VI. PHARMACOLOGICAL STUDIES
EXPERIMENTAL

The nutritional and histopathological studies have already shown that HMG is not only effective in lowering the cholesterol and other lipid levels of serum and liver of normocholesterolemic and hypercholesterolemic rats but also hastens recovery of the fatty liver induced by feeding fat-rich cholesterol diet. During these studies, no toxic or untoward effects of HMG on the cellular architecture of various tissues were observed. The compound was effective orally as well as intraperitoneally. It was therefore, considered worthwhile to undertake a preliminary pharmacological screening of this promising hypocholesterolemic agent.
RESULTS

Effect of HMG on Blood Pressure and Respiration of Dog -

Dogs weighing about 12 Kg were anaesthetized intravenously with sodium pentobarbitone (35 mg/Kg). The carotid blood pressure was recorded. Respiration was also recorded through cannulated trachea by Marey's tambour. HMG was administered through cannulated femoral vein. The kymographic tracings of the blood pressure are shown in Fig.13.

HMG in doses upto 2.0 mg/Kg did not modify the blood pressure, the heart rate and the respiration.

Effect of HMG on Blood Pressure of Spinal Cat - Adult cats were employed for this study. The animal was anaesthetized with intravenous sodium pentobarbitone (30 mg/Kg). The carotid artery was cannulated for recording the blood pressure on a slowly moving kymograph. The spinal cord was sectioned at cervical 7th segment and artificial respiration started through the intubated trachea. The femoral vein was cannulated for intravenous injection of
Fig. 13. Effect of HMG on blood pressure of dog.
See text for details.
HMG. HMG in doses up to 15 mg/Kg did not produce any effect on the blood pressure of the cat. The blood pressure responses to acetylcholine, adrenaline or histamine were not modified by previous administration of HMG in the above doses.

It is clear from the above studies that HMG also failed to produce any effect on blood pressure of spinal cats.

Effect of HMG on the Frog Heart in situ — The experiment was performed in four animals. The brain and the spinal cord of a frog was pithed and the animal laid on its back and firmly fixed to the board. The heart was exposed and a cannula passed through the inferior venacava. The apex of the ventricle was attached to a heart-lever through a hook and thread. A small slit was made in the ventricle to allow the flow of the perfusion fluid. Frog Ringer's solution was perfused from an open reservoir into the heart and the rate of flow adjusted to about 40 drops per minute. Perfusion fluid containing different concentrations of HMG was allowed to flow through the heart.

HMG in concentrations up to 1 in 10,000 did not modify the rate, amplitude and the tone of the heart.
contraction (Fig. 14). It did not modify the responses of the heart to adrenaline or acetylcholine also.

**Effect of HMG on Smooth Muscles** - Effect of HMG was studied on isolated guinea pig ileum, rabbit small intestine and rat uterus, employing routine methods. The effect of HMG was tested against spasms induced by acetylcholine and histamine and relaxation produced by epinephrine. Varying doses of HMG were used against each spasmogen and 3-4 experiments were conducted for each dose.

HMG did not show any effect on the spontaneous contraction of the rabbit intestine, guinea pig ileum or rat uterus. The contraction of the intestine and uterus induced by acetylcholine and its relaxation induced by epinephrine were not modified by the addition of HMG either before or after the above drugs. The contraction induced by histamine was not affected with very high doses of HMG (Fig. 15). HMG (0.4 mg/ml) produces its own spasm. However, at repeated dose no spasmogenic effect was observed.

**Acute Toxicity of HMG** - HMG was tested for acute toxic doses in mice. Oral administration of HMG upto 1 g/Kg did not produce any visible alteration in behaviour or
Fig. 14. Effect of HMG perfusion on frog’s heart. See text for details.
Fig. 15. Effect of HMG on isolated guinea pig ileum. See text for details.
death in a group of 10 mice. In another group of 9 mice receiving the same dose intraperitoneally, 4 animals died within the first 24 hours.
DISCUSSION

In comparison to other known hypocholesterolemic and hypolipemic drugs (Holmes, 1964) which have harmful side effects, HMG, in addition to its hypocholesterolemic and hypolipemic actions, has been found pharmacologically inert. It is pertinent to recall that HMG is a natural metabolite of liver and is known to competitively inhibit hepatic HMG-CoA reductase activity. Therefore as known for bacterial system (Wright, 1957), it may also be considered as an antimetabolite of mevalonic acid for mammalian systems.

Hence, the pharmacological inertness, of HMG, strongly supports the view that it may prove superior to other known hypocholesterolemic and hypolipemic drugs and may find a clinical use, provided in men this substance acts in the same way and is well tolerated. A detailed clinical investigation is, therefore, necessary before the compound can be declared suitable for therapeutic purposes.
EXPERIMENTAL

Chemicals and Reagents — Special chemicals obtained from commercial sources used without further purification included: Coenzyme A (CoA), Crystalline bovine serum albumin and Glutathione (Mann Research Laboratories, (U.S.A.); Versene (E. Merck, Germany); Tris (hydroxy-methyl)-aminomethane (Sigma Chemical Co., U.S.A.); 2,6-dichlorophenol indophenol (Eastman Kodak, U.S.A.).

All other chemicals used were of A.R. grade.

Preparation of special reagents and chemicals was as follows:

HMG-Anhydride — This was synthesized according to the method of Hilz et al. (1958). 902 mg (approximately 62 mmoles) of HMG was refluxed gently for one hour with 30 ml of anhydrous pure benzene and 15 ml of acetic anhydride. Excess acetic anhydride and benzene were removed at 60-70° under vacuum, whereupon the dark residue solidified on cooling. The crude material was charcoaled and crystallized twice from anhydrous benzene by allowing the hot solution
to cool overnight under a soda-lime guard tube. The fine, colorless needles M.P. = 100° (uncorr.); reported M.P. = 101-2° (uncorr.) were stored over anhydrous CaCl₂.

\[ \text{HMG-Hydroxamate} \quad 6.6 \text{ mg (45.8 \, \mu \text{moles}) of HMG-anhydride were dissolved in 0.5 ml of 2 M NH}_2\text{OH solution, pH 7.0, and diluted to 2.00 ml after 10 minutes (Lipmann and Tuttle, 1945).} \]

\[ \text{Nitroprusside Reagent} \quad 1.5 \text{ g of sodium nitroprusside was dissolved in 5.0 ml of 2 M H}_2\text{SO}_4, 95 \text{ ml of absolute methanol and 10 ml of 15 M NH}_4\text{OH solution. The copious white precipitate which resulted was removed by filtration and the clear orange filtrate stored at 0°. Appearance of red spots immediately after treatment with nitroprusside reagent was taken to indicate the presence of free-SH groups (Stadtman, 1957). The very faint color produced with small quantities of -SH group was intensified by treatment with ether. In event of negative test for free -SH, the nitroprusside dip was followed by a treatment with methanolic NaOH solution. Under alkaline conditions the thiol produce a red color. Thus red spots appearing after alkali treatment were assumed to be due to} \]
thiol esters. Here again weak spots were intensified using ether.

HMG-CoA – 100 mg of CoA (approx. 103.0 μmoles, assuming 75% purity) was dissolved in 7.0 ml of ice-cold water and adjusted to pH 7.5 with 1 N KOH (0.5 ml). Maintaining the pH between 8 and 9 by addition of KOH, H₂S gas was bubbled through the solution for 30 minutes to reduce any oxidized CoA. Excess H₂S was then driven off at pH 3-4 by N₂ stream. At this stage the qualitative test for free -SH groups was positive and the test for acyl thiols (Stadtman, 1937) negative. The solution was then buffered to pH 7.5 using saturated KHCO₃ solution (0.6 ml) with continuous bubbling of N₂. HMG-anhydride (approximately 20.0 mg) was then added till the free -SH test was negative and the acyl thiol test positive. The reaction was complete within few minutes. The pH was adjusted to 5.0, and the solution stored at -15°C and used within 3 days (Lynen, 1959).

The purity of HMG-CoA was checked colorimetrically by the method of Durr and Rudney (1960). The HMG-CoA prepared was only as pure as the coenzyme used. Based on 75% purity of CoA used, the yield of HMG-CoA was 80%.
Colorimetric Assay of HMG-CoA - The method is essentially that of Durr and Rudney (1960). 5.0 ml of sample containing HMG-CoA (up to 4 μmoles) was mixed with 0.5 ml of alkaline hydroxylamine (freshly prepared by mixing equal volumes of 2 M NH₂OH.HCL and 2.5 M NaOH solutions). After one minute, 1.5 ml of FeCl₃ reagent (equal parts of 0.37 M FeCl₃, 0.3 M trichloroacetic acid and 0.65 M HCl) was added. The colour intensity was determined in a Klett-Summerson photoclectric colorimeter (green filter). The standard curve used for colorimetric estimation of HMG-CoA was prepared under the above conditions from HMG-hydroxamate (Fig. 16).

Quantitative Measurement of Free Sulfhydryl Groups - The method of Grunertt and Phillips (1951) was used. To a test tube containing 6.0 ml of saturated NaCl solution, a 2 ml of sample (upto 1 μmole) containing 0.4 ml of 9% metaphosphoric acid was added. After 10 minutes at room temperature, 1 ml of 0.067 M sodium nitroprusside and 1 ml of Na₂CO₃ + NaCN solution (equal volumes of 1.5 M Na₂CO₃ and 0.067 M NaCN solutions) was added. The colour density was measured within one minute versus a reagent blank in a Klett photometer using green filter. For the analysis of
Fig. 16.  Colorimetric assay of HMG-hydroxamate.
See text for details.
-SH groups in biological samples, 9% metaphosphoric acid was used as a deproteinizing agent. To quantitate the amount of -SH liberated, GSH was used as a standard (Fig. 17).

**Preparation of Liver Homogenates (10%; w/v)** — All operations were carried out at 5°C. The livers, 0.5 g, from each rat of a particular group, were weighed and pooled together. The pooled samples were cut into fine pieces and 1 g of pooled liver was accurately weighed and transferred to a chilled Potter-Elvehjem all glass homogeniser, containing 5 ml of 0.1 M KCl solution. The homogenisation was carried out at slow speed for two minutes. The resulting thick suspension was diluted with 3.5 ml of 0.1 M KCl and 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4 and stirred mechanically for 30 minutes. After centrifugation at 2,500 r.p.m. for 15 minutes, the supernatant was used for the assay of HMG-CoA hydrolase activity.

**Assay of HMG-CoA Hydrolase (EC 3.1.2.5)** — The enzyme was assayed by the colorimetric method as described (Dekker et al., 1958). The assay system contained,
Fig. 17. Standard curve for -SH estimation.

See text for details.
200 μmoles of Tris buffer, pH 6.8, 20 μmoles of versene, 2 μmoles of synthetically prepared HMG-CoA. The homogenate to be assayed was added to this mixture, the final volume was adjusted to 2 ml with water, and the nitrogen was flushed in the tube, stoppered and incubated at 37° for 15 minutes. At the end of this time the tube was placed in ice and the incubation mixture was deproteinized by 1 ml of 9% metaphosphoric acid. After centrifugation at 5°, a suitable aliquot of the supernatant was used for determination of liberated sulfhydryl groups by nitroprusside reaction as described earlier. Controls without added enzyme and without added substrates were also included to correct for endogenous liberation of sulfhydryl groups. Under the assay conditions the amount of sulfhydryl groups released was found to be dependent over a limited range on the concentration of hydrolase.

HMG-CoA hydrolase was also assayed spectrophotometrically by determining the rate of reduction of 2,6-dichlorophenolph indophenol at 500 μv in a Beckman DU spectrophotometer at 28°. The assay system, in a cuvette in a total volume of 1.5 ml contained, 100 μmoles of phosphate buffer, pH 7.0, 5 μmoles of versene, 0.2 μmoles of 2,6-dichlorophenolph indophenol, the homogenate to be
tested and 2 μmoles of synthetically prepared HMG-CoA. The homogenate was omitted from the control. After a 4 minute preincubation period synthetic HMG-CoA was added to each cuvette as the final constituent. The rate of dye reduction after the addition of thiol ester to the assay mixture was found to be constant for 4 minutes and dependent on concentration of hydrolase over a limited range.

**Protein Estimation** - The method used was that of Lowry et al. (1951). In determining the protein concentrations of homogenates, 3 ml of 10% trichloroacetic acid solution was added to 0.5 ml of rat liver homogenates. The precipitate was centrifuged and washed successively (three times) with 0.1 N potassium acetate in alcohol, alcohol and ether. The washed precipitate was dissolved in 5 ml of 1 N NaOH by heating in boiling water. To 1 ml of aliquot, containing suitable quantity of protein, was added 5 ml of copper reagent and incubated at room temperature for 10 minutes. After incubation, 0.5 ml of diluted Folin reagent (1 N) was added, mixed instantly and color intensity was read after 30 minutes in a Klett photometer using red filter. Crystalline bovine serum albumin solutions were used as
standard (Fig. 18).

Nuclei Counts of Rat Liver Homogenates — The method of Bass et al. (1955) was used for the counting of nuclei. A 10% (W/V) homogenate of liver in 0.85% saline solution was prepared employing homogenisation time of 30 seconds in a Potter - Elvehjem tissue grinder. After homogenisation, a 2 ml aliquot was mixed thoroughly with an equal volume of crystal violet solution (80 mg of crystal violet in 100 ml of 6% acetic acid). A drop of the mixture was placed under the cover slip of a Levey-Hausser-counting chamber and the nuclei were enumerated. Five counts were made on each preparation and the average of these counts was recorded.
Fig. 18. Standard curve for protein determination.
See text for details.
RESULTS

Effect of Dietary Cholesterol on Hepatic HMG-CoA Hydrolase (EC 3.1.2.5) — For determining the effect of dietary cholesterol on hepatic levels of HMG-CoA hydrolase, two groups of young male Albino rats, each group containing five animals were used. In order to produce hypercholesterolemia, the experimental group was maintained for two weeks on an experimental diet as described for Table X. The control group was kept on basal diet only. The rats of both the groups were sacrificed, livers excised and chilled. The livers of all the rats in a particular group were pooled, cut into pieces and homogenized. The cholesterol and protein contents were determined. The nuclei were also counted. The hepatic HMG-CoA hydrolase activity was measured colorimetrically. In mixed assay, equal volumes of supernatants from experimental and control were mixed before enzyme assay. The results are summarized in Table XI. It is important to note that the rats used in this experiment were the same as used for nutritional and histopathological studies.
Table XI

Effect of 2-week Cholesterol Feeding on Hepatic HMG-CoA Hydrolase

<table>
<thead>
<tr>
<th>Status</th>
<th>Weight of animal (g)</th>
<th>Total liver cholesterol (mg %)</th>
<th>Cellularity^a</th>
<th>Supernatant protein^b</th>
<th>Liver activity^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99 ± 5</td>
<td>525 ± 29</td>
<td>272 ± 1</td>
<td>2.9</td>
<td>3.05 (100)^d</td>
</tr>
<tr>
<td>Experimental</td>
<td>85 ± 11</td>
<td>1183 ± 36</td>
<td>230 ± 1</td>
<td>3.86</td>
<td>16.6 (545)</td>
</tr>
<tr>
<td>Mixed</td>
<td>-</td>
<td>-</td>
<td>251 ± 2</td>
<td>3.7</td>
<td>13.9 (455)</td>
</tr>
</tbody>
</table>

^a Expressed in millions of nuclei counted per g of wet weight of tissue;
^b calculated as mg protein per cell x 10^-7; ^c enzyme activities are represented in 
mumoles of sulphydryl liberated per minute per average cell x 10^-7; ^d numbers in 
parentheses express the data in percentages taking the value of respective control 
group as 100.
It is evident that as compared to control group there is a 4-5 fold increase in the HMG-CoA hydrolase activity in animals fed cholesterol, fat and bile salt. In mixed assay also, there is approximately 5-fold increase in the enzyme activity.

In order to see whether cholesterol feeding, alone, also has similar effect on enzyme activity, the rats in experimental group were fed only 2% cholesterol on basal diet (on weight basis). The control animals received basal diet only. From the analysis of hepatic cholesterol levels, it is evident that increase in cholesterol was approximately of the same magnitude as in previous cases where cholesterol and bile salt, were given by intubation. The enzyme activity was measured by colorimetric as well as spectrophotometric methods.

The data presented in Table XII show a 3-4 fold increase in the HMG-CoA hydrolase activity in cholesterol fed animals also as compared to control group.
Table XII

Effect of 3-day Cholesterol Feeding on Hepatic HMG-CoA Hydrolase

<table>
<thead>
<tr>
<th>Status</th>
<th>Weight of animal (g)</th>
<th>Weight of liver (g)</th>
<th>Total liver cholesterol (mg %)</th>
<th>Activity&lt;sup&gt;a&lt;/sup&gt; per g liver</th>
<th>Activity&lt;sup&gt;a&lt;/sup&gt; per mg protein</th>
<th>Activity&lt;sup&gt;a&lt;/sup&gt; per 100 g body weight</th>
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</thead>
<tbody>
<tr>
<td>Spectrophotometric Method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>115 ± 3</td>
<td>5.222</td>
<td>1226 ± 30</td>
<td>51</td>
<td>377</td>
<td>232</td>
</tr>
<tr>
<td>Experimental (3)</td>
<td>109 ± 3</td>
<td>4.896</td>
<td>500 ± 22</td>
<td>19</td>
<td>176</td>
<td>86</td>
</tr>
<tr>
<td>Colorimetric Method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>115 ± 1</td>
<td>4.736</td>
<td>1028 ± 38</td>
<td>150</td>
<td>-</td>
<td>618</td>
</tr>
<tr>
<td>Experimental (5)</td>
<td>114 ± 3</td>
<td>4.392</td>
<td>522 ± 18</td>
<td>40</td>
<td>-</td>
<td>154</td>
</tr>
</tbody>
</table>

<sup>a</sup> One unit of enzyme is the amount which catalyzes the release of one μmole of sulphydryl per minute under standard assay conditions; <sup>b</sup> numbers in parantheses refer to the number of animals in each group.
DISCUSSION

It has been shown by various workers that cholesterol feeding will result in depression of hepatic cholesterol biosynthesis in animals (Gould et al., 1953; Frantz et al., 1954). The enzymatic site most responsible for this diminished activity has been shown to be early in biosynthetic pathway. Gould and Popjak (1957) observed that this reaction site is located prior to MVA. Siperstein et al., (1966) and Siperstein and Fagan (1966) have demonstrated that the HMG synthetic pathway was not impaired by cholesterol feeding although MVA synthesis was diminished. Linn (1967) has provided first direct evidence that HMG-CoA reductase (mevalonate:NADP oxidoreductase) (acylating CoA), EC 1.1.1.34) activity was depressed in cholesterol fed rats. These results, together with the published works of others implicating the reductive step leading to MVA formation, are highly consistent with HMG-CoA reductase being the control enzyme for cholesterol biosynthesis.
It has been suggested that the basic unit which determines the rate of metabolic processes at the cellular level is the enzyme-substrate complex (Krebs, 1957). Thus the size of an enzyme population and the amount of its substrate together limit the maximum velocity which can operate under physiological condition. Since our observations show a 3-5 fold increase in hepatic HMG-CoA hydrolase activity (Table XI and XII), we believe that the rise in activity is primary manifestation of cholesterol feeding; the secondary being the inhibition of HMG-CoA reductase activity by HMG released in vivo due to increase in HMG-CoA hydrolase activity. The increased activity of HMG-CoA hydrolase might be due to substrate level induction because it is well known that HMG-CoA pool increases in cholesterol-fed (Siperstein and Fagan, 1964), as well as fasting rats (Wieland et al., 1960). From the recent work of Linn (1967) a simple inhibition of cholesterol biosynthesis by free cholesterol is eliminated. Furthermore the possibility of a lipoprotein complex being the inhibitor, as suggested earlier (Siperstein and Fagan, 1964) has also been ruled out (Linn, 1967). In light of reported properties and behaviour of cholesterol synthesis inhibitor (Migicovsky, 1962), which is very similar to
HMG, and the fact that HMG is known to inhibit the cholesterol biosynthesis together with our observations, it is quite likely that HMG is the actual inhibitor of cholesterol synthesis. It appears more reasonable that, in vivo, HMG exerts its inhibitory effect in bound form. Support to this hypothesis is again obtained from the work of Migicovsky (1964) who had shown that the inhibitor is bound with protein.

Therefore, it may be concluded that as suggested earlier (Siddiqi, 1962) cholesterol feed-back control might act by increasing the activity of HMG-CoA hydrolase, thereby increasing free HMG and decreasing cholesterol biosynthesis due to simple substrate analog competition.