IV. DISCUSSION
**Nutritional Studies**

Last decade has seen a rapid growth in the understanding of the etiology and mechanics of atherosclerosis. Various dietary measures along with the use of drugs can bring serum lipid levels to a "normal" value and with these measures, a considerable majority of coronary heart disease and atherosclerosis can be prevented. This diseased state is associated with increased lipoprotein concentration which could be the result of either increased release or decreased removal from plasma, in relation to the normal state. The causes of hyperlipidemias are little understood and there is divergence of opinions whether the release of very low density lipoproteins into the plasma is increased or the removal is defective. It is not known whether increased fatty acid and cholesterol biosynthesis can lead to elevated plasma lipoproteins in man and animals although genetically obese animals which have high lipogenic capacity are also hyperlipidemic (Maragoudakis et al., 1972).

The results of present investigation show that HMG exerts a profound hypolipidemic effect in the normal as well as in the cholesterol fed rabbit, as has been shown earlier in rats (Beg and Siddiqi, 1967; 1968). Thus the lipid lowering action of HMG does not appear to be species-specific.
As shown in Tables I and II, HMG treatment significantly decreases serum total cholesterol, ester cholesterol, phospholipids and triglycerides in normally-fed rabbits. Among phospholipids the decrease was more-or-less evenly distributed among the various fractions but with a slightly greater trend with cephalins. However, HMG treatment for one week produced no significant effect on hepatic lipids (data not given).

Almost all serum and aortic lipids were significantly reduced in hypercholesterolemic animals two weeks after return to normal basal diet followed by HMG treatment (Tables III, IV and V). A significant decrease in serum lecithin, sphingomyelin and cephalins is also evident (Table VI). These results suggest that HMG, in addition to alleviating hyperlipemia, reverses lipid deposition in the aorta, a property considered essential for the successful therapy of disorders associated with hypercholesterolemia (Douglas, 1970). The decrease observed in almost all lipid fractions strongly suggests an effect on serum lipoproteins. It has long been recognised that the efficacy of medical approaches to atherosclerosis can best be evaluated in long term studies. The results described in Tables VII to XII provide evidence of the potential of HMG in resisting rise of
various lipid parameters in serum, liver and aorta when given along with cholesterol-corn oil diet for 54 days. Figs. 1 and 2 clearly demonstrate that HMG did significantly check the rise in serum cholesterol and phospholipids. In these regressive studies, HMG markedly suppressed the elevation of serum, liver and aortic triglycerides and cholesterol. Almost all serum and aortic phospholipid fractions, were significantly lower but strikingly so with serum cardiolipin in HMG-treated animals. There was no effect on aortic sphingomyelin. These results show that HMG could effectively counteract the enhanced lipemic response to dietary cholesterol-corn oil combination and does not produce any toxic effects.

It is premature to suggest the mechanism of action of HMG. However, as all serum lipid fractions are affected the action seems likely to be on lipoproteins. Moreover, reduction was most marked in triglycerides in all experiments suggesting an inhibition of triglycerides formation in liver and hence an effect on VLDL and LDL. In addition, the fall in serum triglyceride levels occurred in the absence of a rise in hepatic triglyceride concentrations. This suggests that inhibition of hepatic formation of triglycerides, rather
than assembly of lipoprotein complexes or their release, may be the initial mechanism by which HMG inhibits triglycerides output from liver. However, data do not exclude an additional effect of this agent on lipoprotein synthesis, assembly or release which may contribute to or maintain a hypotriglyceridemic effect. The reduction in fatty acid esterification may also account for the inhibition of triglyceride formation. In addition to inhibiting cholesterol synthesis (Fimognari, 1964; Beg and Lupien, 1972), HMG interferes at some stage of fatty acid synthesis as reported for CPIB and CIBA (Maragoudakis, 1969). The inhibition of hepatic triglyceride output could be attributed to suppress fatty acid synthesis or esterification, decreased lipoprotein formation, or a reduction in release of lipoproteins from liver. However, these experiments do not rule out an effect of HMG at the level of absorption and degradation of lipids.

The ability of HMG to lower all the lipid parameters is of special importance, since elevation in these biochemical parameters have been reported as characteristic of individuals suffering from coronary heart disease (Gertler and White, 1954). It has also been reported that cholesterol is a major
constituent of atherosclerotic lesion (Frants and Moore, 1969). It is also well established that the accumulation of cholesterol in aorta parallels remarkably well with the level of cholesterol found in the serum (Nichols et al., 1971). Furthermore, there is correlation between the severity of atherosclerosis and high levels of cholesterol and triglycerides in aorta. It is now well documented that triglycerides rather than cholesterol play critical role in the etiology of atherosclerosis (Schilling et al., 1966; Ghirardi et al., 1972).

Barlier Day (1962) suggested a role of phospholipids in the development of aortic lesion because its distribution was similar to those of cholesterol and neutral lipids. Peeters et al. (1970) also believed that the structure of phospholipids may play a role in the stability of lipoproteins and their interaction with the arterial wall since the cholesterol to phospholipids ratio is generally considered to be an index of atherogeneity. Cholesterol is supposed to be incorporated into the cells of lesion as cholesterol-phospholipids droplet, the cholesterol becoming esterified within the cell and transformed into amorphous droplets of cholestryl esters (Weller et al., 1968). Furthermore,
the presence of lecithin-cholesterol transacylase in the arterial wall provides evidence for the esterification of cholesterol in aorta since only free cholesterol rather than esterified cholesterol is exchanged with the blood during atherosclerosis. Reduction in the levels of cephalins and sphingomyelin due to HMG treatment may also possibly decrease the chances of atherosclerosis since high concentrations of these parameters are evident in the aortas and plasmas of diseased monkeys (Portman and Alexander, 1970) and in the peripheral occlusive arterial disease (Kunz and Stummvoll, 1971), respectively.

Recently, Lupien et al. (1973) reported the antihyperlipemic and antiatherosclerotic properties of HMG in rabbits. They believed that the mechanism of action of this compound in inhibiting the development of atherosclerosis might involve a shift in lipoprotein spectrum which may also partially be responsible for decreased cholesterol deposition in liver. They suggested that in reducing serum cholesterol, an increased rate of excretion of cholesterol and/or its metabolite can not possibly be excluded. The effect of HMG upon rate of fecal excretion of steroids and turnover of bile acids and lipoprotein metabolism has been postulated by
these workers. Except for hepatic phospholipids, all lipid levels were significantly lower in the HMG-treated animals (Table VIII). Moreover, recent studies of Saleemuddin and Siddiqi (1972) have shown that HMG acts in a physiological control mechanism for cholesterol synthesis.

**Enzymic studies**

Besides acetyl COA carboxylase, G-6-P dehydrogenase, malic enzyme and NADP-isocitric dehydrogenase play critically important role in the lipogenesis. Some lipogenic and glycolytic enzymes as possible locus of action of HMG have been investigated. Enzymic analyses under variety of nutritional conditions provide some insight into the mechanism of hypolipidemic action of HMG. Addition of different concentrations of HMG produced a marked depression in malic enzyme activity. The activities of LDH and G-6-P dehydrogenase were not affected by HMG even at higher concentrations. However, similar doses produce enhancement in the activities of MDH and NADP-isocitric dehydrogenase (Table XIII). The inhibition of malic enzyme is quite specific, since the higher doses of HMG drastically inhibited this enzyme (Table XIV). Moreover, kinetic studies with crude as well partially purified enzyme preparations showed that
HMG behaves allosterically (Figs. 4 and 6). The probable molecular order of participation of HMG in the inhibition of rat liver malic enzyme was obtained by the Hill plot (Fig. 3) where interaction coefficient is approximately two. It is, therefore, quite probable that binding of two HMG molecules per active site was sufficient for inactivation. However, interactions of HMG with the malate binding site of the enzyme may not be so strong as shown by the high dissociation constant, $K_d = 41.96 \times 10^{-3} M$. The significance of this observation is yet to be investigated with more purified enzyme.

The malic enzyme and G-6-P dehydrogenase activities were significantly lowered but NADP-isocitric dehydrogenase and LDH were significantly increased when normally fed rats received i.p injection of HMG (Table XV). The decrease in the activity of malic enzyme and G-6-P dehydrogenase appears to be an expected biochemical effect of HMG which lowers the lipid contents of the blood (Tables I and II). The hypolipidemic activity of the compound could have been exerted by reducing or controlling the NADPH available for lipogenesis since NADPH/NADP ratio is known to play a critical role in the fatty acid synthesis.

Lipid synthesis is depressed on fat feeding and fasting (Bortz et al., 1963; Wieland et al., 1963). Various liver enzymes including G-6-P dehydrogenase, malic enzyme and citrate cleavage enzyme show a pronounced adaptive behaviour.
towards hormonal influences and varied feeding conditions. The activities of these enzymes change in parallel with the extent of lipogenesis (Olsen, 1966; Ballard and Hanson, 1967). The pyruvate carboxylase-malate dehydrogenase-malic enzyme system represents an ATP-requiring trans-hydrogenation and can account for the transfer of the reducing equivalents (NADH formed during the conversion of glucose to pyruvate via Embden-Meyerhof pathway) into NADPH suitable for lipogenesis (Ball, 1966). Since HMG inhibits malic enzyme and also G-6-P dehydrogenase less NADPH is available for lipogenesis and the NADH formed during Embden-Meyerhof pathway brings about an increase in the activity of LDH. The observed increase in the NADP-isocitric dehydrogenase activity could be responsible for decreasing citrate concentration which in turn may diminish fatty acid synthesis through inactivation of acetyl CoA carboxylase.

The mechanism involved in the reduction of enzymatic and lipogenic activity on fat feeding was not clear for long. Several reports have demonstrated that diet induced increase in G-6-P dehydrogenase activity are dependent upon the simultaneous ingestion of the carbohydrate and protein (Niemeyer et al., 1962; Pitot et al., 1964; Mc Donald and
Johnson, 1965). A critical literature survey supports another conclusion that, the changes in the activity of G-6-P dehydrogenase and malic enzyme are secondary to alterations which increase the metabolic demand for reducing equivalents in the form of NADPH. Thus in fasting-refeeding the increase in G-6-P dehydrogenase and malic enzyme activity is preceded by an increase in lipogenesis (Tepperman and Tepperman, 1964). The increase in G-6-P dehydrogenase observed in animals refed a high carbohydrate diet following a fast (Zakim et al., 1967) can be explained on the basis of increased demand for NADPH to support the increase in lipogenesis.

The proposed mechanism is consistent with the decreased enzyme activity in HMG-treated normally fed rats with a resulting decrease in the oxidation of NADPH which could in turn trigger a reduction in G-6-P dehydrogenase and malic enzyme activity. Such a mechanism is in keeping with present concepts concerning the homeostatic control of lipogenesis and related requirements for reduced coenzymes (Leveille, 1966).

In 5% cholesterol fed rats (Table XVI) HMG treatment brings about a significant increase in the activities of malic enzyme (85%), G-6-P dehydrogenase (53%), MDH (60%),
LDH (25%) and NADP-isocitric dehydrogenase (11%). It is interesting to note that under this condition of nutritional stress the activities of malic enzyme and G-6-P dehydrogenase is brought at par with the normal fed control rats (Table XV). The reason for increase in the activities of LDH, MDH and NADP-isocitric dehydrogenase may be same as stated for normal fed rats.

A very large number of enzymes activities are significantly decreased under condition of starvation (Freedland, 1967). It is not certain why the systems behave in this fashion but several possibilities exist. The first possibility is a large scale change in the internal nutrition of the rats, the second possibility is the stability of the template or m-RNA which may decay without replenishment as there is evidence that certain m-RNAs may have life-span rather than a half-life (Pitot, 1965). There is also possibility of change in hormone balance for it has been shown that certain enzymes in starved rats decrease more rapidly in rats with various endocrine removals than in normal rats (Weber, 1963) and again the stability of the turnover time of certain enzymes may be altered significantly during starvation (Shimke, 1964).
The activities of the enzymes thought to be indirectly involved in lipogenesis were the least stable. This may reflect the absence of lipogenesis during starvation. Therefore, G-6-P dehydrogenase and malic enzyme activities are decreased in fasted rats. HMG treatment causes a significant increase in malic enzyme (57%), G-6-P dehydrogenase (42%) and NADP-isocitric dehydrogenase (15%) while the increase in LDH and MDH activities were not statistically significant. It could, therefore, be assumed that HMG is capable of correcting the large scale starvation induced change in internal nutrition of the rats.

When HMG treatment was extended to the fasted rats with the refeeding of a normal basal diet significant depression in the activity of malic enzyme and G-6-P dehydrogenase was found (Table XIX). The other enzymes tested were not influenced by HMG treatment under fasting-refeeding condition. This could be due to actual changes in enzyme content rather than activation or inhibition of preformed enzyme. Thus, in liver the rate of enzyme synthesis or degradation could also be related to explain the mechanism of action of HMG. The enhancement of malic enzyme and G-6-P dehydrogenase due to HMG treatment in cholesterol fed and fasted rats (where as compared to
normally-fed animals, Table XV, the depression in the activity of these enzymes occur) possible role of degradation and synthesis of more enzymes cannot be excluded.

Numerous other factors may influence the activity of acetyl CoA carboxylase, malic enzyme, G-6-P dehydrogenase, citrate cleavage enzyme in vivo. Availability of Krebs cycle intermediate (Atkinson, 1969), inhibition by long chain acyl CoA compounds (Borts and Lynen, 1963) and other as yet unknown factors, could also be related to these complex processes in vivo.

Recently, roles of synthesis and degradation as determinants of the levels of certain lipogenic and glycolytic enzymes have been investigated in the livers of rats subjected to various nutritional states. Craig et al. (1972) have shown that the dramatic increase in levels of hepatic fatty acid synthetase upon refeeding a fat-free diet is due to an increase in the rate of de novo synthesis of the enzyme.

In addition to the fatty acid synthetase a number of other enzymes have been shown to exhibit similar changes in activity with a parallel change in hepatic lipogenesis in response to nutritional stress. Among these are acetyl
COA carboxylase (Allmann et al., 1965; Muto and Gibson, 1970), citrate cleavage enzyme (Kornacker and Lowenstein, 1965; Muto and Gibson, 1970) and malic enzyme (Tepperman and Tepperman, 1964; Muto and Gibson, 1970). A stimulation in malic enzyme upon refeeding a fat-free diet has been reported by Lyons and Gibson (1971). It appears, therefore, that the rate of enzyme synthesis is an important parameter in controlling the levels of these enzymes as well as that of fatty acid synthetase in liver. Thus, the synthesis of these enzymes which are essential for the synthesis of fatty acids may be coordinately controlled, a mechanism which would obviously be of benefit to the organism. This conclusion is further supported by the findings of Muto and Gibson (1970) that the greatly stimulated activities of the lipogenic enzymes, namely, acetyl COA carboxylase, fatty acid synthetase, citrate cleavage enzyme, malic enzyme and G-6-P dehydrogenase in the livers of rats maintained on a fat-free diet are progressively diminished on oral administration of polyunsaturated fatty acids. These results also suggest that polyunsaturated fatty acids are responsible for such adaptive changes in lipogenic enzymes.

In contrast to synthesis, degradation of enzymes also play important role in the control processes. Fatty acid
seems to be degraded more rapidly in the fasted state than in the normal and refeed states (Craig et al., 1972). The degradation of other enzymes has been reported to increase in the fasted state as in acetyl CoA carboxylase (Nakanishi and Numa, 1970), and malic enzyme (Silpananta and Goodridge, 1971). In general, the degradation of total soluble liver proteins is increased on starvation.

Unlike HMG other hypolipidemic agent viz., clofibrate (Zakim et al., 1970) and CIBA (Schacht and Granzer, 1970) increased the activity of malic enzyme under normal feeding conditions. These compounds were inhibitory to acetyl CoA carboxylase in vitro and in vivo (Maragoudakis, 1969; Maragoudakis et al., 1972) however, HMG is only inhibitory to acetyl CoA carboxylase at higher concentrations (Beg, Z. H., personal communication). Therefore, hypolipidemic activity of HMG could not be explained on the basis of acetyl CoA carboxylase inhibition.

CIBA induced increase in malic enzyme appears to be an incomprehensible biochemical effect of a drug which lowers the lipid contents of blood and liver (Schacht and Granzer, 1970). This has been explained on the basis of approximately equal activity increase of microsomal NADPH-cytochrome c reductase. This microsomal enzyme complex
oxidizes the hydrogen of NADPH with atmospheric oxygen to water, no ATP being formed. Through an ATP-dependent reaction which runs between pyruvate, OAA and malate, hydrogen may be transferred from NADH to NADPH (Seubert and Huth, 1965; Kornacker and Lowenstein, 1965) and its oxidation finally achieved by microsomal NADPH-cytochrome c reductase. In this reaction cycle malic enzyme catalyzes the third and last reaction, the NADP-dependent oxidative decarboxylation of malate to pyruvate, NADPH being formed. Therefore, like CIBA, this complex type of enzyme mediation may not possibly be ruled out in case of HMG treatment to cholesterol fed and fasted rats in which activity of malic enzyme and G-6-P dehydrogenase is increasing. Further the enhancement of acetate-1-\textsuperscript{14}C incorporation into hepatic fatty acids (Table XIX) of normally fed rats is an incomprehensible biochemical effect of hypolipidemic action of HMG. This type of synergistic result has also been reported with CPIB (Avoy et al., 1965). Thus acceleration of fatty acid degradation could also be one of the possible way to explain the hypolipidemic action of HMG.

Our studies suggest that HMG has a great potential as hypolipidemic drug; it is effective in both short and long-term administration. The role of triglycerides,
phospholipids and cholesterol in atherogenesis is well
documented (Schilling et al., 1965; Ghirardi et al., 1972;
Peeters et al., 1970; Weller et al., 1968) HMG treatment
lowers all these fractions. As HMG is a natural
metabolite (Dekker et al., 1958), it is well tolerated
and does not produce any side effects. Malic enzyme and
G-6-P dehydrogenase are considered important determinants
of the rate of hepatic fatty acid synthesis (Zakim et al.,
1967; Ballard and Hanson, 1967; Cahill et al., 1958) and
HMG inhibits in vitro and in vivo malic enzyme activity
and in vivo G-6-P dehydrogenase activity. Moreover, recent
studies from this laboratory have shown that HMG acts in
a physiological control mechanism for cholesterol
synthesis (Saleemuddin and Siddiqi, 1972).

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