 mM to 15.0 mM. Inorganic pyrophosphatase has been found in dog aorta (80) with a pH optimum of 7.5. Mg^{+2} is required for activity and the enzyme is inhibited by Ca^{+2}. A nonspecific alkaline monoesterase with a pH optimum of 9.4 was found (76) in the aorta of rabbits, rats and chickens.

Kirk (63) has also found in human arterial tissue 5'-nucleotidase activity. It was observed to have an activity of the same order of magnitude as that of the ATPase found in human arterial tissue.

Zemplényi and Mrhová (132) compared alkaline phosphatase (pH 9.3), acid phosphatase (pH 5.0), adenylpyrophosphatase (pH 9.0) and 5'-nucleotidase (pH 7.5) in different animal species and again there was a pronounced species difference. The highest alkaline phosphatase activity was found in rat aorta as compared to rabbit and cock aorta. The rabbit aorta had the lowest activity while the cock aorta had only slightly higher activity than the rabbit aorta. The highest acid phosphatase activity was again found in rat aorta while both rabbit and cock aorta had lower but very similar activity.

In comparing the alkaline to the acid phosphatase activity Zemplényi and Mrhová found that the ratio was respectively 1:3 in the rabbit, 8:1 in the cock, and 13:1 in the rat aorta. Adenylpyrophosphate activity was again highest in the rat aorta but the cock aorta activity was lower than that of the rabbit aorta. With respect to 5'-nucleotidase the rabbit aorta had the highest, and the cock aorta, the lowest activity; the rat aorta activity was quantitatively halfway between that of the rabbit and cock aorta. In cock aorta, adenylpyrophosphatase and 5'-nucleotidase activity were in the same ratio (1:1), in rabbit aorta the ratio was 1:2, and in rat aorta the ratio was 4:1.

In conclusion, there appear to be several phosphatases in the arterial wall. There is a water-soluble ATPase with a pH optimum of 9.0 which is Mg-activated and is capable of splitting off orthophosphate from ATP and ADP. In animal vascular tissue there is a pronounced species difference with respect to this enzyme. There is also in the arterial wall a myosin-bound ATPase with a pH optimum around 7.0 which is a calcium activated enzyme and which will remove only the terminal phosphate of ATP but will not work on AMP or ADP. There is both acid and alkaline phosphatase in the arterial wall and there is even 5'-nucleotidase activity.

MATERIALS AND METHODS

Chemicals

Potassium monobasic phosphate, potassium dibasic phosphate, potassium hydroxide, petroleum ether (30-60°), sodium sulfate, sodium chloride, sodium carbonate, diethyl ether, acetone, and chloroform were obtained from Mallinckrodt Chemical Works. Potassium acetate, potassium fluoride, potassium chloride, metaphosphoric acid, sodium cyanide, potassium bicarbonate, ammonium sulfate, ammonium molybdate, sodium sulfate, sodium bisulfite, benzene, ethyl acetate, acetic acid, and mineral oil were obtained from Baker and Adamson (Allied Chemicals). Ferric chloride, trichloroacetic acid, nitroprusside, sodium pyrophosphate, sodium fluoride, p-nitrophenol, maleic acid, and fumaric acid were obtained from Matheson, Coleman and Bell. Magnesium chloride and toluene were obtained from J. T. Baker Company. ATP, ADP, AMP, NAD, and coenzyme A were purchased from P-L Biochemicals, Inc. Reduced glutathione, hydroxylamine hydrochloride, Tris(hydroxymethyl)aminomethane (Tris), thioglycerol, and bovine serum albumin were purchased from Sigma Biochemical Corporation. PPO and POPOP were obtained from Packard Instruments Company, Inc. MVA-DBED and p-nitrophenylphosphate were obtained from Mann Research Laboratory, Inc. L-phenylalanine and

aconitic acid were obtained from Calbiochem. Nicotinamide was obtained from Nutritional Biochemical Corporation. Protamine sulfate was purchased from General Biochemicals. Para-chlorophenoxyisobutyric acid was obtained from K and K Laboratories, Inc. Diethyl fumarate was obtained from Aldrich Chemical Company, Inc. Diethyl maleate and Chromagram sheets-silica gel type K301R2 without fluorescent indicator were obtained from Eastman Kodak Company.

Preparation of Crude Homogenate

Aortas were obtained from cattle two to three years old. Immediately upon sacrifice the aortas were placed in ice. Rubber gloves were worn to prevent contamination. First the fat and adventita were removed with scissors and then the aortas (10 to 12 inches long) were placed in ice water in order to wash off any excess blood and fat. Next each aorta was cut up with scissors into 1/4-inch squares which were immediately placed in a large beaker surrounded by ice. The arterial tissue was then ground up in a Waring Blendor at high speed. One hundred and fifty grams of tissue were ground with one hundred and fifty milliliters of cold 0.1 molar phosphate nicotinamide buffer, pH 7.0, (0.067M K_2HPO_4, 0.042M KH_2PO_4, 0.03M nicotinamide, and 0.006M $MgCl_2$). This mixture was immediately placed in another beaker surrounded by ice.

This homogenate was either squeezed through two layers of cheese cloth or centrifuged at 5,000 rpm in a Servall refrigerated centrifuge for 30 minutes. Both methods gave the same results. The supernatant was immediately frozen, and the solid residue, discarded.

This crude homogenate is quite stable if used within a month. It has been known to lose almost all of its activity after being frozen for two months. Thawing and refreezing, if not done more than three times, did not appear to have much effect upon the activity. The activity of the crude homogenate varied somewhat from preparation to preparation.

Preparation of Bovine Aorta Homogenate Using a Duall Homogenizer

Fresh aortas were cut longitudinally into 1/4-inch wide strips. Then these strips were cut into 1/4-inch squares. These squares were homogenized, 2 g/4.0 ml of 0.1 M phosphate nicotinamide buffer pH 7.0, in a 5.0 ml Duall homogenizer.

Preparation of Cell-free System

In order to prepare a cell-free system which was the preparation used in most experiments, the crude homogenate was centrifuged at 60,000 x g in a Beckman Model L Spinco centrifuge for 120 minutes. In most cases the clear cell-free supernatant was used within

hours of centrifugation. The 60,000 x g supernatant can be left at 2-4° C for up to three days with no apparent loss in activity. However, to insure against any variance in activity with a given preparation of aortas, the supernatant was used, unless otherwise stated, within a few hours of preparation. This preparation is referred to in this dissertation as the 60,000 x g supernatant.

Preparation of DL-mevalonic Acid-2-C^{14}

DL-mevalonic acid-2-C^{14} (MVA-2-C^{14}) was obtained from New England Nuclear Corporation of Boston, Massachusetts as the N, N'-dibenzylethylenediamine salt [(MVA-2-C^{14})$_2$-DBED].

The free acid was made by placing 100 mg of "cold" MVA-DBED salt (obtained from Mann Research, Inc.) with 0.1 mc of (MVA-2-C^{14})$_2$-DBED in 50 ml of distilled water. The 50 ml were placed on a column of Dowex-1X4 (chloride form) and eluted with water. A total of 200 ml was collected. The solution of the free acid (0.05 μc/ml) was used for most incubations. This solution was kept frozen and could be stored for months in such a state with no apparent loss of activity.

Standard Assay Procedure

Each preparation of aortas was tested for the incorporation of MVA-2-C^{14} into a nonsaponifiable fraction (NSF) before being used

for any experiments. This was the criterion used for establishing the preparation as being active.

Procedure:

To a 125 ml Erlenmeyer flask were added 1.0 ml ATP (3.3 μmoles), 1.0 ml NAD (6.9 μmoles), 1.0 ml MVA-2-C^{14} (0.05 μc/ml) and 2.5 ml of 0.1M phosphate nicotinamide buffer. Then 5.0 ml of the 60,000 x g supernatant were added. Each flask was swirled in a stream of oxygen (95 percent O_2, 5 percent CO_2) for 15 seconds and then stoppered. The flasks were then incubated for two hours and 50 minutes at 37° C in a Research Specialties oscillating water bath. The incubation time, coenzyme requirements, and concentrations for optimum results had been previously determined in this laboratory (114).

At the end of the incubation period 10 ml of 10% alcoholic potassium hydroxide (25 ml of 10M KOH and 80 ml of 95% ethanol) were added in order to stop the reaction. The incubation mixture was saponified overnight on a steam bath. During saponification a 50 ml glass conical centrifuge tube containing water was placed on top of each flask in order to prevent the evaporation of the ethanol.

The volume of the incubation mixture was kept constant at 10.5 ml. ATP and NAD (both obtained from P-L Biochemicals, Inc.) were routinely added in the concentrations stated above. Any other

substrates which were added to the incubation mixture were made up in 0.1M phosphate nicotinamide buffer or distilled water. The amount of buffer added was adjusted so that the total volume of the incubation mixture was always kept constant.

For some studies large amounts of NSF material were made in the following manner. Into a 250 ml Erlenmeyer flask were placed 10 mg ATP, 50 mg NAD, 10 ml of 0.05 μc MVA-2-C^{14}, and 50 ml of 60,000 x g supernatant in a total volume of 80 ml. After two hours and 50 minutes the reaction was stopped with 80 ml of 10% alcoholic KOH and transferred to a 500 ml Erlenmeyer flask. The incubation mixture was saponified overnight. The saponified material was extracted as described in the next section.

Isolation of the Nonsaponifiable Fraction

After being saponified overnight each mixture was extracted with 200 ml of reagent grade 30-60° petroleum ether according to the method of Wright and Cleland (128). Each flask was swirled with 50 ml of petroleum ether at a time. After being swirled for a few seconds the mixture in each flask was allowed to settle. When this was done four times, the petroleum ether fraction in each flask was decanted into a 250 Erlenmeyer flask containing 30 g of anhydrous sodium sulfate. After the aqueous phase was extracted four times in this manner, the petroleum ether extractions were dried over the

anhydrous sodium sulfate for one hour.

The dried petroleum ether phase was filtered through a Whatman #1 fluted filter into a 250 ml Erlenmeyer flask which contained at least two boiling stones. The sodium sulfate was washed twice with 10 ml of petroleum ether. Each washing was added to the original extraction.

The petroleum ether phase was taken to dryness on a steam bath. The residue remaining after evaporation is known as the non-saponifiable fraction (NSF). If the aorta preparation is left out of the incubation procedure no radioactivity is carried over into the NSF with this extraction procedure.

The large incubation mixture was extracted in the following manner. Each flask was extracted with 800 ml of petroleum ether 30-60°, 200 ml at a time. The petroleum ether 30-60° was dried over 120 gm of Na_2SO_4 in a one liter Erlenmeyer flask for one hour. Then the petroleum ether was filtered through Whatman #1 filter paper into another one liter Erlenmeyer flask which contained three boiling stones. Each flask was rinsed twice with 50 ml of 30-60° petroleum ether and transferred. Then the flasks were evaporated to dryness on the steam bath.

Assay for Radioactivity

The radioactivity of the NSF was assayed by liquid scintillation counting in a Packard "Tri-Carb" liquid scintillation spectrometer Model 3003. The solubility of the NSF lends itself readily to liquid scintillation counting. The advantages of liquid scintillation are its ease of use and its high efficiency. The one disadvantage of this method is that once the material has been picked up in scintillation fluid it cannot be recovered.

Procedure:

The scintillation fluid used for counting was made up of four grams of 2, 5-diphenyloxazole (PPO) and 30 mg of 1, 4-bis-(phenyl-oxazolyl)-benzene (POPOP) dissolved in one liter of reagent toluene. First the boiling stones were placed in a counting vial; then to each flask was added five ml of scintillation fluid. The five ml were quantitatively transferred to the vial; then another five ml were added to each flask and these five ml too were transferred quantitatively. The vials were cooled prior to counting. The spectrometer with a window setting of 50 to 1000 and a gain of eight percent has an efficiency of 85%. All the activity reported in this study has been corrected for background.

Assay for Acetyl-CoA-Kinase--Hydroxamate Method

When acetate is incubated in the presence of ATP, coenzyme A (CoA), acetyl-CoA-kinase, and the necessary cofactors, free acetate is converted to acetyl-CoA. There are two methods for measuring the activity of acetyl-CoA-kinase. The first method measures the amount of acetyl-CoA formed either during or after the reaction. The second method measures the amount of unreacted coenzyme A remaining when the reaction is over.

There are two methods used for measuring the amount of acetyl-CoA formed (60). The first is an enzymatic method whereby acetyl-CoA-kinase is coupled with another enzymatic system. The second method is a chemical method; this is one of the methods used in this study. Both methods are based on the recycling of CoA by transferring the acetyl group from acetyl-CoA to various acceptors. The assay procedure outlined by Jones and Lipmann(60) was used.

Chemically, hydroxylamine in the presence of acetyl-CoA will form hydroxamic acid, liberating coenzyme A and thereby recycling it so that it may again activate free acetate. The hydroxamic acid can then be measured colorimetrically with ferric chloride.

$$\text{acetyl-CoA} + NH_2OH \rightarrow \text{acetyl-NHOH} + \text{CoA}$$

Reagents:

8.1×10^{-4}M coenzyme A

0.1M adenine triphosphate, sodium salt, in 1M phosphate buffer, pH 7.5

0.2M potassium acetate

1M potassium phosphate buffer, pH 7.5

1M potassium fluoride

0.2M magnesium chloride

0.2M reduced glutathione, neutralized to pH 4.5 with 4N KOH

2M hydroxylamine solution, pH 7.4 (this reagent must be freshly prepared by mixing volumes of 4N $NH_2OH \cdot HCl$ and 4N KOH)

ferric chloride reagent (10% $FeCl_3 \cdot 6H_2O$ and 3.3% trichloroacetic acid in 0.66N HCl)

Procedure:

Acetyl-CoA-kinase activity was measured by the intensity at 540 mμ of the colored complex that was formed when the ferric chloride reagent was mixed with the hydroxamic acid. The absorption was measured on a Beckman Model DB Spectrophotometer. To a 3 ml conical centrifuge were added 0.1 ml CoA (0.081 μmoles), 0.1 ml ATP (10.0 μmoles), 0.1 ml KAcetate (20 μmoles), 0.1 ml phosphate buffer (100 μmoles), 0.05 ml KF (50 μmoles), 0.05 ml $MgCl_2$ (10 μmoles), 0.05 ml glutathione (10 μmoles), 0.1 ml hydroxylamine

(200 μmoles), and 0.40 ml of 60,000 x g supernatant. As controls, the 60,000 x g supernatant and cofactors were assayed alone with water replacing whatever was omitted from the assay mixture. Two determinations were made per experiment.

These tubes were placed in a Research Specialties oscillating water bath at 37°C for two hours and 50 minutes; this was the routine incubation time unless otherwise noted. The reaction was stopped with 1.5 ml ferric chloride reagent. The tubes were centrifuged in a refrigerated Servall centrifuge for ten minutes at 10,000 rpm. The supernatants were decanted into another 3 ml conical centrifuge tube and the absorbance read at 540 mμ against a blank composed of 1.0 ml of water and 1.5 ml of ferric chloride reagent. This was the routine assay procedure unless otherwise noted. It was found (9) that one optical density unit is equal to 1.56 μmoles of acetohydroxamic acid per ml.

Assay for Acetyl-CoA-Kinase--Nitroprusside Method

The second method employed in this study of acetyl-CoA-kinase measures the amount of unreacted CoA remaining at the end of the reaction (23).

Reagents:

.0191M coenzyme A

1.6M Tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.0

.048M magnesium chloride

.038M adenine triphosphate, sodium salt, pH 7.5

.24M potassium chloride

.048M potassium acetate, pH 8.0 (titrated to pH 8.0 with Tris-HCl buffer, pH 8.0)

30% metaphosphoric acid

Procedure:

Acetyl-CoA-kinase activity was measured by the intensity of the complex that was formed when nitroprusside was mixed with unreacted coenzyme A. To a 3 ml conical centrifuge tube were added 0.1 ml CoA (1.90 μmoles), 0.1 ml Tris-HCl buffer (160 μmoles), 0.1 ml $MgCl_2$ (4.8 μmoles), 0.1 ml ATP (3.8 μmoles), 0.1 ml KCl (24 μmoles), 0.1 ml KAcetate (24 μmoles) and up to 1.0 ml of protein solution. As controls, coenzyme A, coenzyme A and cofactors, protein solution, and coenzyme A and protein solution were left out of the assay mixture and replaced with H_2O. Two determinations were made per experiment.

These tubes were placed in a Research Specialties oscillating water bath at 37°C for four minutes; this was the routine incubation time unless otherwise noted. The reaction was stopped with 0.4 ml of 30% metaphosphoric acid and then centrifuged for ten minutes at 10,000 rpm. Then 1.0 ml of this mixture was removed for use in the nitroprusside reaction (56). This was the routine assay procedure unless otherwise noted.

the hydroxamate method, the yeast preparation converted 1.55 μmoles of acetate to acetyl-CoA per ml of protein solution per minute, while, with the nitroprusside method, the yeast preparation converted 1.44 μmoles of acetate to acetyl-CoA per ml of protein solution per minute.

Assay of Inhibition of Acetyl-CoA-Kinase Activity by a Factor in the 60,000 x g Supernatant

In order to determine if there were any factors in the 60,000 x g supernatant which would interfere with the formation of acetyl-CoA, acetyl-CoA-kinase was partially purified from yeast. The nitroprusside assay procedure was used in this study.

Procedure:

To a 3 ml conical centrifuge tube were added 0.1 ml CoA (1.9 μmoles), 0.1 ml Tris-HCl buffer (160 μmoles), 0.1 ml $MgCl_2$ (4.8 μmoles), 0.1 ml ATP (3.8 μmoles), 0.1 ml KCl (24 μmoles), 0.1 ml KAcetate (24 μmoles), 0.2 ml of yeast and 0.8 ml of 60,000 x g supernatant. To another 3 ml conical centrifuge tube were added all of the above except the 0.8 ml of 60,000 x g supernatant; the supernatant was replaced by water. To a third 3 ml conical centrifuge tube were added all of the above except the 0.2 ml of yeast; the yeast, too, was replaced by water. The controls were the same as those used in the standard assay procedure for acetyl-CoA-kinase. The rest of the procedure was identical to that previously described.

Concentration of Acetyl-CoA-Kinase Activity

There are several ways of purifying acetyl-CoA-kinase, but the method used in this study was a modification of the method used by Jones and Lipmann (60) in purifying the enzyme from yeast.

Reagents:

fresh yeast

1 M K_2HPO_4

2% protamine sulfate solution

0.05 M potassium bicarbonate

solid ammonium sulfate

Procedure:

Four hundred grams of fresh yeast (RED STAR Brand) were mixed with 400 ml of ether and 600 grams of dry ice. The mixture was stirred for 30 minutes; then the liquid was poured off and the frozen yeast was placed on a cloth and air blown over it to drive off any residual ether. An additional 600 grams of dry ice were added in order to keep the yeast frozen. Thirty-three ml of 1 M K_2HPO_4 were added for every 1000 grams of yeast, and the mixture was stirred overnight at 0 to 5 °C. The mixture was centrifuged at 15,000 rpm in a Servall refrigerated centrifuge for 20 minutes. The supernatant was kept on ice while the residue was discarded.

The ribonucleic acid (RNA) content was determined as outlined by Jones and Lipman (60). For every ml of RNA, 0.025 ml of 2% protamine sulfate was added. The supernatant was centrifuged at 10,000 rpm for ten minutes. The supernatant was made 55% saturated (350 g solid $(NH_4)_2SO_4$/1000 ml of supernatant) with solid ammonium sulfate. After standing for one hour on ice, the supernatant was centrifuged at 15,000 rpm for ten minutes. The precipitate was dissolved in 5 ml of 0.05 M $KHCO_2$ (.066 ml of 0.05 M $KHCO_2$ per ml of supernatant after protamine sulfate precipitation). The resulting supernatant was made 15% saturated with solid ammonium sulfate. After standing on ice for one hour, the supernatant was centrifuged at 15,000 rpm for ten minutes. The precipitate was dissolved in 5 ml of 0.05 M $KHCO_3$. This preparation was used for comparison studies of the acetyl-CoA-kinase from the 60,000 x g supernatant and from yeast. The acetyl-CoA-kinase was partially purified in freshly prepared 60,000 x g supernatant by the same procedure. The yeast preparation was further purified for the inhibition studies in the following manner. The saturation of the solution resulting from the first ammonium sulfate precipitation was increased five to ten percent (or until the precipitate began to appear). This precipitate was again dissolved in 5 ml of 0.05 M $KHCO_3$. This last fraction is known as the partially purified yeast fraction. The final solution was only stable for about two weeks even if kept frozen.

One particular attempt at concentrating activity in the 60,000 x g supernatant was made using the procedure described above up to the point of making the supernatant 55% saturated. In this case, the saturation was brought up to 80% with solid ammonium sulfate. After standing for one hour on ice, the supernatant was centrifuged at 15,000 rpm for ten minutes and the precipitate was dissolved in 8.0 ml of 0.05 M $KHCO_3$. The protein concentration of various samples was determined by the method of Lowry (72), with bovine serum albumin as a standard.

Column Chromatography

The columns used in this study were Kontes Chromaflex, 2.5 x 50 cm.

P-2 and P-100 BioGel, 50-100 mesh, (from Bio-Rad Laboratory) were equilibrated at 2-4° C in 0.1 M phosphate nicotinamide buffer, pH 7.0, and 0.1 M phosphate buffer, pH 7.0, respectively, for at least 24 hours before packing. The P-2 column was packed in the cold (2-4° C) to a height of 10 cm. Thirty ml of 60,000 x g supernatant were placed upon the column. When all the material had drained upon the column, collection of the first fraction began. The first 42 ml were designated as the first fraction (it was the volume necessary in order to remove all the heme-like colored material from the column). The column was eluted with 0.1 M phosphate

nicotinamide buffer. The second fraction was the next 42 ml collected. The P-100 column was also packed in the cold (2-4° C) to a height of 40 cm. The column was eluted with 0.1 M phosphate buffer, pH 7.0. After 12 ml of freeze-dried 60,000 x g supernatant were placed upon the column, the flow rate was adjusted so that 13.0 ml were collected per hour. The fractions were collected with a Technicon Fraction Collector. Six and one half ml were collected per tube. Fifty tubes were collected in about 24 hours. The column was monitored by reading the optical density of each tube at 280 mμ and 260 mμ.

The column used in the acetyl-CoA-kinase experiment was the same as described above. The P-2 column was packed at room temperature to a height of 40 cm after the gel was equilibrated at room temperature overnight. The column was eluted with distilled water. After the sample was placed on the column, the flow rate was adjusted to 16 drops/minute and 6.5 ml fractions were collected.

Dialysis of 60,000 x g Supernatant

In all the dialysis experiments, seamless regenerated cellulose dialysis tubing was employed. All the experiments were carried out in the cold (2-4° C). The 60,000 x g supernatant was dialyzed either against distilled water or 0.1 M phosphate nicotinamide buffer, pH 7.0. The time of dialysis varied somewhat between 20 and 24 hours. In the acetyl-CoA-kinase activity experiment, 30 ml of 60,000 x g

supernatant were dialyzed against four liters of distilled water for 20 hours. In the acetyl-CoA-kinase inhibition experiment, 10 ml of 60,000 x g supernatant were dialyzed against one liter of distilled water overnight. In the ATPase study, 75 ml of 60,000 x g supernatant were dialyzed against five liters of distilled water for 24 hours. In the study on the stability of the 60,000 x g supernatant, 30 ml of 60,000 x g supernatant were dialyzed against one liter of either 0.1 M phosphate nicotinamide buffer or distilled water for 24 hours.

Assay for Phosphomonoesterase

Nonspecific phosphomonoesterase activity can be measured by following the release of p-nitrophenol from p-nitrophenylphosphate at 410 mμ.

Reagents:

5×10^{-3} M p-nitrophenylphosphate in 0.1 M phosphate nicotinamide buffer, pH 7.0.

ATP - 2 mg/ml

NAD - 5 mg/ml

0.1 M phosphate nicotinamide buffer, pH 7.0

10% trichloroacetic acid (TCA)

1.0 N KOH

Procedure:

Phosphomonesterase activity was measured by the intensity at 410 mμ of p-nitrophenol released from p-nitrophenylphosphate. The absorption was measured on a Beckman Model DB Spectrophotometer. To a test tube were added 1.0 ml p-nitrophenylphosphate (5.0 μmoles), 1.0 ml ATP, 1.0 ml NAD, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant. ATP and NAD were not required for the assay but were added in order to be consistent with the standard assay procedure for the incorporation of MVA-2-C^{14} into the NSF. The total assay volume was kept at 10.5 ml. If additional reagents were added to the above assay mixture, less buffer was used; if any of the above reagents were omitted from the assay, they were replaced by buffer. As controls, p-nitrophenylphosphate and 60,000 x g supernatant were assayed alone. Two determinations were made per experiment.

The tubes were placed in a Research Specialties oscillating water bath for two hours and 50 minutes at 37° C; this was the routine incubation time unless otherwise noted. The reaction was stopped with 10% TCA. After the tubes were allowed to stand in ice for five minutes the assay mixture was centrifuged for ten minutes at 10,000 rpm. The supernatants were decanted into another test tube and the solution was neutralized with 1.0 N KOH. Three to four ml of 1.0 N KOH were required to bring the pH to 7.2-7.4. The final volume was

brought to 19.5 ml with water when necessary. The optical density of each sample was read against water at 410 mμ. This was the routine assay procedure unless otherwise noted.

The standard curve for p-nitrophenol was made in the following manner. A stock solution of 3×10^{-4} M p-nitrophenol in 0.1 M phosphate nicotinamide buffer, pH 7.0, was diluted with buffer to known concentrations. Then, to 5.0 ml of p-nitrophenol was added 5.5 ml of 0.1 M phosphate nicotinamide buffer; to this was added 5 ml of 10% TCA followed by 4 ml of 1.0 N KOH. For each concentration two determinations were run. From Figure 8 it can be seen that there is linearity between 3×10^{-4} M and 1.5×10^{-5} M p-nitrophenol. One optical density unit is equal to 1.71 μmoles of p-nitrophenol.

Assay for Adenosinetriphosphatase

Adenosine triphosphatase (ATPase) activity can be followed by measuring the inorganic phosphate released from a suitable substrate. In the presence of ammonium molybdate, orthophosphate will form phosphomolybdic acid. A sulfhydryl compound, acting as a reducing agent, will produce in the presence of sulfite a blue complex which can be measured colorimetrically. A method (55) has been developed whereby pyrophosphate undergoes a similar reaction. The two complexes can be separated and therefore both can be measured in the presence of one another. A modification of this method (55)

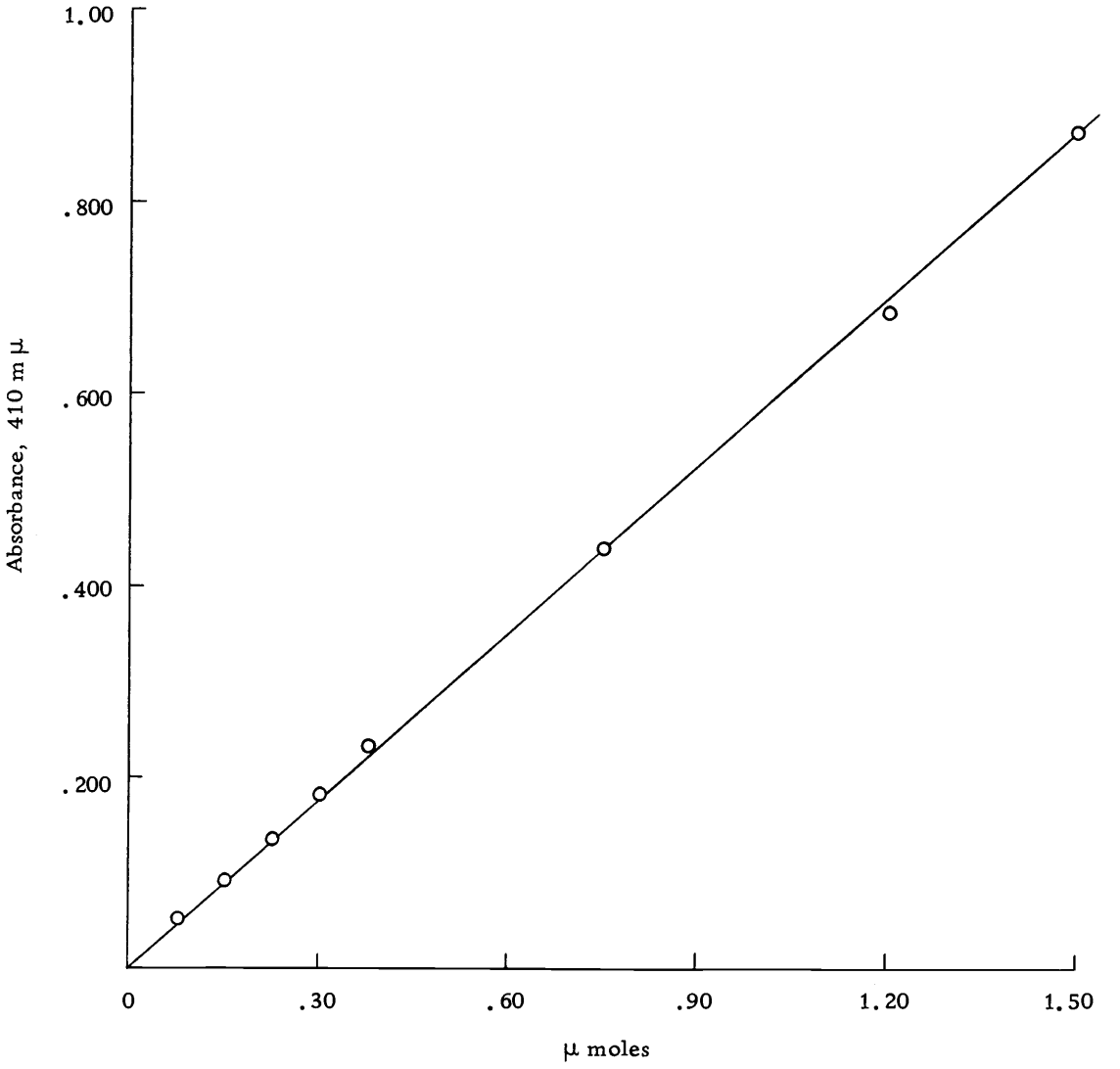


Figure 8. Standard curve for p-nitrophenol.

was used in this investigation.

Reagents:

- ATP - 2 mg/ml (3.3 μmoles/ml)

  .1 M Tris-HCl buffer, pH 7.0

  10% TCA

Procedure:

ATPase activity can be followed by measuring at 775 mμ the blue complex that is formed with the reduction of phosphomolybdic acid by thioglycerol in the presence of sulfite. The pyrophosphate complex can be measured at 575 mμ. The absorption was measured on a Beckman Model DB Spectrophotometer. To a test tube were added 1.0 ml ATP, 4.5 ml .1 M Tris-HCl buffer, and 5.0 ml of dialyzed 60,000 x g supernatant. As controls, ATP and dialyzed 60,000 x g supernatant were assayed alone. Two determinations were made per experiment. Due to the fact that there is endogenous orthophosphate and pyrophosphate in the 60,000 x g supernatant, two complete sets of tubes were run. One set was stopped at zero time while the other was incubated two hours and 50 minutes in a Research Specialties oscillating water bath at 37°C; these were the routine incubation times unless otherwise noted. The reaction was stopped with 5.0 ml of 10% TCA. After the tubes were allowed to stand in ice for five minutes the mixture was centrifuged for ten minutes at 10,000 rpm in a Servall refrigerated centrifuge. The supernatant

was decanted into a test tube. One ml of this mixture was taken and diluted with 2.0 ml of distilled water. Then one ml of this diluted mixture was taken for the determination of orthophosphate and pyrophosphate.

Determination of Orthophosphate and Pyrophosphate

Reagents:

Molybdate Reagent - 2.5 gm $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 100 ml 10 N H_2SO_4

Bisulfite Solution A - 10 gm $NaHSO_3$ and 0.5 gm Na_2SO_3 in 100 ml distilled water

Bisulfite Solution B - dilute solution A 1:15

Thioglycerol Solution - dilute 0.5 ml monothiol glycerol (Sigma Chemical Co.) to 5.0 ml with distilled water and store at 4°C. This solution is unstable and must be freshly prepared daily.

Isoamyl alcohol

95% Ethanol

Procedure:

To the 1.0 ml sample containing orthophosphate and pyrophosphate was added 1.4 ml of distilled water. To this was added 0.15 ml of the molybdate reagent, 0.30 ml of solution A and 0.15 ml of thioglycerol solution. The total volume was 3.0 ml. After ten

minutes, 3.0 ml of isoamyl alcohol were added. The mixture was shaken well and centrifuged immediately in a clinical centrifuge. To 1.4 ml of the upper phase, which contained the orthophosphate complex, were added 0.15 ml of H_2O and 0.60 ml of ethanol. After 20 minutes the optical density of this solution was read at 775 mμ. After removal of the rest of the upper phase, 1.4 ml of the lower phase (containing the pyrophosphate complex) were taken and added to 0.30 ml ethanol, 0.15 ml thioglycerol, and 0.30 ml of solution B. The optical density of this solution was read immediately at 575 mμ. Both complexes were read against a reagent blank in which the 1.0 ml orthophosphate and pyrophosphate solution was replaced by water.

Standard Curves for the Determination of Orthophosphate and Pyrophosphate

Reagents:

$Na_4P_2O_7 \cdot 10\ H_2O$ - 44.61 mg/100 ml distilled water. Add 1 drop chloroform and store at 4° C. This solution contains 1 μmole pyrophosphate per ml.

K_2HPO_4 - 136.13 mg/100 ml distilled water. Add 1 drop chloroform and store at 4° C. This solution contains 10 μmoles orthophosphate per ml.

Procedure:

These stock solutions were diluted to various concentrations

and then 1.0 ml was taken for determination of orthophosphate and pyrophosphate as described previously. As can be seen in Figure 9, for pyrophosphate there is linearity between 0 and 0.3 μmoles of pyrophosphate. One optical density unit equals .296 μmoles of pyrophosphate. There was no contamination of orthophosphate in the pyrophosphate standard. As can be seen in Figure 10, for orthophosphate there is linearity between 0 and 0.4 μmoles of orthophosphate. One optical density unit equals .844 μmoles of orthophosphate. The orthophosphate standard has .049 μmoles of pyrophosphate per μmole of orthophosphate.

Paper Chromatography of the NSF Material

The standard assay procedure and the isolation of the NSF were the same as previously described except that distilled petroleum ether was used. After the NSF sample was evaporated to dryness on the steam bath, the sample was transferred to a 50 ml round bottom flask with 200 ml of distilled chloroform. The chloroform was evaporated to dryness on a rinco evaporator. Just prior to spotting, the sample was dissolved in 0.2 ml of distilled chloroform.

The samples were spotted on 14 x 16 inch Whatman #1 paper which was prepared in the following manner. First the paper was impregnated with 5% mineral oil in petroleum ether. Then the paper was pre-run in 85% acetic acid for 17 hours.

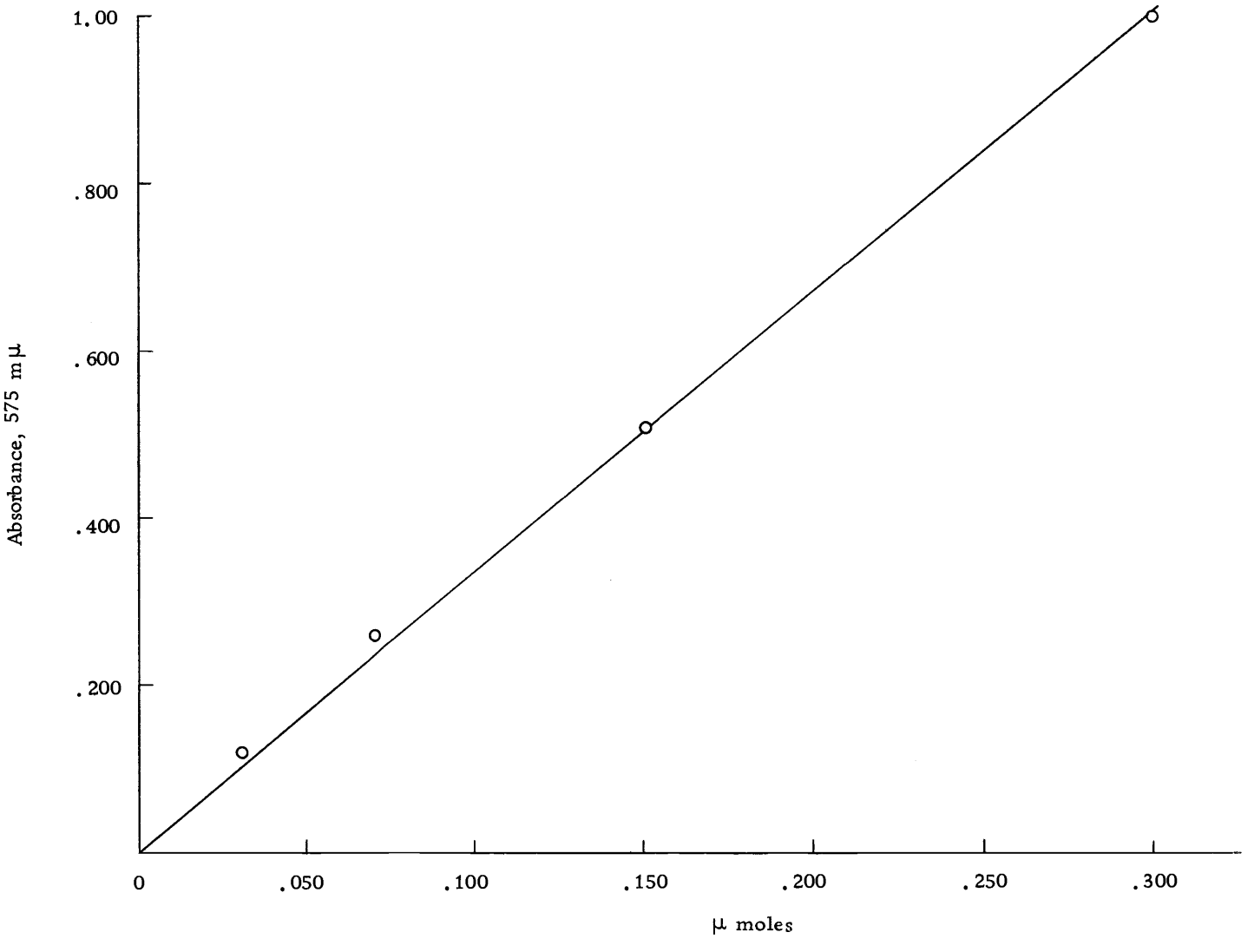


Figure 9. Standard curve for pyrophosphate.

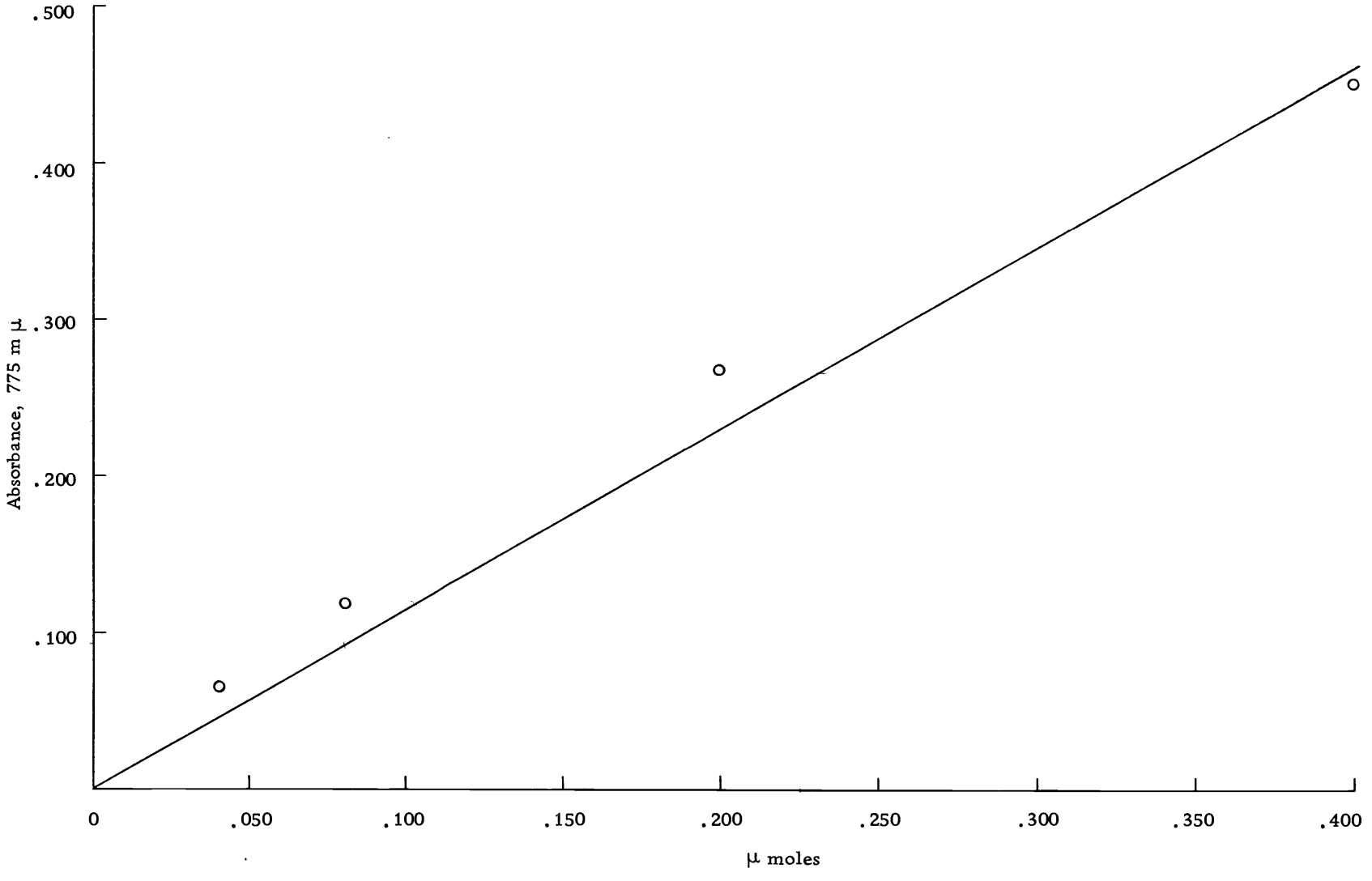


Figure 10. Standard curve for orthophosphate.

Each sample was spotted along with a standard of squalene, cholesterol, and farnesol (50 μg each). Then the paper was run in 85% acetic acid for 17-24 hours. The compounds were detected with ten percent (w/v) phosphomolybdic acid in ethanol after the paper was dried.

The paper in some cases was cut into strips and counted in the liquid scintillation counter. If the NSF compounds are sprayed with phosphomolybdic acid before counting, there will be 80% reduction of the counts by the acid treatment.

Preparation of Rat Liver Homogenate

White Sprague-Dawley rats were obtained without charge from Dr. Frank Dost of Oregon State University. The procedure used for the preparation of rat liver homogenate was that of Wright and Cleland (128).

Immediately upon sacrifice the livers were taken and placed in a distilled water-ice mixture. After the livers were minced with scissors, they were ground in a loose-fitting Potter-Elvehjem homogenizer with three times their volume of 0.1 M phosphate nicotinamide buffer, pH 7.0. The homogenized liver was centrifuged in a clinical centrifuge at 200 x g in the cold for three minutes. The supernatant was decanted and used immediately.

Purification of Silica Gel H

Silica gel H (Brinkmann Instruments) was purified for preparative chromatography according to the method of Parker and Peterson (84). One hundred and twenty-five grams of silica gel H were prepared at a time. All solvents which were reagent grade were distilled prior to use. Two discs of Whatman #2 filter paper were placed in a large Büchner funnel. Over the silica gel H was poured one liter of methanol: formic acid: chloroform, two: one: one (v/v/v). This was followed by two liters of distilled water. The wet gel was placed in a glass container lined with aluminum foil and was dried at approximately 110 °C for 48 hours. Then the gel was stored in a closed container until needed.

Preparation of Preparative Layer Chromatography Plates

A preparative plate, 40 cm by 20 cm, was prepared by mixing 50 grams of purified silica gel H and 85 to 90 ml of distilled water in a Waring Blendor for five minutes at high speed or until a very smooth slurry was formed. The slurry was applied with a Desaga adjustable applicator (Brinkman Instruments). The thickness of the plate was 750 μ. The plate was air dried for at least one hour and then dried one hour at 110 °C. The plates were then stored until needed. This procedure will also make two 20 cm by 20 cm plates

of the same thickness.

Preparation of Alumina Column

A 1/2 inch by 13 inch Pyrex column specifically designed for liquid chromatography (Chromatronix Incorporated) was used for the alumina column.

Approximately 30 grams of aluminum oxide were placed in a beaker. The aluminum oxide was washed three times, each time with one hundred ml of distilled 30-60° petroleum ether. A disk of Whatman #1 filter paper was placed in the bottom of the column. The alumina which had been air dried was poured into the column with slight air pressure. When the column was packed sufficiently, 30-60° petroleum ether was placed on the column to saturate it. A disk of Whatman #3 filter paper was placed on top of the column.

Before any sample was placed on the column, the column was washed with 400 ml of distilled 30-60° petroleum ether. This was followed by 800 ml of distilled acetone:ether, one:one (v/v). Finally the column was washed with 400 ml of distilled 30-60° petroleum ether.

Preparation of Sample for Estimation of Prenols and Isoprenoids

Eighty ml of "ultra" crude aorta homogenate (the material which had been ground in a Waring Blendor but not squeezed through cheese

cloth) or crude aorta homogenate were saponified with 80 ml of 10% alcoholic KOH overnight. Then each flask was extracted with 800 ml of distilled petroleum ether, 200 ml at a time. Four hundred ml of aorta homogenate were extracted at a time; in all, 6400 ml of aorta homogenate were extracted in this manner. The residue in each flask was dissolved in four to eight ml of distilled absolute ethanol; this was centrifuged at 10,000 rpm for ten minutes in a refrigerated Servall centrifuge in order to remove that material which had not gone into solution. In order to precipitate out the cold cholesterol, water was added dropwise until the precipitate started to fall out of solution. The supernatant was placed in ice for 15 minutes and then centrifuged at 10,000 rpm for ten minutes in a refrigerated Servall centrifuge. Then the procedure was repeated until no more precipitate fell out of solution upon the addition of water (repeated at least three times). The supernatant was evaporated to dryness on a rinco evaporator. From 6400 ml of aorta homogenate 1026 mg of cholesterol-free NSF were recovered.

Separation of Prenols and Isoprenoids

The 1026 mg of NSF material were dissolved in 30 ml of distilled chloroform. Two ml of the sample were spotted in a long narrow band on a long (20 x 40 cm) preparative silica gel H plate which had been impregnated with 5% mineral oil in 30-60° petroleum

ether and prerun in 200 ml of 85% acetic acid for 48 hours in order to remove the impurities in the mineral oil. With the sample, cholesterol, farnesol, and squalene were spotted together as standards (50 μg each). In addition, 20 to 40 μl of the sample were spotted next to the standards, both of which were sprayed with 10% phosphomolybdic acid in ethanol after the plate had been run again in 85% acetic acid for 17 hours. A total of 15 plates were spotted and run. The distribution of the cholesterol-free NSF material and the standard are shown in Figure 11.

The component with an R_F the same or close to that of farnesol is the major component of interest because it contains the majority of the activity if a "hot" NSF sample is run in the same manner. The silica gel was removed from the area and extracted with chloroform. The silica gel from each plate was extracted twice with 30 ml of chloroform. Each time, the supernatant was centrifuged at 10,000 rpm for ten minutes in a Servall refrigerated centrifuge. The supernatants were evaporated to dryness.

In order to remove the mineral oil, the sample was again spotted and run on a preparative plate. The sample was dissolved in 16.0 ml of chloroform and spotted on 13 preparative plates (20 x 20 cm) in a long narrow band. The same standards were run and sprayed as those described for the long plates. The plates were run in 200 ml of benzene:ethyl acetate (5:1) for 90 minutes. The

Figure 11. Preparative layer chromatography of cholesterol-free NSF material. A. Cholesterol-free material B. Standards, left to right, squalene, cholesterol, and farnesol.

distribution of this sample and the standards is shown in Figure 12. The component with an R_F just above that of cholesterol-farnesol is the major component of interest. This band was removed and extracted in the same manner as described above for the previous separation. Extraction of the band from the 13 plates produced 57.1 mg of material (prenol sample).

Alumina Chromatography of Prenol Sample

The prenol sample was dissolved in 4.0 ml of petroleum ether and the sample was placed on two separate alumina columns (2.0 ml per column). Then the column was washed with 400 ml of petroleum ether and the flow rate was adjusted so that 1040 drops/tube (10 ml per tube) or 70-80 drops/minute were collected. The fractions were collected with a Packard Fraction Collector. Next the column was eluted with a gradient of acetone in ether (0 to 50% acetone) at the same rate, using a Buchler Varigrad. In order to ascertain in which tube the prenol sample of interest appeared, half of a "hot" NSF sample from a large incubation (three flasks) was placed on an alumina column and the column was run as described above. There was no radioactivity in the petroleum ether fraction, but the radioactivity obtained from eluting the column with ether-acetone is shown in Figure 13.

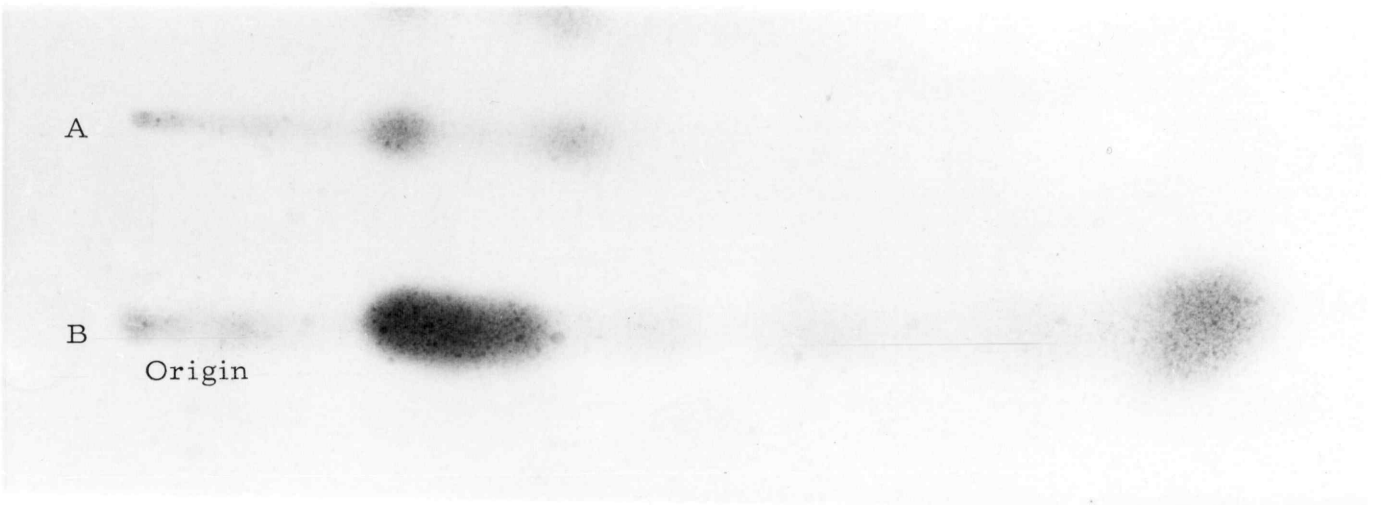


Figure 12. Preparative layer chromatography of prenol sample. A. Prenol sample B. Standards, left to right, cholesterol-farnesol, and squalene.

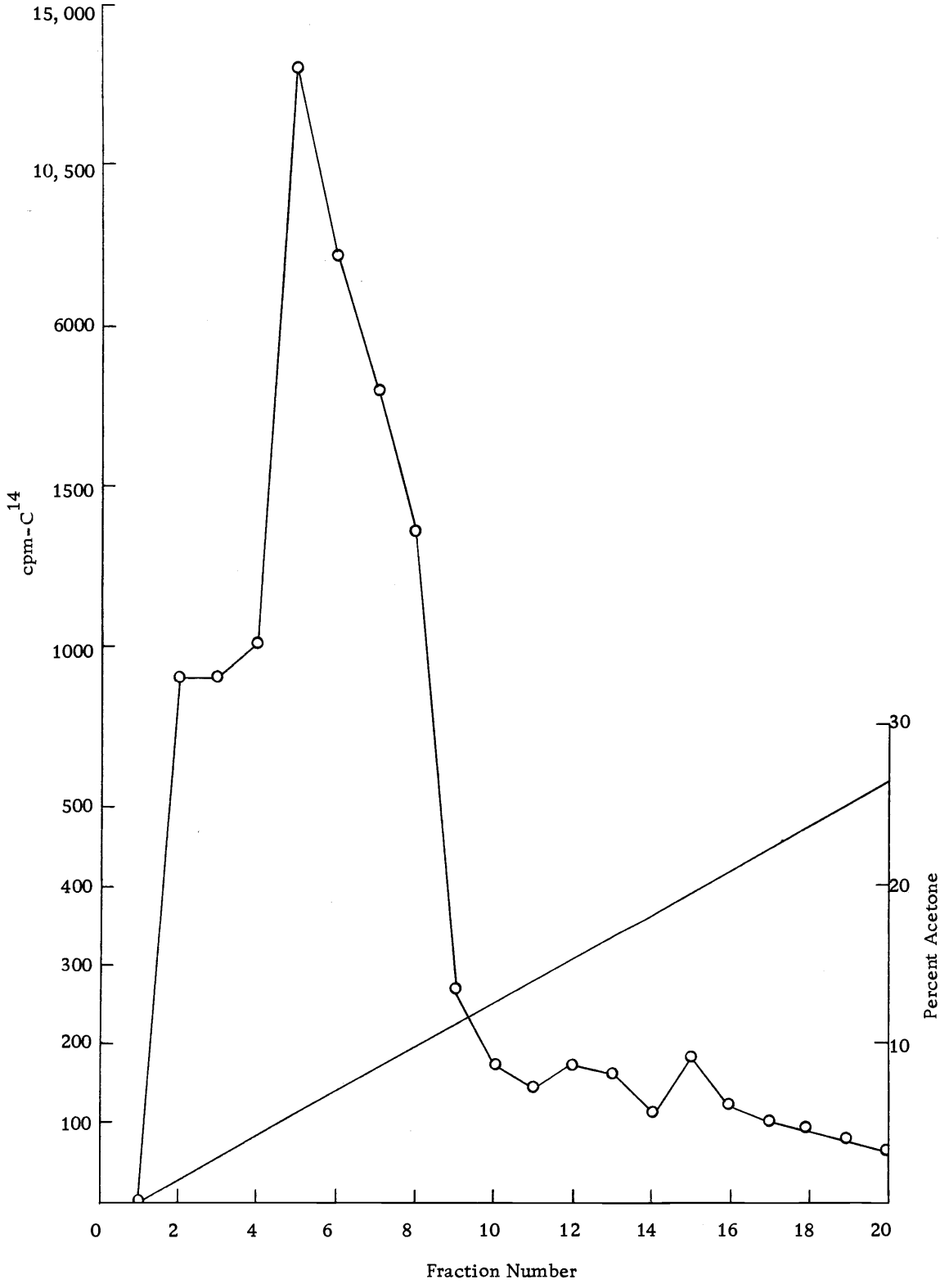


Figure 13. Alumina column chromatography of NSF material.

Gas Chromatography of Prenol Sample

An aliquot of the prenol sample was used for gas chromatography on a Model 402 F and M Gas Chromatograph. Chromatography of the prenol sample was performed on a column (4 feet by 1/4 inch) of six percent w/w diethyleneglycol succinate on Diataport S (80-100 mesh). The prenol sample was eluted from the column employing a temperature gradient of 20° per minute from 130 to 180°C, according to the procedure described by Walsh, Teal and Gamble (115).

Mass Spectrum and Infrared Spectrum of Prenol Sample

The mass spectrum of the prenol sample was determined by Mr. Donald A. Griffin of the Department of Agricultural Chemistry at Oregon State University. The mass spectra were obtained with a direct probe inlet using a CH7 Massenspektrometer. The temperature of the probe was 50°C for the farnesol sample and 150°C for the prenol sample. The results were interpreted by Dr. Leonard M. Libbey of the Department of Food Science and Technology at Oregon State University.

The infrared spectrum of the prenol sample was determined by Dr. R. Hague of the Department of Agricultural Chemistry at Oregon State University. The infrared spectrum was obtained on a Perkin-Elmer Model 457 Infrared Spectrophotometer.

EXPERIMENTAL RESULTS

Stability of 60, 000 x g Supernatant

Stability at 2-4° C

Table 1 summarizes the results of leaving the 60, 000 x g supernatant at 2-4° C for various lengths of time. As can be seen in Table 1, the 60, 000 x g supernatant is stable over a 72 hour time period with apparently only a 17% decrease in activity (as measured in terms of counts per minute NSF) during the last 24 hour period. Even when the 60, 000 x g supernatant is frozen for 24 hours there is no loss in activity (Table 1).

Stability after Lyophylization

Twenty ml of 60, 000 x g supernatant were lyophylized on a VirTis Freeze-Dryer. The dried homogenate was stored overnight at 2-4° C. Then the dried 60, 000 x g supernatant was dissolved back into 20 ml of distilled water and tested for the incorporation of MVA-2-C^{14} into the NSF. It can be seen in Table 2 that there is only about a 33% decrease in activity if the freeze-dried 60, 000 x g supernatant is compared to the 60, 000 x g supernatant which has been kept at 2-4° C for 24 hours.

Table 1. Stability of 60,000 x g supernatant at 2-4° C.[1]

No. of flasks	Time at 2-4° C (hours)	Counts per min. NSF	
		Range	Average
3	0	1736-2089	1918
2	3	1979-1993	1956
3	6	1916-2503	2218
3	24	1958-2051	2008
3	72	1528-1632	1592
3	24 (0° C)	1846-2116	2011

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

Table 2. Stability of lyophylized 60,000 x g supernatant.[1]

No. of flasks	60,000 x g supernatant	Counts per min. NSF	
		Range	Average
3	24 hrs at 2-4° C	3353-3804	3652
3	lyophylized	2294-2731	2450

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

Stability after Dialysis

The 60,000 x g supernatant was dialyzed for 24 hours at 2-4° C. At the end of this time the supernatant was assayed for the incorporation of labeled MVA into the NSF. As can be seen in Table 3, almost all of the activity was lost. As a control, the 60,000 x g supernatant was placed in dialysis tubing and also left for 24 hours at 2-4° C. There was no loss in activity with either control.

In order to determine if the activity of the dialyzed 60,000 x g supernatant could be restored, the dialysate was evaporated to dryness under reduced pressure on a rinco evaporator. Then the dialysate was dissolved in distilled water, in the same volume that was dialyzed. To the 60,000 x g supernatant which had been dialyzed was added back the dialysate in a ratio of 1:1. As can be seen in Table 4, there is a further loss in activity beyond that which one would expect under a simple dilution effect. Therefore it appeared that some factor was added back to the dialyzed 60,000 x g supernatant which caused a further decrease in activity.

In order to find out if there was some factor removed from the 60,000 x g supernatant which was responsible for its initial low activity or if there was some factor added back to it from the dialysate which only caused a further decrease in activity, the experiment summarized in Table 5 was performed. If 0.1 M phosphate buffer

Table 3. Stability of dialyzed 60,000 x g supernatant.[1]

No. of flasks	60,00 x g Supernatant at 2-4° C	Counts per min. NSF	
		Range	Average
3	0 hrs	1254-1590	1422
3	24 hrs	1611-1811	1711
3	24 hrs in dialysis bag	1632-2302	1989
3	Dialyzed 24 hrs against buffer[2]	178-293	191

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 2.5 ml 0.1 M phosphate nicotinamide buffer, 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

[2]0.1 M phosphate nicotinamide pH 7.0.

Table 4. Reconstitution of dialyzed 60,000 x g supernatant.[1]

No. of flasks	60,000 x g Supernatant at 2-4° C	Counts per min. NSF	
		Range	Average
3	0 hrs	3770-3859	3800
3	24 hrs	3697-3976	3876
3	24 hrs diluted with H_2O (1:1)	1803-1989	1925
3	Dialyzed 24 hrs against H_2O	1438-1666	1552
3	Dialyzed 24 hrs and diluted with dialysate (1:1)	116-130	124

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.5 μc MVA-2-C^{14}, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

Table 5. Effect of reconstitution of 60,000 x g supernatant.[1]

No. of flasks	60,000 x g Supernatant at 2-4° C	Counts per min. NSF	
		Range	Average
3	24 hrs	3388-3529	3460
3	24 hrs diluted with H_2O (1:1)	1417-1509	1476
2	24 hrs diluted with dialyzed buffer (1:1)[2]	1452-1600	1504
3	24 hrs diluted with dialysate (1:1)	594-732	673

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,00 x g supernatant in a total volume of 10.5 ml.

[2]0.1 M phosphate nicotinamide buffer, pH 7.0

Table 6. Effect of boiling dialysis bag prior to dialysis.[1]

No. of flasks	60,000 x g Supernatant at 2-4° C	Counts per min. NSF	
		Range	Average
3	24 hrs	1680-2578	2181
3	Dialyzed 24 hrs against H_2O	2121-2595	2310

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

is placed in a dialysis bag and dialyzed 24 hours (against water) and the dialysate is added back to the 60,000 x g supernatant which has been standing at 2-4° C for 24 hours, there is a loss in activity beyond a simple dilution effect.

Some investigators routinely boil the dialysis tubing prior to dialysis in order to prevent contamination of their sample by contaminators that can be washed out of the bag. Thus, at this point in this study it was suspected that the instability of the 60,000 x g supernatant to dialysis was caused by the washing of some contaminator from the bag into the supernatant. The bag was boiled in 100 ml of distilled water three times. The 60,000 x g supernatant was then placed in the bag and dialyzed and tested. As can be seen in Table 6, there was no loss in activity. Thus, the 60,000 x g supernatant is stable to dialysis.

Stability on BioGel P-2 and P-100

The stability of the 60,000 x g supernatant to gel filtration was examined. In Table 7 it can be seen that mere exposure to polyacrylamide has no effect on the stability of the 60,000 x g supernatant.

Eighty ml of 60,000 x g supernatant were lyophylized, dissolved in 12.0 ml of distilled water and placed on a BioGel P-100 column. The protein was monitored by following the absorbance at 260 mμ

Table 7. Effect of BioGel P-2 on the 60, 000 x g supernatant.[1]

No. of flasks	Fraction	Counts per min. NSF	
		Range	Average
2	Control[2]	667-860	764
3	First	634-1027	840
3	Second	11-16	13

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60, 000 x g supernatant in a total volume of 10.5 ml.

[2]60, 000 x g supernatant left standing 3 hrs at 2-4 °C.

Table 8. Effect of BioGel P-100 on the 60, 000 x g supernatant.[1]

No. of flasks	Fraction	Counts per min. NSF	
		Range	Average
3	Peak I	214-294	257
3	Peak II	37-53	46
3	Peak I and II (1:1)	82-120	104

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60, 000 x g supernatant in a total volume of 10.5 ml.

(Figure 14). Both peaks were assayed for activity. The results are summarized in Table 8. There is still some activity after separation on P-100, most of it appearing in the first peak.

Acetyl-CoA-Kinase

Time Study

The formation with time of acetyl-CoA, measured as the number of μmoles of hydroxamic acid formed, is summarized in Figure 15. In three hours approximately 0.400 μmole of hydroxamic acid is formed.

ATP Dependence

The dependence on the amount of ATP required was investigated by the hydroxamate method. As can be seen in Table 9, endogenous ATP is not enough for full activity, but, at the same time, there is no significant increase in activity with high levels of ATP. There is no difference in activity when the sodium or potassium salt of ATP is used with the 60,000 x g supernatant.

CoA and Acetate Dependence

The dependence upon added acetate and CoA was investigated. As Table 10 shows, the μmoles of hydroxamic acid formed appear to be independent of either exogenous CoA or exogenous acetate. Thus

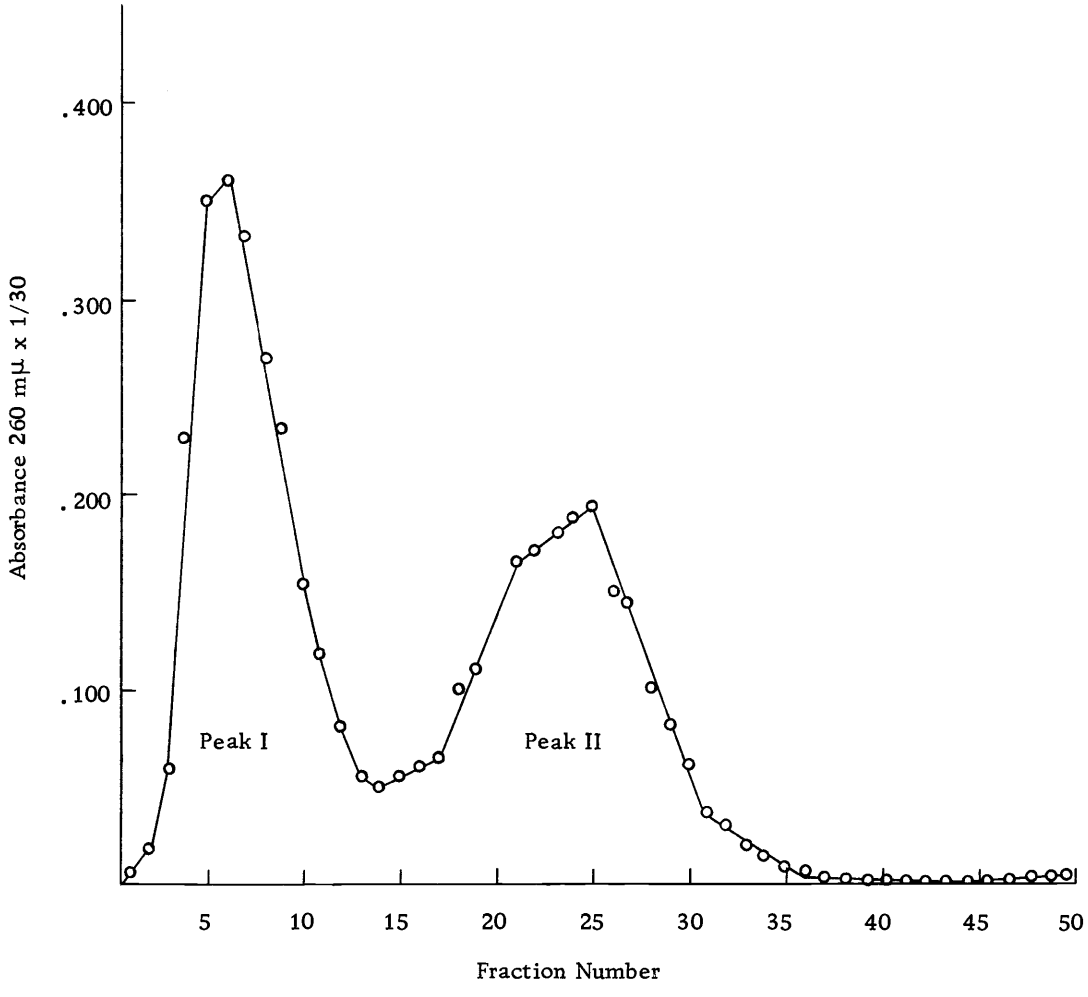


Figure 14. BioGel P-100 column chromatography of lyophylized 60, 000 x g supernatant.

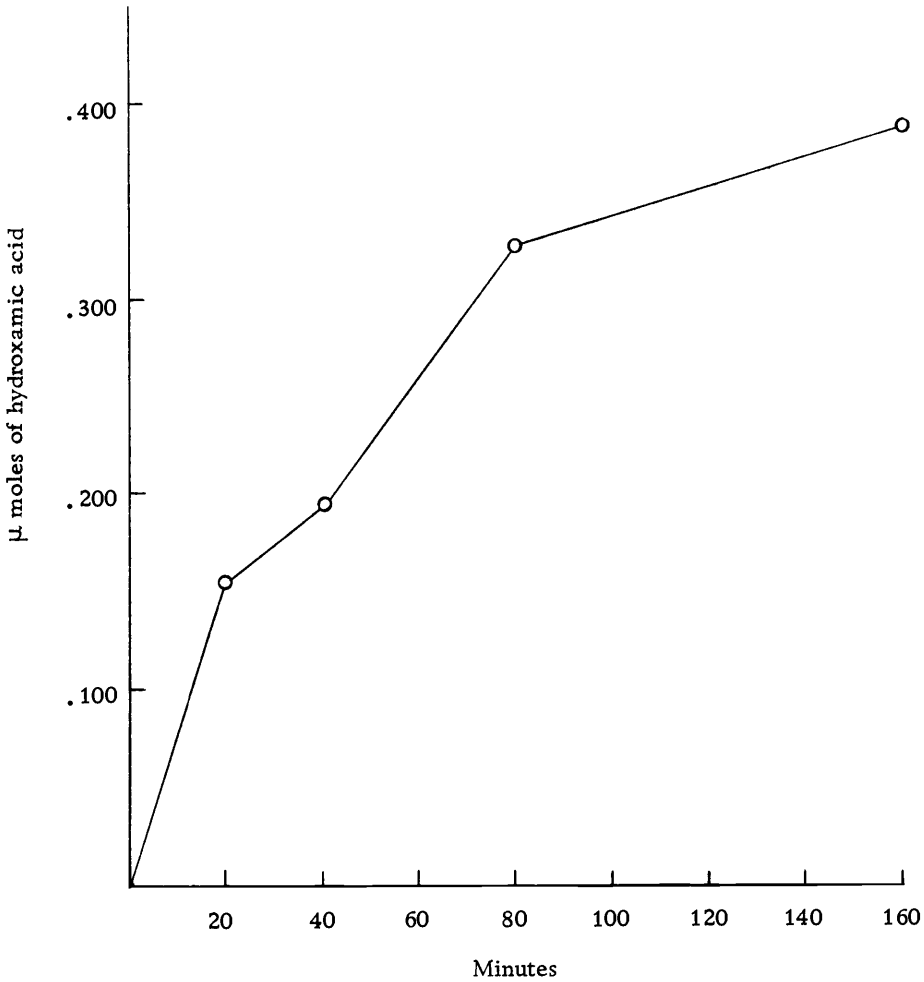


Figure 15. Formation of acetyl-CoA.

Table 9. Dependence of the formation of acetyl-CoA upon ATP.[1]

ATP (μmoles)	Hydroxamic acid (μmoles)
0	0.222
0.6	0.348
1.0	0.380
10.0	0.440

[1]Each tube contained (μmoles): CoA (0.08), KAcetate (20.0), phosphate buffer pH 7.5 (100), KF (50), $MgCl_2$ (10), glutathione (10), hydroxylamine (200), and 0.4 ml of 60,000 x g supernatant. Total volume: 1.05 ml.

Table 10. Dependence of the formation of acetyl-CoA upon CoA and acetate.[1]

Assay Mixture	Hydroxamic acid (μmoles)
complete	0.400
minus KAcetate	0.300
minus CoA	0.312
minus CoA and KAcetate	0.312

[1]Each tube contained (μmoles): CoA (0.08), ATP (10.0), KAcetate (20.0), phosphate buffer pH 7.5 (100), KF (50), $MgCl_2$ (10), glutathione (10), hydroxylamine (200), and 0.4 ml of 60,000 x g supernatant. Total volume: 1.05 ml.

it would appear that only added ATP is required for acetyl-CoA-kinase activity as measured by the hydroxamate method in the 60,000 x g supernatant.

Effect of Dialysis Upon Activity

The effect of dialysis of the 60,000 x g supernatant upon acetyl-CoA-kinase activity was investigated. As can be seen in Table 11, about half the activity present is lost upon dialysis as measured by the hydroxamate method.

Acetyl-CoA-Kinase Activity in a Bovine Aorta Homogenate

In order to determine if the activity of acetyl-CoA-kinase could be increased, a different method of preparing the aorta was tried.

An homogenate of bovine aorta was made using a Duall homogenizer. The homogenate was tested for activity by both the nitroprusside and hydroxamate methods. No activity was found by either method. The homogenate was then decanted and the decanted supernatant tested for activity. No activity was found by either the nitroprusside or hydroxamate method. Then the decanted supernatant was centrifuged in a Servall refrigerated centrifuge at 10,000 rpm for ten minutes. No activity was observed in the resulting supernatant with either the nitroprusside or hydroxamate method.

Table 11. Effect of dialysis upon the formation of acetyl-CoA.[1]

60,000 x g Supernatant	Hydroxamic Acid (μmoles)
Not dialyzed	0.440
Dialyzed	0.248

[1]Each tube contained (μmoles): CoA (0.08), ATP (10.0), KAcetate (20.0), phosphate buffer pH 7.5 (100), KF (50), $MgCl_2$ (10), glutathione (10), hydroxylamine (200), and 0.4 ml of 60,000 x g supernatant. Total volume: 1.05 ml.

Table 12. Formation of $C^{14}O_2$.[1]

No. of flasks	Enzyme Fraction	Counts per min. $C^{14}O_2$	
		Range	Average
2	Decanted Supernatant	8-17	13
2	Centrifuged Supernatant	0-28	14

[1]Each flask contained (μmoles): CoA (0.40), ATP (50.0), phosphate buffer pH 7.5 (500), KF (250), $MgCl_2$ (50), glutathione (50), hydroxylamine (1000), 2.5 μc acetate-1-C^{14} and 5.0 ml of homogenate in a total volume of 8.75 ml.

Effect of Acetate-1-C^{14}

Both of these supernatants were incubated in the presence of acetate-1-C^{14} in order to see if acetate was activated and utilized. The reaction flasks and procedure for trapping the $C^{14}O_2$ were those of Saba and Di Luzio (96). The $C^{14}O_2$ was counted in Bray's solution (19). As can be seen in Table 12, no $C^{14}O_2$ was recovered. At the same time, 2.0 ml of the reaction mixture were mixed with 3.0 ml of the ferric chloride solution. After centrifugation the resulting solution was read at 520 mμ. There was no activity, as measured by this method (hydroxamate).

Comparison of Acetyl-CoA-Kinase Activity in Yeast and Aorta

As can be seen in Table 13, acetyl-CoA-kinase can be partially purified from yeast by ammonium sulfate precipitation. The same purification scheme was applied to the 60,000 x g supernatant; the results are summarized in Table 14. Since there was no increase in the number of μmoles of hydroxamic acid with this purification scheme, the 60,000 x g supernatant protein was concentrated with 80% ammonium sulfate in order to see whether there would be any increase in activity if the protein were concentrated. If the protein concentration is increased by a factor of four, there is only a 30%

Table 13. Partial purification of acetyl-CoA-kinase in a yeast preparation.[1, 2]

Fraction	Protein (mg/ml)	Hydroxamic Acid (μmoles)
Crude	73.25	2.60
Ammonium sulfate precipitate, 0-15%	210.00	5.36

[1]Each tube contained (μmoles): CoA (0.08), ATP (10.0), KAcetate (20.0), phosphate buffer pH 7.5 (100), KF (50), $MgCl_2$ (10), glutathione (10), hydroxylamine (200), 0.2 ml H_2O, and 0.2 ml of yeast preparation. Total volume: 1.05 ml.

[2]Incubation time of 20 minutes.

Table 14. Partial purification of acetyl-CoA-kinase in the aorta homogenate.[1]

Fraction	Protein (mg/ml)	Hydroxamic Acid (μmoles)
60,000 x g supernatant	10.9	0.648
Ammonium sulfate precipitate, 0-15%	17.7	0.428

[1]Each tube contained (μmoles): CoA (0.08), ATP (10.0), KAcetate (20.0), phosphate buffer, pH 7.5 (100), KF (50), $MgCl_2$(10), glutathione (10), hydroxylamine (200) and 0.4 ml of aorta homogenate. Total volume: 1.05 ml.

increase in the number of μmoles of hydroxamic acid (Table 15).

Inhibition Study of Acetyl-CoA-Kinase

In order to determine if there was a factor in the 60,000 x g supernatant which could be preventing the formation of acetyl-CoA, the partially purified yeast fraction was assayed in the presence and in the absence of the 60,000 x g supernatant. As can be seen in Table 16, there is about a 37% inhibition (in terms of percent CoA converted to acetyl-CoA) when the 60,000 x g supernatant is present. As Table 17 shows, even if the 60,000 x g supernatant has been heated for 15 minutes at 100° C, it still exhibits an inhibitory effect. However, when the 60,000 x g supernatant has been dialyzed overnight (21 hours), this inhibitory effect disappears (Table 18). If the dialysate is reduced to the same volume as the material which was dialyzed, not only will it inhibit the conversion of acetate to acetyl-CoA but the dialysate by itself will cause the reduction of free sulfhydryl groups (Table 19).

In the light of these results it was thought that there was some contaminator in the nicotinamide (used in making the buffer) which might be causing the inhibition. Therefore nicotinamide was run in the same system. As can be seen in Table 20, nicotinamide had no effect on the reduction of free sulfhydryl groups.

The non-dialyzed 60,000 x g supernatant causes no reduction

Table 15. Acetyl-CoA-kinase activity after concentration of protein by means of ammonium sulfate precipitation.[1]

Fraction	Protein (mg/ml)	Hydroxamic Acid (μmoles)
60,000 x g supernatant	7.23	0.440
Ammonium sulfate precipitate, 0-80%	28.35	0.580

[1] Each tube contained (μmoles): CoA (0.08), ATP (10.0), KAcetate (20.0), phosphate buffer, pH 7.5 (100), KF (50), $MgCl_2$ (10), glutathione (10), hydroxylamine (200) and 0.4 ml of aorta homogenate. Total volume: 1.05 ml.

Table 16. Inhibition of acetyl-CoA-kinase activity in yeast by the 60,000 x g supernatant.[1]

Enzyme System	CoA remaining (μmoles)	% converted to acetyl-CoA
Yeast	.040	96
Yeast and aorta[2]	.386	59
Aorta	.875	6
None	.935	0

[1]Each tube contained (μmoles): CoA (1.90), Tris-HCl buffer (160), $MgCl_2$ (4.8), ATP (3.8), KCl (24), KAcetate (4.8), 0.2 ml of yeast and/or 0.8 ml 60,000 x g supernatant. Total volume is 1.6 ml.

[2]60,000 x g supernatant

Table 17. Inhibition of acetyl-CoA-kinase activity in yeast by heat-treated 60,000 x g supernatant.[1]

Enzyme System	CoA remaining (μmoles)	% converted to acetyl-CoA
Yeast	.250	69
Yeast and aorta-[2] heated 15 min. at 100° C	.570	30
Aorta heated 15 min. at 100° C.	.775	6
None	.810	0

[1]Each tube contained (μmoles): CoA (1.90), Tris-HCl buffer (160), $MgCl_2$ (4.8), ATP (3.8), KCl (24), KAcetate (4.8), 0.2 ml of yeast and/or 0.8 ml 60,000 x g supernatant. Total volume is 1.6 ml.

[2]60,000 x g supernatant

Table 18. Inhibition of acetyl-CoA-kinase activity in yeast by dialyzed 60,000 x g supernatant.[1]

Enzyme System	CoA remaining (μ moles)	% converted to acetyl-CoA
Yeast	.304	66
Yeast and aorta-[2] dialyzed overnight	.410	55
Aorta-dialyzed overnight	.880	0
None	.900	0

[1]Each tube contained (μmoles): CoA (1.90), Tris-HCl buffer (160), $MgCl_2$ (4.8), ATP (3.8), KCl (24), KAcetate (4.8), 0.2 ml of yeast and/or 0.8 ml 60,000 x g supernatant. Total volume is 1.6 ml.

[2]60,000 x g supernatant

Table 19. Inhibition of acetyl-CoA-kinase activity in yeast by the dialysate of the 60,000 x g supernatant.[1]

Enzyme System	CoA remaining (μ moles)	% converted to acetyl-CoA
Yeast	.258	76
Yeast and Dialysate	.585	45
Dialysate	.655	38
None	1.060	0

[1]Each tube contained (μmoles): CoA (1.90), Tris-HCl buffer (160), $MgCl_2$ (4.8), ATP (3.8), KCl (24), KAcetate (4.8), 0.2 ml of yeast and/or 0.8 ml of dialysate. Total volume is 1.6 ml.

Table 20. Effect of nicotinamide upon acetyl-CoA-kinase activity in yeast.[1]

Enzyme System	CoA remaining (μ moles)	% converted to acetyl-CoA
Yeast	.525	51
Yeast and 0.03 M Nicotinamide	.541	50
0.03 M Nicotinamide	1.080	0
None	1.080	0

[1]Each tube contained (μmoles): CoA (1.90), Tris-HCl, pH 8.0 (160), ATP (3.8), KCl (24), KAcetate (4.8), 0.2 ml of yeast and/or 0.8 ml of 0.03 M nicotinamide. Total volume is 1.6 ml.

Table 21. Effect of dialysate and 60,000 x g supernatant on Coenzyme A.[1]

Enzyme System	Absorbance 520 mμ
60,000 x g supernatant	.485
Dialysate	.300
Dialyzed 60,000 x g supernatant and dialysate	.425
None	.450

[1]Each tube contained (μmoles): CoA (1.90), Tris-HCl buffer (160), ATP (3.8), KCl (24), KAcetate (4.8), and 1.0 ml enzyme solution. Total volume is 1.6 ml.

in free sulfhydryl groups but the dialysate by itself will cause the reduction of free sulfhydryl groups. This result implies that the inhibitor may be associated with something in the 60,000 x g supernatant which prevents the inhibitor from interacting directly with free sulfhydryl groups. If this assumption is true, then, if the dialysate is dissolved in the dialyzed 60,000 x g supernatant, there should not be any reduction in free sulfhydryl groups. As can be seen in Table 21, the reconstituted 60,000 x g supernatant (dialyzed 60,000 x g supernatant and dialysate) shows no decrease in absorbancy at 520 mμ.

Control Experiments on Inhibitor

As can be seen in Table 22, the inhibition by the dialysate is not affected by any of the cofactors, that is, by ATP, potassium acetate, or KCl. In addition, glutathione can replace CoA in the assay mixture (Table 23).

In order to show that there was no contaminator from the dialysis bag which could cause the reduction of free sulfhydryl groups, the following experiments were performed. First the dialysis bag was filled with 10 ml of distilled water and dialyzed overnight, and then the dialysate was evaporated down and picked up in 10 ml of distilled water and then tested. There was no decrease in absorbance at 520 mμ (the nitroprusside method). Then in another experiment,

Table 22. Inhibition in the absence of cofactors.[1]

Assay Mixture	Absorbance 520 mμ
Complete	.290
Minus dialysate	.520

[1]Each tube contained (μmoles): CoA (1.90), Tris-HCl (160), 0.6 ml H_2O, and 0.8 ml of dialysate. Total volume is 1.6 ml.

Table 23. Inhibition in the absence of CoA.[1]

Assay Mixture	Absorbance, 520 mμ
Complete	.260
Minus dialysate	.390

[1]Each tube contained: 0.64 mg glutathione, Tris-HCl buffer (160 μmoles), 0.6 ml H_2O and 0.8 ml dialysate. Total volume is 1.6 ml.

Table 24. Effect of boiling dialysis bag.[1]

Assay Mixture	Absorbance, 520 mμ
Dialysate	.320
Boilings	.395
Control	.405

[1]Each tube contained 0.64 mg glutathione, Tris-HCl buffer (160 μmoles), 0.6 ml H_2O and 0.8 ml of dialysate or boilings. Total volume is 1.6 ml.

the bag was boiled in 100 ml of 10^{-4} M EDTA, pH 7.0, twice and then boiled twice in 100 ml of distilled water. Ten ml of 60,000 x g supernatant were then dialyzed overnight in the boiled bag. Both the boilings and dialysate were evaporated to dryness and then dissolved in 10 ml of distilled water and tested. The results are summarized in Table 24. Both of these experiments conclusively prove that there is no contaminator from the dialysis bag which causes the reduction of free sulfhydryl groups.

BioGel P-2 Column Chromatography of Dialysate

Ten ml of 60,000 x g supernatant were dialyzed overnight. After the dialysate was reduced to a volume of 10 ml, it was placed on a BioGel P-2 column. The inhibitor came off in the first peak, i.e., fractions 11-24 (Figure 16) which is the void volume. There was no activity in any of the other peaks. These results indicate that the inhibitor may be associated with a peptide or some other large compound.

Nature of Inhibitor

Ten ml of 60,000 x g supernatant were dialyzed overnight. The dialysate was reduced to a volume of 10 ml. This solution was diluted with distilled water and tested without cofactors and with glutathione replacing CoA. The results, as seen in Figure 17, show

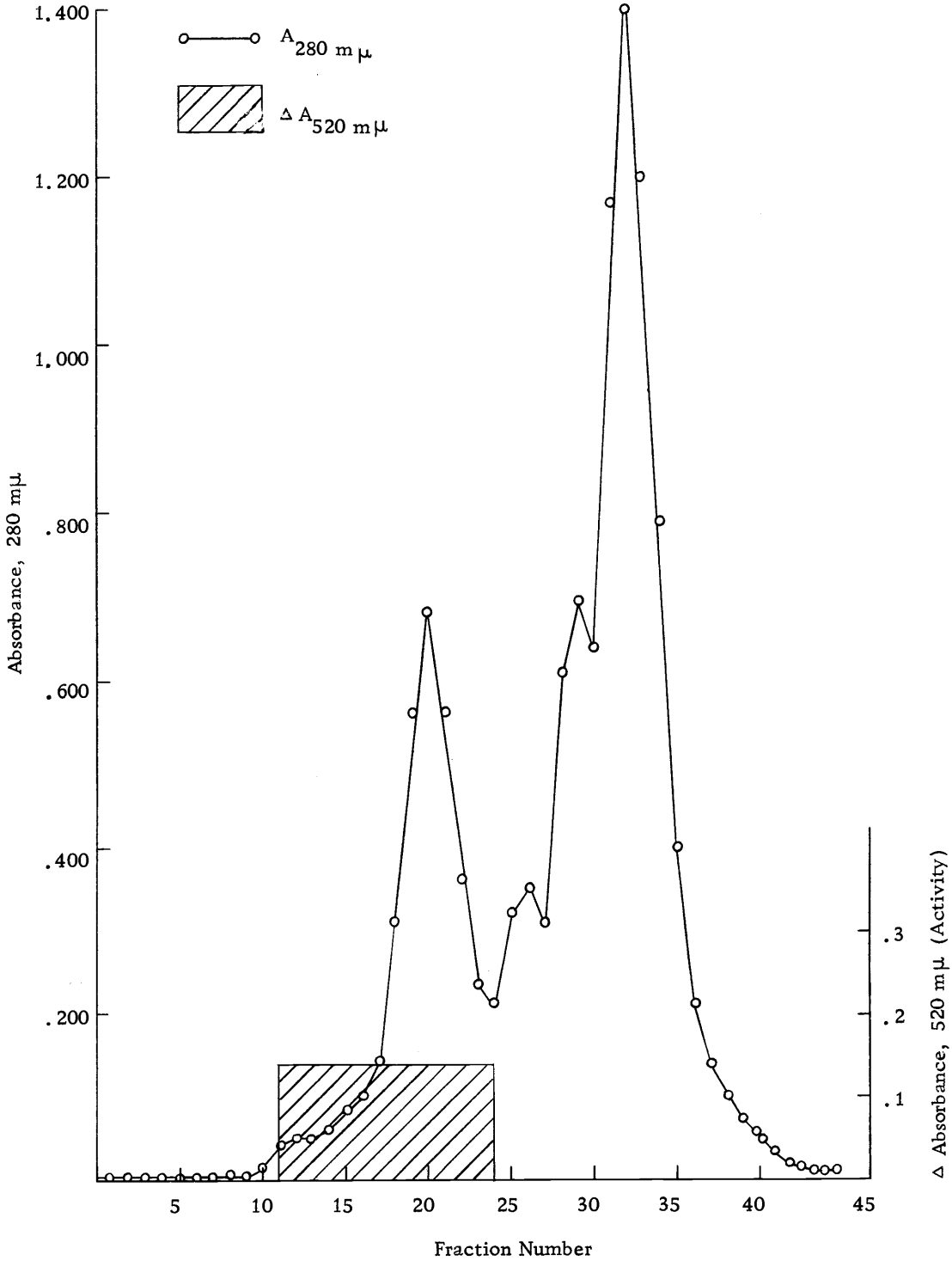


Figure 16. BioGel P-2 column chromatography of the dialysate from the 60,000 x g supernatant.

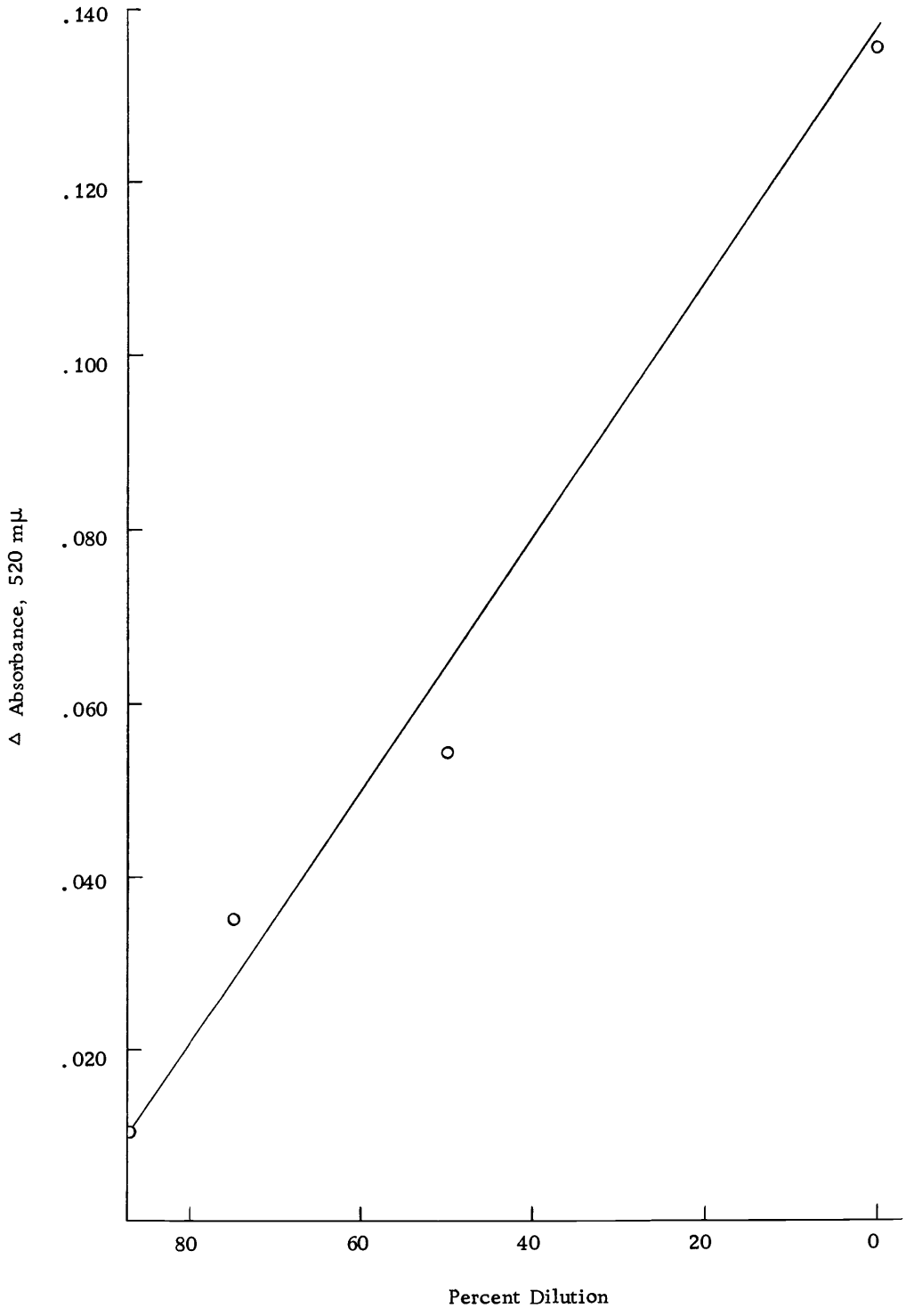


Figure 17. Concentration dependence of the inhibitor.

that the reduction of free sulfhydryl groups is linear with dilution.

Next the pH dependence of the reaction between the inhibitor and glutathione was examined. As can be seen in Figure 18, the reaction between the inhibitor and glutathione is pH dependent. The inhibitor was adjusted to pH 8 and pH 4. Then the dialysate, in both cases, was left at room temperature and frozen overnight. The dialysate was then adjusted back to pH 7.0 and tested. The percent decrease in absorbance at 520 mμ was the same as that for the original sample. Thus it appears that the inhibitor is stable to high and low pH. In addition, two samples were exposed to acid (HCl) and base (KOH) for 24 hours. The samples were then neutralized to pH 7.0 and tested. Again the percent decrease in absorbance at 520 mμ was the same as that for the original sample. Thus it would appear that the inhibitor is stable to both acid and base.

Model System for Inhibition

Maleic acid, fumaric acid, diethyl fumarate, and diethyl maleate were run in the assay system in place of the inhibitor in order to see if they would react with glutathione under the assay conditions employed. As can be seen in Tables 25, 26, and 27, maleic acid, fumaric acid, diethyl fumarate and diethyl maleate will react with glutathione. In the reaction with glutathione, maleic acid was more reactive than fumaric acid. With the corresponding esters,

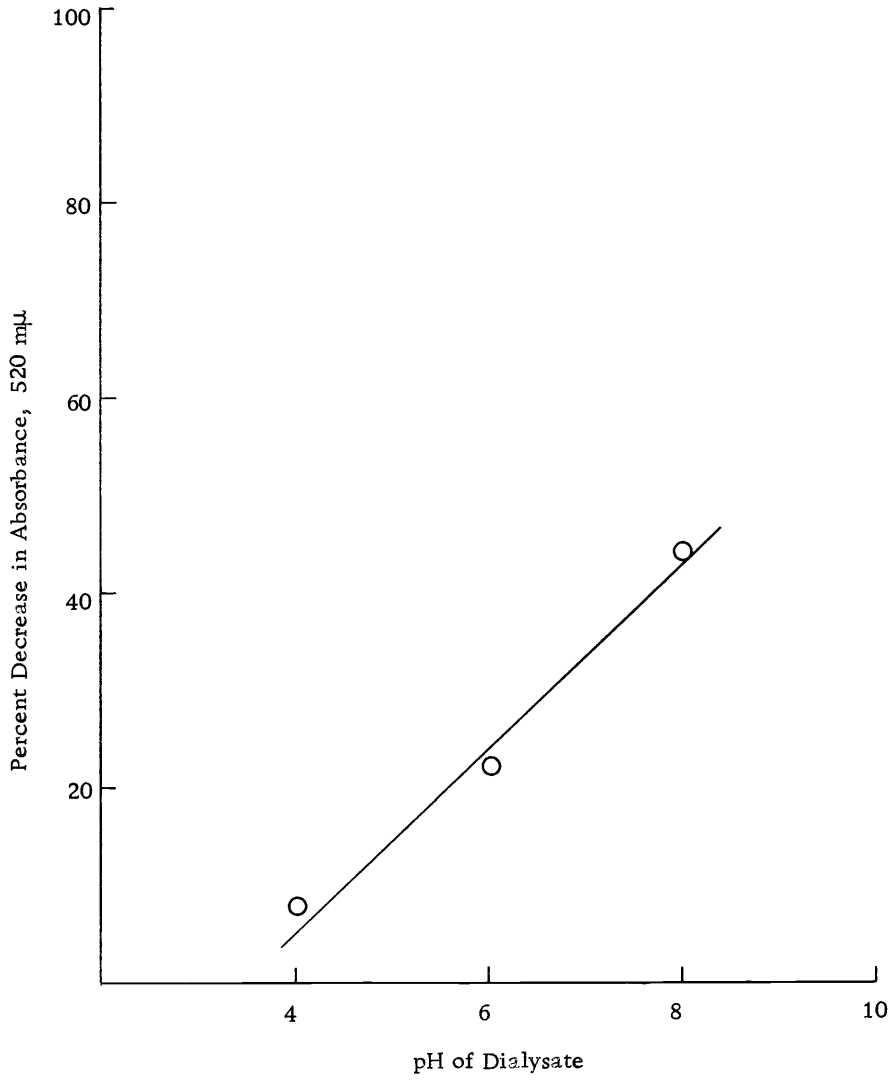


Figure 18. Effect of pH on dialysate.

Table 25. Effect of maleic and fumaric acid.[1]

Amounts (μ moles)	% Decrease in Absorbance, 520 mμ	
	Maleic acid	Fumaric acid
80	15	0
320	50	10
800	82	--

[1]Each tube contained 0.64 mg glutathione, Tris-HCl buffer (160 μmoles), 0.6 ml H_2O, and 0.8 ml maleic or fumaric acid, pH 7.0. Total volume is 1.60 ml.

Table 26. Effect of diethyl fumarate.[1]

Amounts (μmoles)	% Decrease in Absorbance, 520 mμ Diethyl Fumarate[2]
0.8	0
4.0	30
8.0	38
16.0	70
32.0	89
80.0	89

[1]Each tube contained 0.64 mg glutathione, Tris-HCl buffer (160 (μmoles), 0.6 ml H_2O, and 0.8 ml diethyl fumarate. Total volume is 1.60 ml.

[2]Made up in ethanol and diluted with ethanol.

Table 27. Effect of diethyl maleate.[1]

Amounts (μ moles)	% Decrease in Absorbance, 520 mμ Diethyl Maleate[2]
20	43
40	50
80	79

[1]Each tube contained 0.64 mg glutathione, Tris-HCl buffer (160 μmoles), 0.6 ml H_2O, and 0.8 ml diethyl maleate. Total volume is 1.60 ml.

[2]Made up in ethanol and all dilution with ethanol.

for reaction with glutathione diethyl fumarate was more reactive than diethyl maleate. Boyland and Chasseaud (18), studying the enzyme-catalyzed conjugations of diethyl maleate and glutathione in rat liver supernatant, have made similar observations. However, 1.0 M, 0.4 M, and 0.1 M aconitic acid, pH 7.0, did not show the same effect when they were run in the assay system.

Phosphatase Activity

Nonspecific phosphomonoesterase activity was assayed in the 60,000 x g supernatant by measuring the release of para-nitrophenol from para-nitrophenylphosphate. The release of p-nitrophenol with time at pH 7.0 is shown in Figure 19.

As can be seen in Table 28, the crude homogenate and the 60,000 x g supernatant release the same number of μmoles of p-nitrophenol in the same time period. In Table 29 is a summary of the stability of the 60,000 x g supernatant with respect to orthophosphatase activity. The 60,000 x g supernatant can be dialyzed, frozen, or kept at 2-4°C for 24 hours with no apparent loss in activity.

In order to study ATPase and inorganic phosphatase activity in the 60,000 x g supernatant, the added phosphate must be removed. Ninety-nine percent of the added phosphate can be removed in 24 hours by dialysis.

As can be seen in Table 30 there is ATPase activity in the 60,000 x g supernatant. The ATPase in the 60,000 x g supernatant will remove the first and second phosphate of ATP and the initial

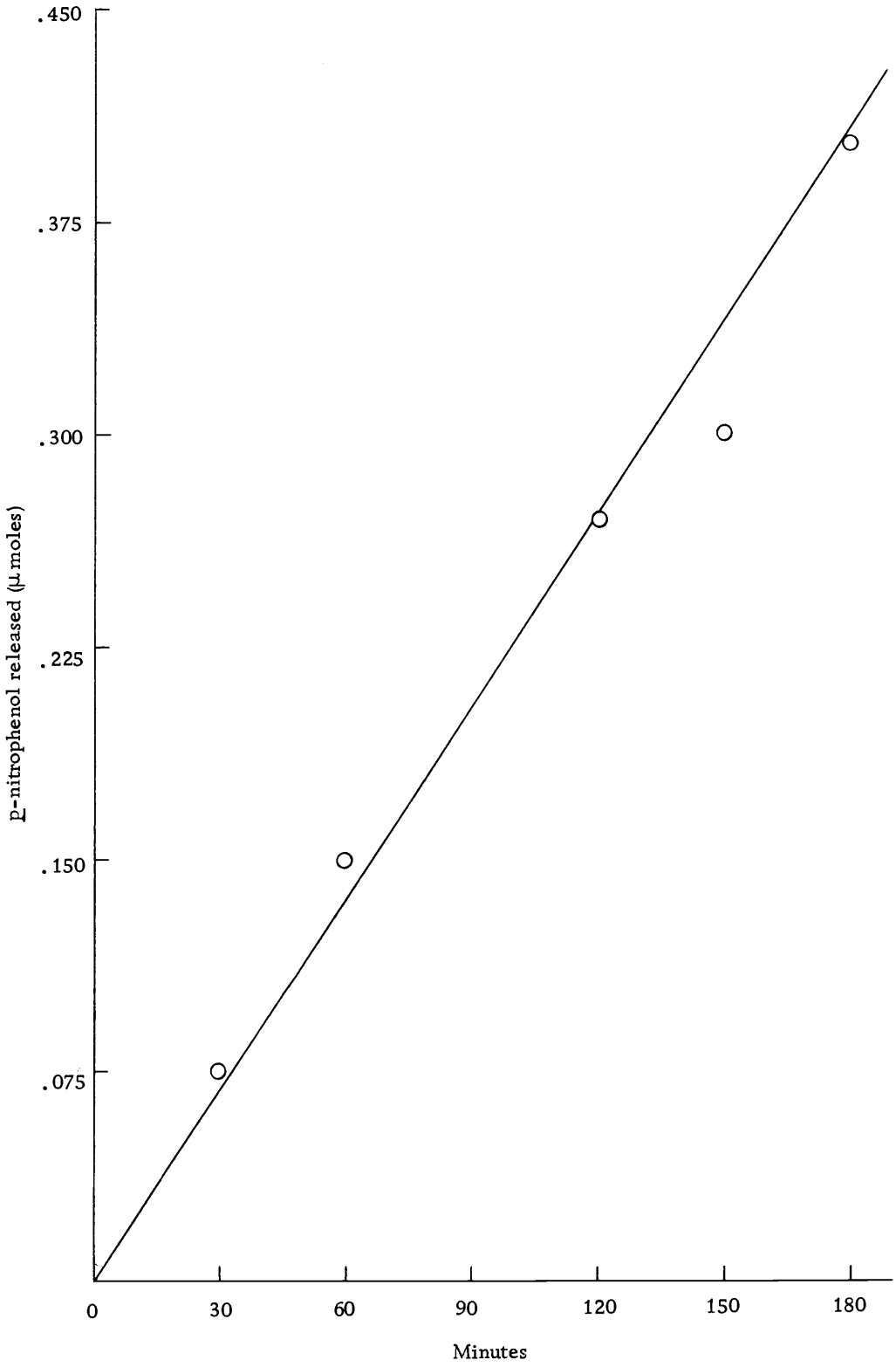


Figure 19. Rate of release of orthophosphate.

Table 28. Comparison of orthophosphatase activity in crude and 60,000 x g supernatant.[1]

Fraction	p-nitrophenol released (μmoles)
Crude	.480
60,000 x g supernatant	.400

[1]Each tube contained 1.0 ml of p-nitrophenolphosphate, 2.0 ml H_2O, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of homogenate.

Table 29. Stability of orthophosphatase activity of the 60,000 x g supernatant.[1]

60,000 x g supernatant	p-nitrophenol released (μmoles)
0 hrs at 2-4° C	.400
24 hrs at 2-4° C	.440
24 hrs at 0° C	.430
Dialyzed 24 hrs at 2-4° C	.400

[1]Each tube contained 1.0 ml of p-nitrophenylphosphate, 2.0 ml H_2O, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant.

Table 30. ATPase activity in the 60,000 x g supernatant.[1]

ATP	ADP (μmoles)	AMP	Orthophosphate released (μmoles)
3.3	-	-	6.88
-	6.6	-	2.16
-	-	9.9	0

[1]Each tube contained: 5.0 ml of dialyzed 60,000 x g supernatant and 4.5 ml .1 M Tris-HCl buffer in a total volume of 10.5 ml.

Table 31. Inorganic pyrophosphatase activity in the 60,000 x g supernatant.[1]

$Na_4P_2O_7 \cdot 10H_2O$ (μmoles)	Orthophosphate released (μmoles)
7.0	12.0

[1]Each tube contained: 5.0 ml of dialyzed 60,000 x g supernatant and 4.5 ml 0.1 M Tris-HCl buffer in a total volume of 10.5 ml.

phosphate of ADP but will not remove the terminal phosphate of AMP. In addition, there is inorganic pyrophosphatase activity in the 60,000 x g supernatant (Table 31).

Effect of NaF and BSA on Phosphatase Activity in the 60,000 x g Supernatant

In Tables 32 and 33 are summarized the effects of sodium fluoride (NaF) upon orthophosphatase and ATPase activity in the 60,000 x g supernatant. NaF has no effect on orthophosphatase at pH 7.0. At 10 mM NaF inhibited ATPase to 50% of its activity in the absence of NaF.

Bovine serum albumin (BSA) will inhibit orthophosphatase activity in the 60,000 x g supernatant. There is a linear relationship between inhibition and the amount of BSA added (Table 34) between 0 and 6 mg/ml BSA.

Effect of NaF and BSA on MVA-2-C^{14} Incorporation into the NSF

The effect of BSA upon the incorporation of MVA-2-C^{14} into the NSF is summarized in Table 35. With only 0.4 mg/ml BSA there is approximately 50% inhibition of incorporation of labeled MVA into the NSF. The effect of NaF on the incorporation of MVA-2-C^{14} into the NSF is shown in Table 36. At 0.5 mM NaF there is 60% inhibition of incorporation of labeled MVA into the NSF.

Table 32. Effect of sodium fluoride upon orthophosphatase activity in the 60,000 x g supernatant.[1]

NaF (mM)	p-nitrophenol released (μmoles)
0	.480
.1	.505
1.0	.530
10.0	.460

[1]Each tube contained 1.0 ml of p-nitrophenylphosphate, 1.0 ml H_2O, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

Table 33. Effect of sodium fluoride upon ATPase activity in the 60,000 x g supernatant.[1]

NaF (mM)	Orthophosphate released (μmoles)
0	7.45
.05	7.45
10.0	3.92

[1]Each tube contained 5.0 ml of dialyzed 60,000 x g supernatant, 4.5 ml .1 M Tris-HCl buffer and 3.3 μmoles of ATP in a total volume of 10.5 ml.

Table 34. Effect of bovine serum albumin upon orthophosphatase activity in the 60, 000 x g supernatant.[1]

BSA (mg/ml)	p-nitrophenol released (μ moles)	% inhibition
0	.510	0
2	.405	21
4	.330	35
6	.250	50

[1] Each tube contained 1.0 ml p-nitrophenylphosphate, 1.0 ml H_2O, 2.5 ml 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60, 000 x g supernatant in a total volume of 10.5 ml.

Table 35. Effect of BSA on the incorporation of MVA-2-C^{14} into the NSF.[1]

No. of flasks	BSA (mg/ml)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Inhibition
2	0	3406-3719	3613	0
3	0.4	1621-1817	1738	48
3	2.0	315-352	335	89

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.5 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

Table 36. Effect of NaF on the incorporation of MVA-2-C^{14} into the NSF.[1]

No. of flasks	NaF (mM)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Inhibition
3	0	2871-3489	3241	0
3	0.5	1287-1491	1388	57
3	1	332-458	389	88
3	10	50-65	58	98

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

The NSF material from assays containing 0.4 mg/ml BSA and 0.5 mM NaF and the NSF material from a standard assay were spotted on Whatman #1 paper impregnated with 5% mineral oil in petroleum ether and run in 85% acetic acid in order to see if there was any change in the distribution of the counts per minute in the NSF fraction in the presence of BSA and NaF. The migration of the standards and the areas cut out for counting are shown in Figure 20. As can be seen in Table 37, there is a change in the distribution of activity in band IV, V, and VI after incubation with NaF and BSA. The presence of NaF causes the distribution of activity to be shifted to those compounds whose R_F will be the same as that of farnesol, while the presence of BSA causes the distribution of activity to be shifted to those compounds whose R_F will be closer to that of cholesterol. There are areas (Figure 20 and Table 37) which contain radioactivity but do not stain with phosphomolybdic acid. This result will occur when there is some trailing of material or when there is not enough material present. The samples in Table 37 were not sprayed but were cut according to the pattern exhibited by the standards and NSF marker shown in Figure 20. As can be seen in Table 38, increasing the concentration of ATP cannot overcome the inhibition by NaF.

Key to Figure 20

Spotted left to right:

(1) NSF material, (2) Mixture of standards, bottom to top, cholesterol, farnesol and squalene.

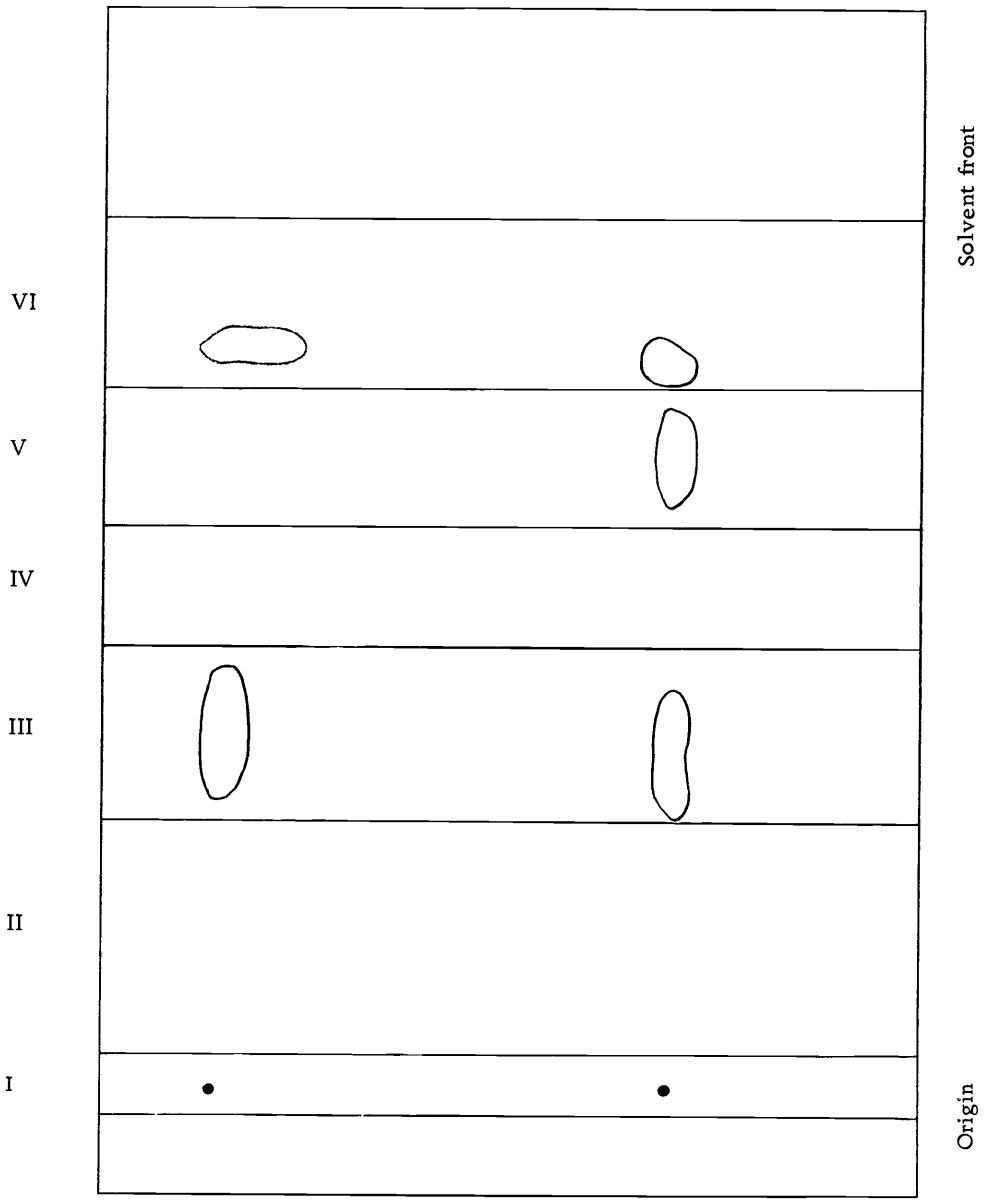


Figure 20. Paper chromatography of NSF material.

Table 37. Distribution of NSF after paper chromatography.

Band[1]	Counts per min. NSF			Distribution of counts per min. (%)		
	control	NaF (0.5 mM)	BSA (0.4 mg/ml)	control	NaF (0.5 mM)	BSA (0.4 mg/ml)
I	186	55	141	10	5	9
II	19	21	43	1	2	3
III	59	44	116	3	4	8
IV	316	47	645	16	4	42
V	1164	597	523	60	58	35
VI	212	265	73	11	26	5

[1] See Figure 20 for description of bands.

Table 38. Effect of ATP on NaF inhibition of the incorporation of MVA-2-C^{14} into the NSF.[1]

No. of flasks	ATP (mM)	NaF (mM)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Inhibition
3	1.65	-	1105-1444	1239	0
3	1.65	0.5	681-694	688	55
3	3.30	-	1410-1798	1565	0
3	3.30	0.5	567-995	831	53
3	16.5	-	1866-2431	2228	0
2	16.5	0.5	1096-1488	1282	56

Effect of L-Phenylalanine on the Incorporation of MVA-2-C^{14} into the NSF

The effect of L-phenylalanine on the incorporation of MVA-2-C^{14} into the NSF is summarized in Table 39. With 90 μmoles of L-phenylalanine there is about 40% inhibition of labeled MVA into the NSF. If the ATP concentration is increased, the inhibition by L-phenylalanine can be overcome (Table 40). Potassium acetate (Table 39) did not inhibit the incorporation of labeled MVA into the NSF.

Anion Effect on the Incorporation of MVA-2-C^{14} into the NSF

The effect of various anions on the incorporation of MVA-2-C^{14} into the NSF is summarized in Tables 41 through 47.

In Table 41 it can be seen that, if the concentration of NaF is lowered to 0.05 mM in the standard assay, there is a 35% increase in the counts per minute in the NSF. In Table 42 it can be seen that maximum stimulation by NaF occurs at an ATP concentration of 3.3 mM.

It can be seen in Table 43 that, at a concentration greater than 1 mM, *para*-nitrophenylphosphate will inhibit the incorporation of MVA-2-C^{14} into the NSF whereas at a concentration of 0.5 mM or lower there is a stimulation of the incorporation of MVA-2-C^{14} into the NSF (Table 44). The same effect can be seen for *p*-nitrophenol

Table 39. Effect of L-phenylalanine on the incorporation of MVA-2-C-^{14} into the NSF.[1]

No. of flasks	L-phenylalanine (μmoles)	Counts per min. NSF Range	Average
3	0	1509-1712	1596
2	90	978-1002	986
3	180	301-501	377
3	270	186-241	206
2	360	130-183	155
3	540	35-48	43
3	100 (KAcetate)	1078-1686	1410

[1]Each flask contained 0.05 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, 5.0 ml of 60,000 x g supernatant, 2 mg ATP, and 5 mg NAD in a total volume of 10.5 ml.

Table 40. Effect of ATP on L-phenylalanine inhibition of the incorporation of MVA-2-C^{14} into the NSF.[1]

No. of flasks	ATP (μmoles)	L-phenylalanine (μ moles)	Counts per min. NSF Range	Average
3	3.3	--	1342-1684	1563
3	3.3	90	959-1143	1019
2	6.6	--	1150-1718	1434
3	6.6	90	1203-1603	1428
3	16.5	--	1524-2041	1701
3	16.5	90	1237-1783	1573

[1]Each flask contained 0.05 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, 5.0 ml of 60,000 x g supernatant, and 5 mg NAD in a total volume of 10.5 ml.

Table 41. Stimulatory effect of NaF on the incorporation of MVA-2-C^{14} into the NSF.[1]

No. of flasks	NaF (mM)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Stimulation
3	0	2871-3489	3241	0
3	0.1	3807-4124	3947	22
3	0.05	4170-4470	4363	35
3	0.01	3815-4373	4142	28

[1] Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

Table 42. Effect of ATP on NaF stimulation of the incorporation of MVA-2-C^{14} into the NSF.[1]

No. of flasks	ATP (mM)	NaF (mM)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Stimulation
3	1.65	--	1371-1543	1480	0
3	1.65	0.05	1560-1580	1728	17
3	3.30	--	1349-1901	1659	0
3	3.30	0.05	2213-2634	2493	50
3	16.5	--	2260-2963	2701	0
3	16.5	0.05	3182-3339	3242	20

[1] Each flask contained 0.05 μc MVA-2-C^{14}, 1.5 ml 0.1 M phosphate nicotinamide buffer, 5.0 ml of 60,000 x g supernatant, and 5 mg NAD in a total volume of 10.5 ml.

Table 43. Effect of p-nitrophenylphosphate on the incorporation of MVA-2-C^{14} into the NSF - Part I.[1]

No. of flasks	p-nitrophenylphosphate (mM)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Inhibition
3	0	1964-2316	2151	0
3	5.0	320-644	515	86
3	3.75	622-976	852	60
3	2.5	1080-1361	1231	43
2	1.25	2047-2137	2092	3

[1]Each flask contained 0.05 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, 5.0 ml of 60,000 x g supernatant, 2 mg ATP, and 5 mg NAD in a total volume of 10.5 ml.

Table 44. Effect of p-nitrophenylphosphate on the incorporation of MVA-2-C^{14} into the NSF - Part II.[1]

No. of flasks	p-nitrophenylphosphate (mM)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Stimulation
3	0	1588-1709	1637	0
2	0.5	1964-2131	2048	25
3	0.25	2136-2222	2168	32
3	0.05	2261-2481	2366	45

[1]Each flask contained 0.05 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, 5.0 ml of 60,000 x g supernatant, 2 mg ATP, and 5 mg NAD in a total volume of 10.5 ml.

(Table 45).

The effects of CPIB (p-chlorophenoxyisobutyric acid) and L-phenylalanine on the incorporation of MVA-2-C^{14} into the NSF are shown in Table 46. Some other anions were run in the standard assay system. As can be seen in Table 47, sulfate, chloride, and acetate weakly stimulate the incorporation of MVA-2-C^{14} into the NSF.

Effect of Phosphate on the Incorporation of MVA-2-C^{14} into the NSF

Since anions can stimulate the incorporation of MVA-2-C^{14} into the NSF, it was of interest to know how necessary phosphate is to the system of enzymes in the 60,000 x g supernatant that converts labeled MVA to the NSF material; thus the following experiment was performed. Three different crude homogenates were prepared from bovine arota, made up in 0.1 M phosphate nicotinamide buffer, pH 7.0, 0.1 M Tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.2 (Tris-HCl), and 0.1 M phosphate buffer, pH 7.0. The 60,000 x g supernatants from the three preparations were assayed in the presence of the buffer in which they were prepared and also in the presence of each of the other buffers at the same concentration as if the 60,000 x g supernatant had been prepared and incubated with that buffer.

The incorporation of MVA-2-C^{14} into the NSF by the 60,000 x g

Table 45. Effect of p-nitrophenol on the incorporation of MVA-2-C^{14} into the NSF.[1]

No. of flasks	p-nitrophenol (mM)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Stimulation (+) or Inhibition (-)
3	0	1588-709	1637	0
3	5.0	756-999	880	-46
3	0.07	2348-2429	2378	+45

[1]Each flask contained 0.05 μc MVA-2-C^{14}, 0.1 M phosphate nicotinamide buffer, 5.0 ml of 60,000 x g supernatant, 2 mg ATP, and 5 mg NAD in a total volume of 10.5 ml.

Table 46. Effect of CPIB and L-phenylalanine on the incorporation of MVA-2-C^{14} into the NSF.[1]

No. of flasks	CPIB (mM)	L-phenylalanine (mM)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Stimulation
3	0	0	1173-1217	1197	0
3	0.5	--	2084-2109	2098	75
3	0.05	--	1809-1965	1897	58
3	--	1.0	1417-1654	1554	30
3	--	0.5	1432-1678	1585	32
3	--	0.05	1514-1585	1555	30

[1]Each flask contained 0.05 μc MVA-2-C^{14}, 1.5 ml 0.1 M phosphate nicotinamide buffer, 5.0 ml of 60,000 x g supernatant, 2 mg ATP, and 5 mg NAD in a total volume of 10.5 ml.

Table 47. Effect of anions on the incorporation of MVA-2-C^{14} into the NSF.[1]

No. of flasks	Anion	Conc. (mM)	Counts per min. NSF Range	Counts per min. NSF Average	Percent Stimulation
3	None		1573-1741	1656	0
3	Na_2SO_4	5.0	1748-2199	2014	22
3	Na_2SO_4	0.05	2028-2105	2072	25
3	KCl	5.0	1988-2252	2154	30
3	KAcetate	5.0	1884-2045	1986	20

[1]Each flask contained 0.05 μc MVA-2-C^{14}, 1.5 ml 0.1 M phosphate nicotinamide buffer, 5.0 ml of 60,000 x g supernatant, 2 mg ATP, and 5 mg NAD in a total volume of 10.5 ml.

supernatants of the three crude homogenates is summarized in Table 48. The data in Table 48 show that the best incorporation of labeled MVA into the NSF occurs with the homogenates that are made up in either 0.1 M phosphate or 0.1 M phosphate nicotinamide.

The low incorporation of MVA-2-C^{14} into the NSF by the 60,000 x g supernatant of the 0.1 M Tris-HCl homogenate (Table 48) cannot be overcome by the addition of 0.3 M phosphate nicotinamide buffer nor can the incorporation of labeled MVA by the 60,000 x g supernatants of the homogenate made with 0.1 M phosphate and 0.1 M phosphate nicotinamide be significantly depressed by the presence of 0.3 M Tris-HCl buffer. Nicotinamide is not a necessary requirement for activity.

Utilization of Aorta NSF by a Rat Liver Homogenate

The NSF material from a standard assay procedure was collected and pooled. This material was dissolved in 2.0 ml of ethanol and then centrifuged in order to remove that material (cholesterol) which did not go into solution (90% of the original activity remained in the ethanol supernatant). This material was used as the substrate in an NSF assay.

In Table 49 are summarized the results of incubating the NSF in the 60,000 x g supernatant. After two hours and 50 minutes at 37° C, 85% of the original NSF activity was still found in the NSF.

Table 48. Incorporation of MVA-2-C^{14} into the NSF by three different aorta preparations.[1]

No. of flasks	Buffer Added (2.5 ml)	0.1 M Phosphate nicotinamide 60,000 x g supernatant		0.1 M Tris-HCl 60,000 x g supernatant		0.1 M Phosphate 60,000 x g supernatant	
		Counts per min. NSF		Counts per min. NSF		Counts per min. NSF	
		Average	Range	Average	Range	Average	Range
3	0.1 M Tris-HCl, pH 7.2			464	445-480		
3	0.3 M phosphate nicotinamide, pH 7.0			607	520-694		
3	0.1 M phosphate nicotinamide, pH 7.0	1987	1898-2032				
3	0.3 M Tris-HCl, pH 7.2	2699	2579-2935			1897	1684-2029
3	0.1 M phosphate, pH 7.0					2650	2590-2692

[1] Each flask contained 2 mg ATP, 5 mg NAD, 0.05 μc MVA-2-C^{14}, 0.72 μmoles $MgCl_2$, 5.0 ml of 60,000 x g supernatant and 2.5 ml buffer in a total volume of 10.5 ml.

Table 49. Assay for the NSF material using the NSF as the substrate in the 60,000 x g supernatant.[1]

No. of flasks	Substrate	Counts per min. NSF Range	Average
3	MVA-2-C^{14}[2]	2560-2780	2701
3	MVA-2-C^{14}[3]	2049-2295	2138
3	NSF[4]	1513-1559	1551
3	NSF	1301-1323	1312

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 ml.

[2]0.05 μc

[3]0.1 ml of ethanol added

[4]Stopped at zero time.

Table 50. Assay for the NSF material using the NSF as the substrate in a rat liver homogenate.[1]

No. of flasks	Substrate	Counts per min. NSF Range	Average
3	MVA-2-C^{14}[2]	27658-28617	28278
3	MVA-2-C^{14}[3]	23784-25421	24527
3	NSF[4]	1159-1298	1228
3	NSF	55-83	69

[1]Each flask contained 2 mg ATP, 5 mg NAD, 0.1 M phosphate nicotinamide buffer, and 5.0 ml of 60,000 x g supernatant in a total volume of 10.5 mls.

[2]0.05 μc

[3]0.1 ml of ethanol added

[4]Stopped at zero time.

The distribution of the new NSF material after paper chromatography in 85% acetic acid is shown in Table 51.

The original NSF material was also incubated for the same length of time in a rat liver homogenate. The results of this experiment are shown in Table 50. In this case, only 5% of the original NSF activity remained in the NSF at the end of the incubation period. When the saponified incubation mixture was made acid (pH 2.0) and then extracted with petroleum ether, the remaining activity was then found in the petroleum ether fraction.

The material extracted with petroleum ether after the acidification of the saponified mixture was subjected to thin-layer chromatography on silica gel Eastman Kodak Chromagram sheets in 55 parts diethyl ether, five parts chloroform, 40 parts hexane, and 0.1 part acetic acid. Then the sheets were cut and counted as shown in Figure 21. Myristic, steric, and oleic acid migrate to the same spot as palmitic acid. The distribution of the activity is shown in Table 52. It can be seen that the distribution of the NSF product is different from that of the NSF substrate.

Mass Spectrum of Prenol Sample

In order to obtain some estimation of the types of compounds that are present in the arterial tissue and presumably also in the 60,000 x g supernatant, the nonsaponifiable material was extracted

Table 51. Paper chromatography of the re-extracted NSF material from the 60,000 x g supernatant.

Band[1]	Counts per minute		Distribution of counts per minute	
Substrate	MVA-2-C^{14}	NSF	MVA-2-C^{14}	NSF
I	30	55	11	22
II	0	0	0	0
III	0	10	0	4
IV	26	85	10	34
V	187	97	71	39
VI	20	5	8	2

[1] See Figure 20 for description of bands

Key to Figure 21

Spotted left to right:

(1) Mixture of standards, bottom to top, cholesterol, farnesol and squalene, (2) Palmitic acid, (3) Re-extracted NSF material, (4) NSF material.

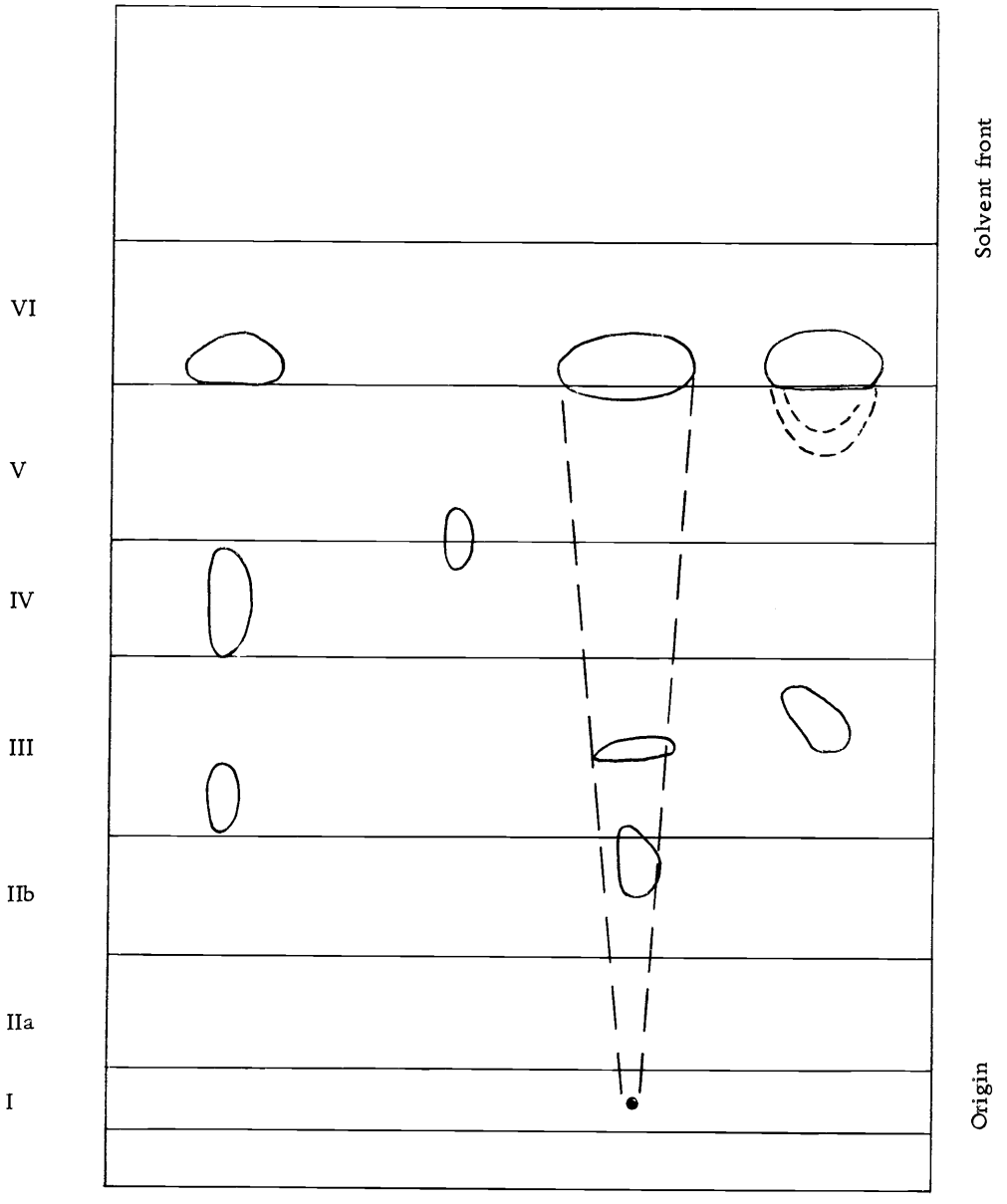


Figure 21. Thin-layer chromatography of NSF material.

Table 52. Distribution of the rat liver NSF material after thin-layer chromatography.

Band[1]	Counts per minute		Distribution of counts per minute	
	NSF substrate	NSF product	NSF substrate	NSF product
I	25	9	2	3
IIa	23	52	2	18
IIb	37	93	2	32
III	260	38	18	13
IV	941	30	64	11
V	176	60	12	21
VI	8	5	0	2

[1] See Figure 21 for description of bands

from 6400 ml of aorta homogenate. This material was separated into four major bands by preparative layer chromatography on silica gel H. The NSF material which migrates to the same area as "hot" NSF material was removed and separated from the mineral oil on another preparative thin-layer plate. This sample was further separated on an alumina column. The material of interest appeared in the third tube as ascertained by previous column chromatography of the NSF and confirmed by paper and thin-layer chromatography and gas chromatography.

A spectrum of farnesol was obtained because the prenol sample was suspected of having a structure similar to that of farnesol. The spectrum of the prenol sample suggested that it probably contained more than one compound. In the m/e region of 40 to 125 the spectra of the prenol sample and farnesol are very similar with some notable exceptions. The most abundant ion in the prenol sample is at m/e = 43 which is indicative of saturated hydrocarbons (C_3H_7-). The next most abundant ions (in decreasing order) are at m/e = 59, 57, 41, 69, 71, and 149. The m/e = 59 ion is indicative of C_3H_7O- while the ion at m/e = 41 is indicative of unsaturated hydrocarbons (C_3H_5-). The ion at m/e = 69 is indicative of the isoprene unit $CH_3\text{-}\underset{CH_3}{C}{=}CH\text{-}CH_2^-$. The m/e = 71 ion will arise from the saturation of this isoprene unit. There is a series of peaks regularly distributed 14 mass units apart, from m/e = 69 to m/e = 163, corresponding to the loss of CH_2 groups

which is typical of hydrocarbon chains. The ion at m/e = 149 may be a C_{11} fragment ($-CH_2-\underset{CH_3}{C}=CH-(CH_2)_2-\underset{CH_3}{C}=CH-CH_2-CH-$). There are no cyclic compounds in the prenol sample. In addition, there are mass peaks from m/e = 437 to m/e = 521, 28 mass units apart. The parent peak may be m/e = 521. In conclusion, it appears that the compounds in the prenol sample are high molecular weight straight chain hydrocarbons with some branching. These compounds contain oxygen, most probably as an alcohol, and have some degree of unsaturation.

Infrared Spectrum of Prenol Sample

The prenol sample was placed on a thin disc and run in the absence of any solvents. Figure 22 shows the spectrum of the prenol sample. Strong absorption bands were present at 2920 cm^{-1}, 2830 cm^{-1}, and 1740 cm^{-1}. Less intense bands were present at 1470 cm^{-1}, 1380 cm^{-1}, and 1075 cm^{-1}. Weak and broad bands were present at 1180-1110 cm^{-1} and 950-850 cm^{-1}.

The absorption in the 2920-2830 cm^{-1} is due to C-H stretching; this is indicative that $-CH_3$, $-CH_2$, and C-H groups are present in the prenol sample. This finding is supported by the band at 1470 cm^{-1} which is due to aliphatic $-CH_2$ groups. In addition, the 1380 cm^{-1} band is due to C-H bending and the 1290 cm^{-1} band, to single bond bending. The broad band in the region of 950-850 cm^{-1} is due

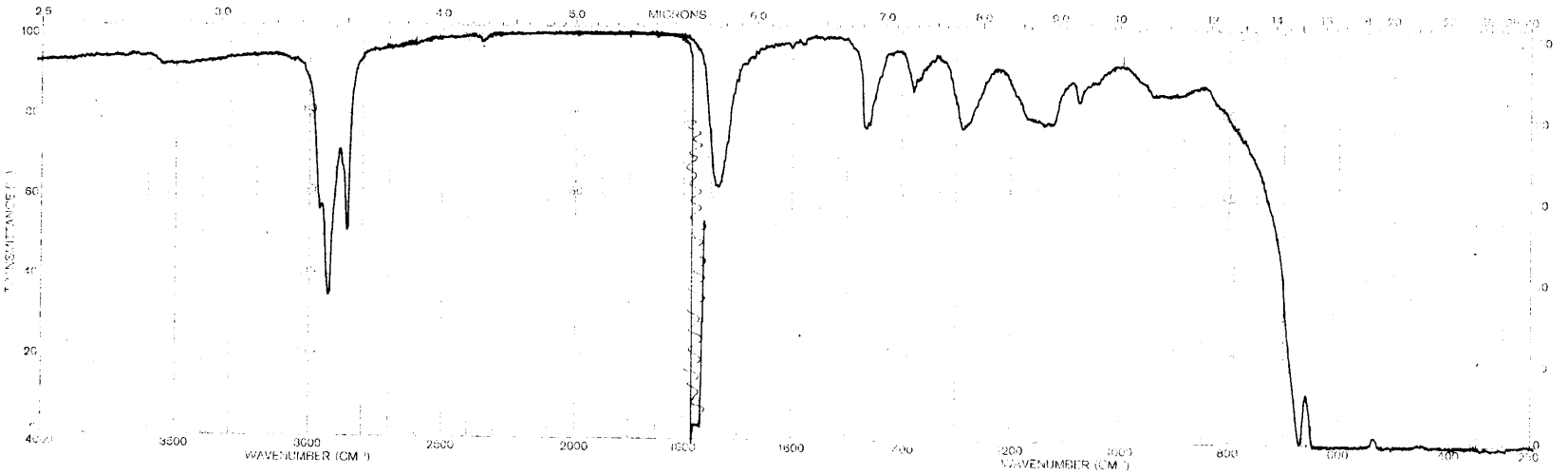


Figure 22. Infrared spectrum of the prenol sample.

to out of plane bending of C-H in a C=C-H group.

The strong band at 1740 cm^{-1} is indicative of the presence of a carbonyl group in the sample. In addition, the band at 1075 cm^{-1} would indicate the presence of a primary alcohol while the broad band at 1180-1110 cm^{-1} would indicate the presence of secondary and/or tertiary alcohols.

The results obtained from the infrared spectrum support the conclusion made from the mass spectrum of the prenol sample. Furthermore, the sharp absorption bands indicate that the compounds in the prenol sample are homongeneous with respect to the types of compounds, and it is this fact which makes their separation difficult.

DISCUSSION

The biosynthesis of sterols by arterial tissue has been mainly investigated either with intact aorta or aorta slices, but a few investigators have used an aorta homogenate. A cell-free homogenate has been made in this laboratory which shows low but significant incorporation of MVA into a nonsaponifiable fraction. The cofactor requirements for the incorporation of MVA into the NSF have been established along with the proof that mevalonic acid-5-phosphate and mevalonic acid-5-pyrophosphate are formed. In addition, it has been shown in this laboratory that acetyl-CoA, but not acetate, can be incorporated into the NSF by the cell-free preparation. Thus this study was undertaken to investigate why acetate cannot be activated by the cell-free preparation from bovine aorta and to characterize further the cell-free preparation with respect to isoprenoid biosynthesis.

In order to work with and study any part of the system of enzymes involved in incorporating mevalonic acid into sterol-related compounds in bovine aorta, it is advantageous to know how stable the whole system is. The 60,000 x g supernatant was found to be stable at 2-4°C for at least three days. Freezing for 24 hours did not cause any loss in activity in the 60,000 x g supernatant. The 60,000 x g supernatant could be lyophylized with only about a 33% decrease

in activity. If the dialysis bag was boiled in distilled water prior to dialysis, there was no loss in activity after dialysis. If the boiling process was omitted, one or more contaminators which prevent MVA-2-C^{14} from being converted to nonsaponifiable material were leeched from the bag. The 60,000 x g supernatant retained some activity after being fractionated on BioGel P-100.

The 60,000 x g supernatant is sufficiently stable in the cold, to lyophylization, to gel filtration, and to dialysis which means that it may be possible to isolate some of the enzymes involved in the incorporation of MVA into the NSF so that individual enzymes and reactions can be studied. In addition, stability after lyophylization means that the 60,000 x g supernatant can be stored in a dry state.

Many investigators (46, 103, 113, 120, 122) have used acetate-C^{14} as the substrate in investigations of sterol-synthesizing systems in arterial tissue. Walsh, Teal and Gamble (115) found that acetyl-C^{14}-CoA but not acetate-C^{14} could be incorporated into the NSF by the 60,000 x g supernatant. Thus the question arose as to whether or not the enzyme (acetyl-CoA-kinase) for activating acetate to acetyl-CoA was present in the 60,000 x g supernatant.

The 60,000 x g supernatant exhibited very little acetyl-CoA-kinase activity after three hours of incubation at 37°C. This activity appeared to be independent of exogenous acetate and CoA but did show some dependency upon added ATP, although there was no significant

increase in activity with high levels of ATP. Upon dialysis, the 60,000 x g supernatant showed no increase, but rather, a decrease in activity. Attempts at using a gentler method of preparing a tissue homogenate failed to produce any increase in activity. In an attempt to increase acetyl-CoA-kinase activity in the 60,000 x g supernatant, the protein was precipitated out of solution with ammonium sulfate and redissolved in a smaller volume of $KHCO_3$. There was no increase in activity when the protein from the 60,000 x g supernatant was concentrated as much as four-fold.

In order to determine if there was a factor in the 60,000 x g supernatant which could be preventing the formation of acetyl-CoA, the enzyme partially purified from yeast was assayed in the presence and in the absence of the 60,000 x g supernatant. There was a 37% inhibition of the conversion of acetate to acetyl-CoA with the yeast enzyme when the 60,000 x g supernatant was present. The heat-denatured 60,000 x g supernatant still exhibited an inhibitory effect. However, when the 60,000 x g supernatant was dialyzed overnight, this effect disappeared. When the dialysate was brought to the same volume as the dialyzed 60,000 x g supernatant, it caused the disappearance of free sulfhydryl groups as measured by the nitroprusside method. If the dialyzed 60,000 x g supernatant and dialysate were recombined and incubated with CoA, there was no reduction of free sulfhydryl groups.

The reaction between the dialysate and CoA does not require ATP, potassium acetate, or KCl. In addition, reduced glutathione could replace CoA as the substrate. The inhibitor is not a contaminator from the dialysis bag, since, even if the bag were boiled, the inhibitor could still be extracted from the 60,000 x g supernatant. In addition, the boilings from the bag had no effect upon reduced glutathione.

If the dialysate was placed on a BioGel P-2 column, the inhibitor came off in the void volume. The reduction of free sulfhydryl groups by the dialysate was shown to be concentration- and pH-dependent. In addition, the inhibitor appears to be stable to both acid and base.

No acetyl-CoA-kinase activity can be detected in the 60,000 x g supernatant by the nitroprusside assay method. Some acetyl-CoA-kinase activity is measured by the hydroxylamine assay method whereby the amount of hydroxamic acid formed is a measurement of activity. Since the activity appears to be independent of added substrate, the hydroxamic acid may arise from another reaction. Jones and Lipmann (60) have found, with crude yeast extracts, the acetylation of hydroxylamine to be, in part, enzymatic. Therefore, in addition to acetyl-CoA-kinase activity they observed "aceto-hydroxylamine kinase" activity. It may be the latter activity which is measured in the 60,000 x g supernatant with the hydroxylamine

method. The acetylation of hydroxylamine may require ATP. This would explain the dependency of the formation of hydroxamic acid upon added ATP. Dialysis failed to produce any increase in activity. It has been shown (60) that acetyl-CoA-kinase is unstable to dialysis; if the enzyme were present in the 60,000 x g supernatant, it would lose some activity after dialysis. Attempts at using a milder method of preparing a tissue homogenate failed to produce any increase in activity. Not only did the supernatants fail to exhibit acetyl-CoA-kinase activity, but the whole homogenate, including the particulate fraction, does not show any activity. Concentrating the protein in the 60,000 x g supernatant failed to produce any appreciable increase in activity. From these results it must be concluded that there is little or no acetyl-CoA-kinase activity in the 60,000 x g supernatant.

Acetyl-CoA-kinase is found in the soluble portion of the homogenate of yeast (60), ox brain (4), rabbit heart muscle (102), and bovine heart mitochondria (23). Thus the low activity in the 60,000 x g supernatant may be due to the fact that the enzyme is not in the soluble portion but remains in the particulate fraction when the method of preparation of the aorta homogenate described in this study is employed. However, assay of the entire homogenate (particulate and soluble fractions) did not show any acetyl-CoA-kinase activity. There is some evidence (1, 4, 101, 117) that acetyl-CoA-kinase is not a very stable enzyme; thus another reason for the low

activity of acetyl-CoA-kinase in the 60,000 x g supernatant may be that, once the enzyme is extracted from the tissue, it immediately becomes unstable and therefore inactive. A third explanation of the low activity of acetyl-CoA-kinase in the 60,000 x g supernatant may be that there is some inhibitor in the 60,000 x g supernatant which will interact with either the substrate (CoA) or the substrate enzyme complex and prevent the activation of acetate to acetyl-CoA. There is indeed an inhibitor in the 60,000 x g supernatant which prevents the condensation of acetate and CoA.

The inhibitor in the 60,000 x g supernatant is heat-stable and dialyzable. The inhibitor after dialysis will react directly with free sulfhydryl groups, but, in the presence of the 60,000 x g supernatant, it will react with the enzyme (acetyl-CoA-kinase). After dialysis the inhibitor will react not only with CoA but with glutathione. The reaction between the inhibitor and glutathione is pH-dependent. The inhibitor is not readily oxidizable, a fact which suggests that the reaction between the inhibitor and CoA is not one of oxidation-reduction. Furthermore, it was found that maleic acid, fumaric acid, diethyl fumarate, and diethyl maleate would react with glutathione under the same assay conditions as those under which the inhibitor reacts with glutathione. It has been shown that glutathione will react nonenzymatically with maleic acid (5), fumaric acid, diethyl fumarate, and diethyl maleate (18) to form addition products. Thus the inhibitor may be some compound such as fumaric acid which is extracted out of the bovine aorta in high enough concentration to interfere with the condensation of acetate and CoA. Therefore the inability of the 60,000 x g supernatant to incorporate acetate-C^{14} into the NSF may

be due in large part to this dialyzable inhibitor.

A number of investigators (7, 8, 62, 80, 132) have found ATPase, inorganic pyrophosphatase, and alkaline and acid phosphatase activity in the soluble portion of arterial tissue homogenates. Thus it appears that phosphatases are easily extracted from arterial tissue. Since there are many phosphorylated intermediates between mevalonic acid and squalene, the presence of phosphatase activity in a sterol- and isoprenoid- system would be important. There have been few studies of phosphatases in arterial tissue and none involving bovine aorta. Thus it was of interest to know if there was any phosphatase activity in the 60,000 x g supernatant.

When para-nitrophenylphosphate was used as the substrate there appeared to be nonspecific phosphomonoesterase activity at pH 7.0 in the 60,000 x g supernatant; this activity appeared to be stable. In addition, there was adenylpyrophosphatase and inorganic pyrophosphatase activity in the 60,000 x g supernatant.

It has been shown (28) that NaF and BSA will inhibit allyl pyrophosphatase activity in the soluble supernatant of rat liver homogenates. Thus it was of interest to know what effect NaF and BSA would have on the phosphatase activity of the 60,000 x g supernatant and on the incorporation of MVA-2-C^{14} into the NSF. NaF at 10 mM inhibited ATPase but not orthophosphatase activity, while BSA inhibited orthophosphatase activity in the 60,000 x g supernatant. In

addition, both NaF and BSA inhibited the incorporation of MVA-2-C^{14} into the NSF. When the NSF material from an assay in the presence of NaF and BSA was run on mineral oil impregnated paper, the distribution of the radioactivity was slightly different from that of the radioactive material obtained in the absence of both NaF and BSA. In the presence of NaF there appeared to be more polar compounds such as farnesol formed, while, in the presence of BSA less polar compounds were formed. Thus it appears that not only did NaF and BSA inhibit the incorporation of MVA-2-C^{14} into the NSF, but both influenced the type of compounds that were made by the 60,000 x g supernatant. The inhibition by NaF could not be prevented by increasing the ATP concentration.

Recently it was reported (100) that phenylalanine inhibits sterol biosynthesis *in vitro* in rat brain and liver. Due to the similarity in structure of phenylalanine and *para*-chlorophenoxyisobutyric acid (CPIB) and the fact that CPIB will inhibit the incorporation of MVA-2-C^{14} into the NSF by competing with ATP for a common binding site on MVA kinase (115), it was of interest to see what effect phenylalanine would have on the incorporation of labeled MVA into the NSF. L-phenylalanine was found to inhibit the incorporation of MVA-2-C^{14} into the NSF, but this inhibition was overcome by increasing the ATP concentration. CPIB inhibition can also be suppressed by increasing the ATP concentration (115).

In the investigation of the inhibitory effects of NaF it was found that, by lowering the concentration of NaF, the incorporation of MVA-2-C^{14} into the NSF could be increased. Maximum stimulation by NaF occurs at an ATP concentration of 3.3 mM. Other anions such as p-nitrophenylphosphate and p-nitrophenol were tested and found either to stimulate or inhibit the incorporation of MVA-2-C^{14} into the NSF. CPIB and L-phenylalanine at low concentrations stimulated the incorporation of MVA into the NSF. Other anions such as sulfate, chloride, and acetate weakly stimulated the incorporation of MVA-2-C^{14} into the NSF. In the light of these results on anion stimulation, it was of interest to know how necessary phosphate is to the system of enzymes in the 60,000 x g supernatant that converts MVA-2-C^{14} to the nonsaponifiable material. It was found that the arterial tissue must be prepared using phosphate in order to obtain any appreciable activity. Adding phosphate buffer to a 60,000 x g supernatant made with Tris-HCl buffer did not increase the amount of MVA-2-C^{14} incorporated into the NSF. On the other hand, adding Tris-HCl buffer to a 60,000 x g supernatant made with phosphate buffer did not decrease the amount of MVA-2-C^{14} incorporated into the NSF. Thus it must be concluded that phosphate must be present when the tissue is homogenized in order to extract active enzymes. If phosphate is not present but added later, there is no restoration of activity which implies that phosphate stabilizes rather than activates the enzyme(s) necessary for incorporating MVA-2-C^{14} into the NSF.

The "hot" NSF material has been shown (115) to have some radioactive cholesterol and lanosterol, but the majority of the labeled

products have been shown (115) to have chromatographic properties which are similar to, but distinct from those of cholesterol. No squalene or farnesol has been detected in the "hot" NSF material. It appears (115) that the major labeled material is a 20-25 carbon atom polyprenol which has a similar retention time to that of squalene when gas chromatographed and an R_F similar to that of farnesol when thin-layer chromatographed.

In order to determine if the "hot" NSF material could be further converted in the 60,000 x g supernatant, the "hot" NSF from one set of assays was used in place of MVA-2-C^{14} in another assay. The distribution of activity, after paper chromatography, of the new nonsaponifiable material when compared to that of the original NSF material shows only some redistribution of activity, which implies that the radioactive compounds formed in three hours in the 60,000 x g supernatant cannot be further converted under the assay conditions employed in this study. On the other hand, if the assay conditions are kept constant with the exception of the substitution of a rat liver homogenate for the 60,000 x g supernatant, only five percent of the original NSF activity remains in the nonsaponifiable material. When the saponified mixture is acidified, the remaining activity can be extracted with petroleum ether. This result implies that the original NSF material can be converted to acidic compounds. The material which was extractable with petroleum ether after acidification of the

saponified mixture was compared to the original NSF material using thin-layer chromatography. There was considerable difference between the two samples (in terms of distribution of radioactive material) after thin-layer chromatography. Thus it must be concluded that the 60,000 x g supernatant can synthesize biologically active compounds from mevalonic acid which are not precursors of cholesterol.

In an attempt to identify the compounds that are present in the arterial tissue and presumably also in the 60,000 x g supernatant after incubation with MVA-2-C^{14}, the nonsaponifiable material was extracted from 6400 ml of aorta homogenate. The sample of interest was separated by preparative layer chromatography and alumina column chromatography. After separation, a mass spectrum of the prenol sample was obtained. From this it was found that the compounds in the prenol sample are high molecular weight straight chain hydrocarbons with some branching. Also, the mass spectrum showed that the compounds contain oxygen and have some degree of unsaturation. A possible parent ion was found at m/e = 521. An infrared spectrum of the prenol sample supported the conclusions derived from the mass spectrum of the prenol sample. In addition, the infrared data showed that oxygen is present in the compounds as an alcohol and as a carbonyl. Furthermore, the sharp absorption bands indicate that the compounds in the prenol sample are homogeneous with respect to the types of compounds, and it is this fact which makes their separation difficult.

SUMMARY

This study was undertaken to investigate why acetate cannot be activated by the cell-free preparation from bovine aorta and to characterize further the cell-free preparation with respect to isoprenoid biosynthesis.

The 60,000 x g supernatant is stable at 2-4° C for at least three days. If the 60,000 x g supernatant is lyophylized, there is only a 33% decrease in activity. The 60,000 x g supernatant is stable to dialysis if the dialysis tubing is boiled prior to dialysis.

The 60,000 x g supernatant exhibited very little acetyl-CoA-kinase activity. This activity appeared to be independent of exogenous acetate and CoA but did show some dependency upon added ATP. Since the activity is independent of added substrate, the hydroxamic acid may arise from the direct acetylation of hydroxylamine. There is an inhibitor of acetyl-CoA-kinase in the 60,000 x g supernatant which is heat-stable and dialyzable. The inhibitor, after dialysis, can react directly with free sulfhydryl groups. Reduced glutathione can replace CoA as the substrate. The reduction of free sulfhydryl groups by the dialysate was shown to be concentration- and pH-dependent.

At pH 7.0 there is nonspecific phosphomonoesterase, adenyl-pyrophosphatase, and inorganic pyrophosphatase activity in the

60,000 x g supernatant.

NaF will inhibit ATPase but not orthophosphatase activity while BSA will inhibit orthophosphatase activity in the 60,000 x g supernatant. In addition, both NaF and BSA inhibit the incorporation of MVA-2-C^{14} into the NSF in the 60,000 x g supernatant.

L-phenylalanine was found to inhibit the incorporation of MVA-2-C^{14} into the NSF in the 60,000 x g supernatant. The inhibition by L-pyenylalanine can be overcome by increasing the ATP concentration.

NaF, p-nitrophenylphosphate, p-nitrophenol, p-chlorophenoxyisobutyric acid, and L-phenylalanine in low concentrations were shown to stimulate the incorporation of MVA into the NSF in the 60,000 x g supernatant. Other anions such as sulfate, chloride, and acetate weakly stimulated the incorporation of MVA-2-C^{14} into the NSF. It was also found that, at pH 7.0, phosphate stabilizes the enzyme(s) necessary for incorporating MVA-2-C^{14} into the NSF to a greater extent than does Tris.

Rat liver homogenate converted the radioactive material isolated from the 60,000 x g supernatant to acidic material.

A prenol sample was isolated from the aorta homogenate and determined to be a high molecular weight straight chain hydrocarbon with some unsaturation and branching. In addition, the prenol compounds contain oxygen as a carbonyl.

Thus, this study has shown that there is a heat-stable and dialyzable inhibitor of acetyl-CoA-kinase present in the 60,000 x g supernatant. In addition, at pH 7.0 there is phosphatase activity in the 60,000 x g supernatant. Certain anions, at high and low concentrations, respectively, can inhibit or stimulate the incorporation of MVA-2-C^{14} into the NSF. The radioactive NSF material was converted to acidic material in a rat liver homogenate, suggesting that the NSF material is not a precursor of cholesterol. There are high molecular weight prenols present in bovine aorta.

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