EXPERIMENTAL
## A. MATERIALS

### I. Chemicals:

The chemicals used in the present study were obtained from the sources given against their names.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2,4-Amino naphthosulfonic acid</td>
<td>Centron Res. Lab., Bombay.</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Sigma Chem. Co., USA.</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>J.T. Baker Chem. Co., USA.</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>E. Merck, India.</td>
</tr>
<tr>
<td>Dextran sulphate</td>
<td>Sigma Chem. Co., USA.</td>
</tr>
<tr>
<td>Digitonin</td>
<td>Sigma Chem. Co., USA.</td>
</tr>
<tr>
<td>Diphenyl carbazide</td>
<td>Wilson Laboratories, India.</td>
</tr>
<tr>
<td>Diphenyl carbazone</td>
<td>Veb Jenapharm Laborchemie, Apolda, Glaxo</td>
</tr>
<tr>
<td>Heptane</td>
<td>Laboratories, India.</td>
</tr>
<tr>
<td>Hydrogenated vegetable oil</td>
<td>Hindustan Lever Ltd., India.</td>
</tr>
<tr>
<td>3-Hydroxy-3-methyl glutaric acid</td>
<td>Mann Research Laboratories, USA.</td>
</tr>
<tr>
<td>Orthophosphoric acid</td>
<td>Glaxo Laboratories, India.</td>
</tr>
<tr>
<td>Perchloric acid</td>
<td>E. Merck, India.</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
<td>B.D.H., India.</td>
</tr>
<tr>
<td>Silicic acid</td>
<td>Sigma Chem. Co., USA.</td>
</tr>
<tr>
<td>Sodium metaperiodate</td>
<td>Loba-Chemie, India.</td>
</tr>
</tbody>
</table>
II. Animals:

Male albino rats, used in the present study, were purchased from a local animal supplier.

III. Diet:

The following diets were used during the study,

(i) Basal diet: Hind Lever Rat Feed was obtained from Hindustan Lever Ltd. India.

(ii) Fat-rich cholesterol diet: Basal diet was mixed with hydrogenated vegetable oil (5%), cholesterol (2%) and cholic acid (1%) (Beg and Siddiqi, 1968).

B. METHODS

Animals were conditioned for two weeks on basal diet prior to their transfer on experimental diet. All the diets and water were given ad libitum. Twenty rats were made hyperlipidemic by feeding them...
fat-rich cholesterol diet for three weeks. These hyperlipidemic rats were divided into two groups each of ten rats and were returned to basal diet. Animals in the treated group received 20 mg HMG/kg body weight/day, i.p., in 1ml saline for twelve days. Animals in the corresponding control group were given an equal volume of saline for the same period. The animals were fasted overnight, anaesthetized with ether, and blood was withdrawn by cardiac puncture. The heart and liver were excised out, quickly washed, weighed and dropped into ice cold 0.25 M sucrose solution. Blood was allowed to stand for 4 h. at room temp and centrifuged at 3,000 rpm for 5 min at 4°C.

I. Fractionation of Serum Lipoproteins:

Serum lipoproteins were fractionated by the precipitation method as described by Onanogbu and Lewis (1976). To 1 ml of serum, in a glass centrifuge tube, were added 0.05 ml of 22.2% calcium chloride and 0.04 ml of 5% dextran sulphate. The contents of the tube were mixed thoroughly and the tube was left at 4°C for 24 h. The contents of the tube were centrifuged at 2,000 rpm for 25 min at 4°C. Supernatant was separated out and used for analysis of HDL lipids. The precipitate was redissolved in 1 ml of 0.9% sodium chloride and treated with 0.1 ml of 13.0% potassium oxalate and kept at 4°C for 24 h. After centrifugation, at 3,000 rpm for 10 min, the supernatant containing LDL and VLDL was used for lipid analysis.
II. Quantitative Determination of Triglycerides:

Triglycerides were estimated in serum and lipoprotein fractions according to the method of Gottfried and Rosenberg (1973) and Levy (1972). 0.5 ml of the test samples were taken in glass test tubes. 2.0 ml of heptane, 3.5 ml of isopropanol and 1.0 ml of 0.08N sulfuric acid were added to all tubes. At the same time 0.5 ml of water was taken as the control sample and was treated in a similar way as the test samples.

The contents of each tube were mixed for 20 sec on a cyclomixer and allowed to stand for 5 min at room temperature. The upper heptane layer was carefully transferred to clean tubes. 0.5 ml of this extract was treated with 4.0 ml of isopropanol and 2 drops of 6.25 M potassium hydroxide and mixed well. The samples were heated at 70°C for 10 min followed by the addition of 0.4 ml of periodate reagent and 2.0 ml of acetylacetone reagent. The tubes were again heated for 10 min at 70°C, cooled to room temperature and the absorbance was read at 425 nm against the blank set at zero absorbance in a "spectronic 20" spectrophotometer.

For standardization stock standard of 1000 mg triolein/100 ml isopropanol was prepared. Calibration curve was drawn using working standards of 50, 100, 200, 300 and 350 mg/100 ml concentrations. The
standard samples were analyzed exactly like the test one, except that 3.0 ml of isopropanol and 0.5 ml of water were added in the extraction step instead of 3.5 ml of isopropanol. It was found that an optical density of 0.5 was equivalent to 300 mg/100 ml triglyceride concentration.

III. Quantitative Determination of Total Lipids:

The serum and lipoprotein total lipids were determined by the method of Frings et al. (1972). 0.1 ml of the test sample and 2.0 ml of concentrated sulfuric acid were added to a glass test tube. At the same time 0.1 ml of standard solution (700 mg olive oil/100 ml of absolute ethanol) and 2.0 ml of concentrated sulfuric acid were added to another tube. The contents of the tubes were mixed well and heated in a boiling water bath for 10 min and then cooled to room temperature. 0.1 ml aliquot of each tube was then transferred to a clean tube. 0.1 ml of concentrated sulfuric acid was taken as 'Blank'. To all tubes 6.0 ml of phosphovanillin reagent (1.2 g of vanillin dissolved in 200 ml of water and made up to 1 lit with orthophosphoric acid) was added with thorough mixing and the tubes were incubated at 37 C for 15 min. The absorbance was read at 540 nm in a "spectronic 20" spectrophotometer using Blank to set zero absorbance.
The amount of total lipids was calculated as follows.

$$\text{Total lipids (mg/dl)} = \frac{\text{absorbance of Test sample}}{\text{absorbance of Standard}} \times 700$$

IV. Quantitative Determination of Phospholipids:

Phospholipids were determined by the method of Bartlett (1959) as modified by Marinetti (1962). The lipoprotein or serum sample (0.3 ml) was delivered into centrifuge tubes containing 3.0 ml of water. Five ml of freshly prepared 10% TCA (w/v) solution were added to all tubes. The tubes were allowed to stand for 10 min and centrifuged at 3,000 rpm for 15 min. The supernatant was decanted and the tubes were inverted on filter paper until practically all the supernatant was removed. The precipitates were digested with 1.0 ml of perchloric acid (70%) on an electric digestion unit for about 45 min. On cooling to room temp., 7.0 ml of distilled water were added to all tubes followed by the addition of 1.5 ml of ammonium molybdate (2.5%, w/v) and 0.2 ml ANSA reagent (0.25%, w/v). The samples were mixed on cyclomixer, and heated in a boiling water bath for exactly 7 min, cooled and read after 20 min at 660 nm in a "Spectronic 20" spectrophotometer. A calibration curve was prepared using potassium dihydrogen orthophosphate (KH_2PO_4) as standard (2 mg phosphorus/100 ml). Suitable aliquots of 0.1 ml-1.0 ml were diluted to 8.0 ml with 1.0 ml of perchloric acid and 6.9-6.0 ml of distilled
water and treated in the same manner as the test samples. Under the experimental conditions, an optical density of 0.5 was equivalent to 16 μg of phosphorus. The phospholipid values were obtained after multiplying the phospholipid phosphorus by a factor of 25.

V. Quantitative Determination of Cholesterol:

Total, free and esterified cholesterol were estimated by the method of Loeffler and Mc Dougald (1963) in serum and lipoproteins. To glass centrifuge tubes, containing 4.8 ml isopropanol, were added 0.2 ml of the test sample and 0.2 ml of the standard (200 mg cholesterol/100 ml isopropanol). The contents were mixed, allowed to stand for 5 min and centrifuged at 3,000 rpm for 10 min.

For total cholesterol, 1.0 ml of the test supernatant was taken in glass test tubes and treated with 2.0 ml of ferric chloride reagent (70 mg of ferric chloride hexahydrate/100 ml of glacial acetic acid) mixed immediately followed by the addition of 2.0 ml of conc. sulfuric acid with thorough mixing. For reagent blank, 1 ml of isopropanol was used instead of test supernatant. After 5 min the absorbance was read at 550 nm in a "Spectronic 20" spectrophotometer using Blank to set zero absorbance.

For the determination of free cholesterol and cholesterol ester 2.0 ml of the supernatant was treated with 4.0 ml of acetone and 2.0
ml of digitonin solution (1.0 g of digitonin dissolved in 60 ml of absolute ethanol, diluted to 100 ml with water and mixed well). The tubes were allowed to stand in an ice bath for 30 min and then centrifuged for 10 min at 4,000 rpm. The supernatant was decanted completely. The precipitate was again washed with 5.0 ml of acetone and dissolved in 1.0 ml of isopropanol. At the same time, 1.0 ml isopropanol was taken in a clean tube to serve as 'Blank'. To all the tubes, 2.0 ml of ferric chloride reagent and 2.0 ml of conc. sulfuric acid were added and the contents were mixed well. After 5 min, the absorbance was read at 550 nm in a "Spectronic 20" spectrophotometer using blank to set zero absorbance.

The total, free and esterified cholesterol were calculated as follows:

Total cholesterol (mg/dl) = \frac{\text{absorbance of Test sample}}{\text{absorbance of Standard}} \times 200

Free cholesterol (mg/dl) = \frac{\text{absorbance of Test sample}}{\text{absorbance of Standard}} \times 100

Cholesterol esters (mg/dl) = \text{Total cholesterol} - \text{Free cholesterol}

VI. Determination of the Activity of Hepatic Lipase:

The livers, excised from the animals, were homogenized in a medium containing 0.25 M sucrose and 10 mM Tris/HCl, pH 7.4. The protein was estimated in the homogenates by the method of Lowry et al. (1951). For the assay of lipase activity, the substrate used had the
following composition: 0.2 M Tris, 0.75 M NaCl, 0.4% gum arabic, 0.5% BSA and 2.26 mM triolein. Prior to use it was emulsified by sonication in MSE "Soniprep 150". 0.01 ml aliquots of liver homogenate were added to stoppered glass tubes containing 0.09 ml of preincubated substrate. The tubes were incubated on a shaking water bath maintained at 37 °C for 25 min. The reaction was stopped by adding 6 ml of the solvent containing chloroform-heptane-methanol (200:150:7, v/v/v) to the reaction mixture. Free fatty acids were extracted and estimated in the reaction mixture by the method of Hron and Menahan (1981).

About 330 mg of silicic acid was added to each reaction mixture and the tubes were shaken 30 times by hand and allowed to stand for 15 min at room temperature. The tubes were again shaken 6 times and centrifuged at 4,000 rpm for 5 min. The supernatant containing the extracted free fatty acids was decanted into another tube containing 2.0 ml of freshly prepared Cu-TEA solution [0.05 M Cu(NO₃)₂, 0.1 M triethanolamine, pH 8.1, saturated with NaCl (app. 33 g/100 ml)]. The tubes were shaken vigorously on a mechanical shaker for 20 min and centrifuged for 5 min at 4,000 rpm. 4.0 ml of the upper organic layer was then treated with 2.0 ml of the colour reagent (0.5% solution of a mixture of diphenylcarbazone and diphenyl carbazide (5:95) in methanol). The resulting colour was read after 10 min at 550 nm in "Spectronic 20" spectrophotometer against a reagent blank (containing no substrate but buffer) run in the same way as the test sample. The amount of free fatty acids was calculated from the calibration curve,
prepared by using standard solution of palmitic acid in chloroform (5 μ moles/ml). The activity of hepatic lipase was expressed as μ moles FFA/mg/hr.

VII. Determination of the Activity of Myocardial Lipase:

The hearts were homogenized in a medium containing 0.25 M sucrose and 10 mM Tris/HCl, pH 7.4. The protein was estimated in heart homogenates by the method of Lowry et al. (1951). The aliquots of this homogenate served as the source of lipase.

The reaction mixture had the following composition: 20 mg Triolein, 5.0 ml, 20% BSA and 2.5 ml, 0.1 M NaCl (Vajreswari and Tulpule, 1980). It was emulsified by sonication in a MSE "Soniprep 150". The lipolytic activity was determined by adding 0.025 ml of homogenate to glass stoppered tubes containing 0.075 ml of preincubated reaction mixture at 37°C. The tubes were shaken for 25 min on a shaking water bath maintained at 37°C. The reaction was stopped by adding 6 ml of the chloroform-heptane-methanol (200:150:7, v/v/v) solvent. The enzyme activity was measured by estimating amount of FFA released, spectrophotometrically as described earlier by the method of Hron and Menahan (1981). The activity was expressed as μ moles FFA/mg/hr.
C. TREATMENT OF DATA:

Unless mentioned otherwise, the percent reduction has been calculated in the following manner.

\[
\text{Percent reduction} = \frac{\text{Value of control group} - \text{Value of HMG treated group}}{\text{Value of control group}} \times 100
\]

Statistical significance was calculated by Mann-Whitney U-test, using a microcomputer software.