CHAPTER II

ANALYTICAL PROCEDURES
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1. Estimation of blood sugar.

Blood sugar was estimated by the method of Asatoor and King.61

Reagents:

1. Isotonic sodium sulphate, copper sulphate solution.

A mixture of 320 ml of 3% sodium sulphate and 30 ml of 7% copper sulphate.

2. Sodium tungstate

10 g /100 ml water

3. Low alkaline copper reagent:

12 g of Rochelle salt and 24 g of anhydrous sodium carbonate were dissolved in 250 ml water. 4 g of CuSO₄. 5H₂O in water was added with stirring, followed
by 16 g of sodium bicarbonate. 180 g of anhydrous sodium sulphate in 500 ml water was boiled and the two solutions were mixed and diluted to one litre.


25 g of ammonium molybdate was dissolved in 450 ml water and to this solution, 2 ml conc. $\text{H}_2\text{SO}_4$ was added. 3 g of sodium arsenate, dissolved in 25 ml water was then added and the mixture was kept in a 37°C water bath for 2 days and preserved in a brown bottle. One volume of the reagent was diluted to 2 volumes for use.

**Procedure:**

0.05 ml blood was pipetted out into 3.9 ml of isotonic sodium sulphate-copper sulphate solution. 0.05 ml sodium tungstate was added and mixed well. It was centrifuged and 2 ml of the supernatent was used for estimation. 2 ml of the standard glucose solution and 2 ml of isotonic sodium sulphate-copper sulphate solution were taken as the standard and blank respectively. To all tubes, 2 ml low alkaline copper reagent was added and heated for 10 minutes in a boiling water-bath. The tubes were cooled in ice and 1 ml of arsenomolybdate reagent was added to each tube. The volume was made up to 10 ml with water and the absorbance read at 670 nm.
2. Estimation of cholesterol, triglycerides and phospholipids in serum and tissues.

a. Extraction of serum for lipid estimation.

The serum was extracted according to the procedure of Folch et al. 62

'n' ml of sample was added dropwise to 5 'n' volume of methanol in a stoppered tube. Then 5 'n' volume of chloroform was added and mixed. This mixture was incubated at 55°C for 15 minutes. At the end, another 5 'n' volume of chloroform was added, so that the proportion of chloroform to methanol was 2:1 (v/v). It was filtered and the residue washed with chloroform : methanol (2 : 1) at least 3 times. The filtrates were combined.

To the filtrate, 0.7% KCl or 0.02% CaCl₂ (20% of the total volume of the extract) was added in a stoppered tube, mixed vigorously and allowed to stand. The aqueous upper phase was removed with a pasteur pipette and the lower layer was washed with chloroform : methanol : KCl (or CaCl₂ solution) 3 : 48 : 47 v/v. The chloroform layer was evaporated to dryness and the lipids redissolved in a known volume of chloroform. From this, aliquots were used for the analysis of lipids.
b. Extraction of tissues for estimation of lipids.

The tissue was homogenised with washed, powdered glass and extracted with chloroform; methanol (2 : 1) and processed as described for serum. 0.25 g of the tissue except the aorta corresponds to 25 ml. In the case of aorta, the aortas from two rats (65.0 mg) were pooled and the extract was made upto 10.0 ml.

c. Estimation of total cholesterol

The cholesterol was estimated by the method of Abell.\textsuperscript{63}

Reagents:  i) 33% KOH

  ii) Absolute ethanol

  iii) Ethanolic KOH - 6.0 ml of 33% KOH in water was added to 94 ml of absolute ethanol.

  iv) Petroleum ether (AR) (60-80\textdegree C)

  v) Colour reagent.

20.0 ml of acetic anhydride was chilled in ice. 1.0 ml of conc. H\textsubscript{2}SO\textsubscript{4} was added to this with mixing. It was again chilled for 10 minutes and 10.0 ml of glacial acetic acid was added and was allowed to attain room temperature.

Procedure:

An aliquot of the lipid extract was pipetted out into a glass stoppered centrifuge tube and was evaporated
to dryness. 5.0 ml of ethanolic KOH was added, stoppered and was shaken well. It was then warmed in a waterbath at 37-41°C for 55 minutes. After cooling to room temperature 10.0 ml of petroleum ether (60-80°C) was added and mixed. 5.0 ml of water was then added to this and was shaken vigorously for 1 minute. It was then centrifuged at a low speed for 5 minutes. 4.0 ml of petroleum ether layer was pipetted out into a test tube and evaporated to dryness at 60°C. A standard was also treated in the same manner. 6.0 ml of colour reagent was added to each tube and kept at 25°C after thorough shaking. 6.0 ml of colour reagent was taken as blank. After 30-35 minutes the optical density was read at 620 nm.

d. Estimation of triglycerides.

Triglycerides were estimated by the method of Van Handel and Zilversmit, with the modification that florisil was used to remove phospholipids.

Reagents

i) Chloroform (AR)

ii) Florisil

iii) Ethanolic KOH (0.4%)

2.0 g of KOH was dissolved in 100 ml ethanol. This was then diluted 5 times with ethanol.
iv) 0.2 N $\text{H}_2\text{SO}_4$

v) 0.05 M sodium metaperiodate

vi) 0.5 M sodium arsenite

vii) Chromotropic acid

2.0 g of chromotropic acid (or 2.24 g sodium salt) was dissolved in 200 ml distilled water. 600 ml of conc. $\text{H}_2\text{SO}_4$ was added slowly to 300 ml of distilled water which was already chilled in ice. This diluted acid was then added to the chromotropic acid solution (0.05 mg/ml).

Procedure:

2.0 g of florisil was taken in a glass stoppered tube and 3.0 ml of chloroform was added. An aliquot of the extract was layered on the top of florisil and mixed, chloroform was then added to this to a total volume of 10.0 ml. It was then stoppered and was shaken intermittently for about 10 minutes. After filtration, 1.0 ml was pipetted out into each of 3 tubes, 1.0 ml of the working standard was similarly pipetted out into each of 3 tubes. The solvent was evaporated at 60-60°C. 0.5 ml of ethanolic KOH was added to 2 out of each 3 tubes and 0.5 ml of ethanol was added to the third tube. The tubes were closed and kept at 60-70°C
for 10 minutes. To each tube, 0.5 ml 0.2 N H₂SO₄ was added and then placed in a gently boiling waterbath for about 15 minutes to remove alcohol. They were then cooled to room temperature, 0.1 ml sodium metaperiodate was added to each tube and kept for 10 minutes. 0.1 ml sodium arsenite solution was added. Anyellow colour of iodine appeared and vanished within few minutes. To each tube 5.0 ml of chromotropic acid was then added and mixed. The tubes were closed and heated in a boiling waterbath for 30 minutes. The tubes were cooled and the absorbance was read at 570 nm.

e. Estimation of phospholipids

Phospholipids were estimated by the method of Zilversmit and Davis.⁶⁵

Reagents

i) 5N H₂SO₄

ii) 2.5% ammonium molybdate

iii) ANSA

0.2 g of 1-amin-2-naphthol 4-sulfonic acid was mixed with 1.2 g of sodium bisulphite and 1.2 g of sodium sulphite. 0.25 g was taken from this mixture and dissolved in 10 ml water.
Procedure:

An aliquot of the extract was pipetted out into a kjeldahl flask and evaporated to dryness. 1.0 ml of 5 N H$_2$SO$_4$ was added and digested in a digestion rack till it became light brown. It was then cooled to room temperature. 1 or 2 drops of 2N HNO$_3$ were added and digested again till it became colourless. The kjeldahl flask was cooled, 1.0 ml of water was added and heated in a boiling waterbath for 5 minutes. 1.0 ml of 2.5% ammonium molybdate and 0.1 ml of ANSA were added and the volume made up to 10.0 ml with distilled water. The absorbance was measured at 660 nm within 10 minutes.

f. Estimation of free fatty acids

Free fatty acids were estimated by the method of Falholt et al.\textsuperscript{66}

Reagents

i) Extraction solvent
Chloroform : Heptane : Methanol (5:5:1)

ii) Phosphate buffer pH 6.4
Mix 2 volume KH$_2$PO$_4$ (4.539 g/litre) and 1 volume disodium hydrogen phosphate (5.938 g/litre)
iii) Stock copper solution 12.07 g Cu(NO₃)₂·3H₂O made up to 100 ml.

iv) Triethanolamine 1 mole / litre
10 ml triethanolamine in 100 ml water

v) Sodium Hydroxide. 1 mole/litre.
4 g NaOH in 100 ml water.

vi) Copper Reagent

10 ml of copper solution, 10 ml of triethanolamine and 6 ml sodium hydroxide were mixed and diluted to 100 ml. 33g sodium chloride was added and the pH adjusted to 8.1. The solution was used fresh.

vii) Diphenyl carbazide solution Prepared immediately before use by adding a solution of 40 mg in 10 ml ethanol to 0.1 ml triethanolamine solution.

Procedure:

An aliquot of the lipid extract was pipetted out into a glass stoppered tube and evaporated to dryness. 1 ml phosphate buffer, 6 ml of extraction solvent and 2.5 ml copper reagent were added. The standard solution was also treated similarly. The tubes were shaken vigorously for 90 seconds and allowed to stand for 15 minutes. It was then centrifuged at 4000 rpm for
5 minutes and 3 ml of the upper layer was transferred to a tube containing 0.5 ml diphenyl carbazide solution and mixed carefully. The tube was kept for 15 minutes and the absorbance read at 550 nm.

3. Estimation of serum lipoproteins

Total cholesterol was estimated in the whole serum by the method of Abell as described above.\textsuperscript{63}

a. Serum HDL and LDL + VLDL were estimated by the heparin-manganese precipitation method according to the procedure of Warnick and Albers.\textsuperscript{67} LDL + VLDL were precipitated from the serum by treating with heparin and manganese chloride (final concentration of heparin 0.144\% and MnCl\(_2\) 0.091 M). After keeping at room temperature for 10 minutes, it was centrifuged for 30 minutes at 4000 rpm at 4°C. The supernatent which contained HDL was analysed for cholesterol to obtain HDL cholesterol. The difference between total cholesterol and HDL cholesterol gave LDL + VLDL cholesterol.

The supernatant from heparin - Mn\(^{++}\) precipitation was also analysed for the triglycerides by the method of Van Handel and Zilversmit.\textsuperscript{64}
4. Activity of enzymes involved in lipoprotein catabolism.

a. Lipoprotein lipase (EC 3.1.1.3) activity of adipose tissue.

Lipoprotein lipase activity of adipose tissue was estimated according to the procedure of Krauss et al. Acetone dry powder of the tissue was extracted with 0.025 M NH₄OH-NH₄Cl buffer, pH 8.6 containing 1 unit of heparin/ml and the extract was used as the enzyme. Protamine inhibited activity is taken as measure of lipoprotein lipase activity. Protein in the enzyme extract was determined after TCA precipitation by the method of Lowry et al. The enzyme activity is expressed as micromoles of glycerol liberated per hour per g protein.

b. Assay of plasma lecithin: cholesterol acyl transferase activity (LCAT, EC 2.3.1.43)

Blood was collected in heparinised tubes maintained at O°C and immediately centrifuged at O°C to separate the plasma. An aliquot was immediately extracted with acetone: ethanol (1:1) to extract the lipids. Another aliquot of the plasma was incubated at 37°C for 3 hours, and at the end it was extracted with acetone : ethanol (1:1). Ester
cholesterol and unesterified cholesterol were estimated in the lipid extract by the method of Schoenheimer and Sperry. The extent of increase in the ester Cholesterol/unesterified cholesterol ratio was taken as a measure of LCAT activity.

5. Assay of $\beta$-hydroxy $\beta$-methyl glutaryl-CoA reductase (HMG CoA reductase, EC 1.1.1.34).

HMG CoA reductase of the liver and small intestine was estimated according to the procedure described by Rao and Ramakrishnan, by determining the ratio of HMG CoA to mevalonic acid.

Reagents

a. Saline arsenate

1.0 g of sodium arsenate/litre physiological saline.

b. Dilute perchloric acid

50 ml/litre

c. Hydroxylamine hydrochloride reagent

138.98 g/litre

d. Hydroxylamine hydrochloride reagent for mevalonate - Equal volume of hydroxylamine hydrochloride reagent and water are mixed freshly before use.
e. Hydroxylamine hydrochloride reagent for HMG CoA
Equal volumes of hydroxylamine hydrochloride reagent and sodium hydroxide solution (180.0 g/litre) were mixed freshly before use.

f. Ferric chloride reagent.

5.2 g of trichloroacetic acid (TCA) and 10.0 g ferric chloride were dissolved in 50.0 ml of 0.65 mol/litre HCl and diluted to 100 ml with the latter.

Procedure

Equal volumes of fresh 10% tissue homogenate and dilute perchloric acid were mixed, kept for 5 minutes and centrifuged at 2000 rpm for 10 minutes. To 1.0 ml of the filtrate, 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG CoA) was added, mixed and kept for 5 minutes. 1.5 ml of ferric chloride was added and shaken well and kept for 10 minutes. The readings were taken at 540 nm against a similarly treated saline arsenate blank. The ratio of HMG CoA to mevalonate is taken as an index of enzyme activity which catalyses the conversion of HMG CoA to mevalonate.
6. **In vivo** incorporation of (1,2-$^{14}$C) acetate into free and ester cholesterol in the serum, liver and small intestine. 

The rats were deprived of food overnight and were injected intraperitoneally with 0.5 ml solution (1,2-$^{14}$C) sodium acetate (10 $\mu$ci/100 g body weight) at 09.00 hrs. After 3 hrs, the rats were killed by decapitation. The serum, liver and small intestine (0.5 cm below pylorus and 0.5 cm above cecum) were quickly removed to ice-cold containers. The intestine was cut open laterally and the contents removed by rinsing in cold physiological saline and the mucosa scrapped out. The tissue in each case was gently blotted and weighed. The serum and tissues were extracted with chloroform : methanol according to the procedure of Folch et al. Free cholesterol and ester cholesterol in the extract were separated by TLC over silica gel (Silica gel G; solvent system - n-hexane : ether : acetic acid 80 : 20 : '1', v/v) and activity counted in Packard's Priyas Liquid Scintillation Counter. The scintillant fluid used was [6.0g 2,5-diphenyl oxazole (PPO) and 0.2 g 1,4-bis [2-(5-phenyl oxazolyl)] benzene (POPOP)]/litre toluene.
7. Extraction of bile acids from liver and small intestine.

The procedure followed was that of Okishio et al. The intestine after cleaning by dipping in physiological saline and removing the intestinal contents by cutting open the wall laterally, was used. The tissue was homogenised with 95% (v/v) ethanol containing 0.1% ammonium hydroxide (v/v, specific activity 0.88), refluxed for 30 min and filtered. The residue in each case was reextracted with the same volume of solvent and filtered. The combined filtrate was then concentrated in vacum, made alkaline (pH 10.0) by the addition of NaOH. An equal volume of water was added and extracted 3 times with petroleum ether to remove neutral sterols. The aqueous solution after acidification with HCl to pH 1-2 was extracted with CHCl₃ : CH₃OH (1:1) 3 times. The chloroform layer was washed with little water and dried over anhydrous Na₂SO₄. After filtration and evaporation, the bile acids were dissolved in a known volume of chloroform.

8. Estimation of total bile acids

The bile acids were estimated by the enzymatic procedure described by Palmer. An aliquot of the chloroform solution of the bile acid was evaporated to dryness and
dissolved in the pyrophosphate buffer mentioned below.

Reagents

a. 0.1 M sodium pyrophosphate buffer pH 9.5.
b. Methanol—double distilled.
c. Cholic acid (0.75 mg/ml) in methanol.
d. 5 mM NAD\(^+\), kept in ice, stored frozen
e. 3α-hydroxy steroid dehydrogenase

[Prepared from pseudomonas testosteroni (ATCC 11996) as described in Methods in Enzymology].\(^76\)

The system consisted of 0.1 ml NAD\(^+\), 0.02 ml of the bile acid and 2.88 ml of pyrophosphate buffer in a 10 mm light path spectrophotometer cuvette. The reaction was started by adding 20 μl of enzyme solution and stirring rapidly. At 30 seconds and 15 seconds thereafter, optical density measurements at 340 nm were taken against a control cuvette containing all components except the bile acid and compared with a standard of cholic acid.


24 hr stool samples were collected from the rats in metabolic cages and lyophilised. Approximately 600 mg of the sample was treated with 10.0 ml of 1.0 N NaOH in
90% ethanol for 2 hrs at 80°C. After centrifugation, the residue was again treated with 10.0 ml of 1.0 N NaOH in 90% ethanol at 80°C for 2 hrs. The combined extract was then shaken with 200 ml hexane. The hexane layer was collected and washed with a little water. It was evaporated to dryness and redissolved in a known volume (20.0 ml) of chloroform for neutral sterol estimation.

The solution left after extraction with hexane was acidified to pH 2.0 and extracted with ethyl acetate. The ethyl acetate layer was collected, washed with a little water and evaporated to dryness. The bile acids were redissolved in a known volume of ethyl acetate (20.0 ml).

GLYCOSAMINOGLYCANS AND ENZYMES INVOLVED IN THE METABOLISM OF GLYCOSAMINOGLYCANS

1. Estimation of different glycosaminoglycans (GAG) fractions in the tissues.

   a) Preparation of defatted tissue.

   The tissue (approximately 500 mg wet weight for tissues other than aorta; in the case of aorta about 60 mg) was defatted by successive extraction at 60°C with ethanol : ether (3 : 1, v/v) twice followed by chloroform : methanol (1 : 1, v/v) each time for 2 hr. The defatted tissue was dried under vacuum to constant weight.
b) Papain digestion:

The procedure of Laurent as described by Scott was used. A known amount of the dry defatted tissue was digested with papain (crystalline papain, one third the dry weight of the tissue) in 0.1 M phosphate buffer (pH 6.5), containing 0.005 M EDTA and 0.005 M cysteine hydrochloride for 48 hr at 65°C. Fresh papain was added every 16 hr.

c) Fractionation and estimation of glycosaminoglycans.

The papain digest was deproteinized with trichloroacetic acid (final concentration of TCA 10%) and the supernatant was dialysed till free of TCA. Total GAG was precipitated from the solution by the addition of 4-5 volumes of 95% ethanol (v/v) containing 1-2% potassium acetate (w/v). The precipitate was collected by centrifugation, and dissolved in a known volume of water. An aliquot of this solution was used to determine total GAG by estimating uronic acid content by the modified procedure of Bitter and Muir.

Another aliquot of the GAG solution (approximately 100 µg uronic acid) was digested with testicular hyaluronidase (Sigma chemicals, 100 units) in 0.25 M acetate buffer (pH 5.3) containing 0.75 M NaCl for 16 hr under a layer of
toluene. The undigested GAG [containing heparan sulphate (HS), dermatan sulphate (DS) and heparin (H)]* was precipitated from the solution by 95% ethanol containing potassium acetate as described above. The precipitate was collected after centrifugation. It was washed with ethanol solution, dissolved in a known volume of water and the uronic acid estimated.

A third aliquot of the GAG solution (approximately 100 μg uronic acid) was subjected to digestion with chondroitinase ABC (Sigma chemicals, 0.02 units) in tris-HCl buffer (0.25 M, pH 8.0) containing bovine serum albumin, sodium acetate and sodium chloride (50.0 mg, 2.4 g and 1.4 g respectively/100 ml of the solution) for 16 hr under toluene. The undigested GAG (containing HA, HS and H) was precipitated with 95% ethanol as described above. The precipitate was dissolved in a known volume of water and the solution passed through a column of Dowex 1 x 2 (Cl⁻ form), 0.5 x 5 cm. The column was washed with water and eluted successively with 0.25 M, 1.7 M and 3.5 M NaCl solution. These eluates contained HA, HS and H respectively. The uronic acid in each eluate was estimated as described above. From these the amount of HA, HS, Ch-4-S + Ch-6-S,*

*HA - Hyaluronic acid, HS - Heparan Sulphate  
Ch-4-S Chondroitin-4-Sulphate  
Ch-6-S Chondroitin-6-Sulphate  
DS - Dermatan Sulphate and H - Heparin
DS and H were obtained. No attempt was made to estimate separately, Ch-4-S and Ch-6-S.

d) Estimation of Uronic Acid.

1. 0.025 M sodium tetraborate in conc. H₂SO₄
2. 0.125% carbazole in methanol

Procedure:

5.0 ml of sodium tetraborate was taken in a test tube and cooled to 4°C by keeping it in an ice bath. 1.0 ml of the sample was layered over this and stirred thoroughly with a glass rod. It was allowed to attain room temperature and then heated in a boiling water bath for 10 minutes. After cooling to room temperature, 0.2 ml of carbazole was added and heated again for 15 minutes in a boiling waterbath. It was then cooled and the optical density was read at 530 nm.

2. Assay of the activity of enzymes involved in the degradation of glycosaminoglycans

The tissue was homogenised in aqueous 0.1% Brij-35 solution at 0°C and was centrifuged at 4000xg at 4°C. The supernatant was diluted with an equal volume
of appropriate double strength buffer, and was used as the enzyme source.

The procedure of Kawai and Anno\textsuperscript{81} was followed for the assay of $\beta$-glucuronidase, (EC 3.2.1.31) and $\beta$-N-acetyl glucosaminidase (EC 3.2.1.30). P-nitrophenyl $\beta$-D-glucuronide in 0.1 M acetate buffer (pH 4.5) was used as the substrate for the assay of $\beta$-glucuronidase and p-nitrophenyl $\beta$-D-N-acetyl glucosaminide for $\beta$-N-acetyl glucosaminidase. The enzyme activity in each case is expressed in terms of micrograms of p-nitrophenol liberated per hour per milligram protein. Hyaluronoglucosaminidase (EC. 3.2.1.35) was assayed by the procedure described by Kawai and Anno,\textsuperscript{81} using hyaluronic acid (Na Salt) as substrate in 0.1 M acetate buffer (pH 4.5) and estimating the N-acetyl hexosamine liberated by the method of Reissig \textit{et al.}\textsuperscript{82} (pH of the acetate buffer was 3.8 in the case of hepatic enzyme). The enzyme activity is expressed in terms of micrograms of N-acetyl glucosamine liberated per hour per milligram protein. The procedure described by Roy\textsuperscript{83} was used for assaying the activity of aryl sulphotase (EC 3.1.6.1) using p-nitrocatechol sulphate in 0.1 M acetate buffer (pH 4.5) as the substrate. The enzyme activity is expressed in terms of micrograms of p-nitrocatechol liberated per hour per milligram protein.
Cathepsin-D (EC 3.4.23.5) was assayed using 2% hemoglobin as substrate in 0.1 M acetate buffer (pH 4.5) and determining the amount of tyrosine liberated by the method of Folin and Ciocalteu. The enzyme activity is expressed in terms of micrograms of tyrosine liberated per hour per milligram protein.

Protein content of the enzyme solution was estimated by the method of Lowry et al. as mentioned above.


a) D-glucosamine-6-phosphate isomerase (glutamine forming) (EC 5.3.1.19).

The procedure of Pogell and Gryder was used to estimate the activity of D-glucosamine-6-phosphate isomerase in the liver. The tissue was homogenised in a solution containing KCl (0.154 M), EDTA (0.001M) and glucose-6-phosphate (0.012 M), pH 7.2 (2 ml/g) and centrifuged at 2000xg at 0°C for 10 minutes. This supernatant was used as the enzyme source. The enzyme activity is expressed in terms of micromoles of hexosamine liberated per hour per gram protein.
b) UDPG dehydrogenase (UDP glucose: NAD\(^{+}\) Oxidoreductase) (EC 1.1.1.22)

The procedure of Strominger et al.\(^86\) was used to determine the activity of UDPG dehydrogenase (EC 1.1.1.22) in the liver. The tissue was homogenised in glycine buffer (0.1 M, pH 8.7) and centrifuged at 2000 xg at 0°C. The supernatant was used as the enzyme source. One unit of enzyme activity is expressed as the amount of enzyme required to give an increase in optical density of 0.001/minute at 340 nm under the conditions of assay.

4. Sulphate metabolism

The method of Van Kempen and Jansen\(^87,88\) using methyl umbelliferone was used to determine the concentration of PAPS (3'-phosphoadenosine-5'-phosphosulphate) and the activity of sulphate activating system (which includes sulphate adenylyl transferase (EC 2.7.7.4) and adenylyl sulphate kinase (EC 2.7.1.25)) as well as that of aryl sulfotransferase (EC 2.8.2.1) of the liver. Details of the procedure are given below.

a. Estimation of 3'-phosphoadenosine-5'-phosphosulphate (PAPS)

The PAPS present in a heat inactivated aliquot of the tissue extract was estimated by using methyl umbelliferone (MU) in the presence of phenol sulphotransferase
present in normal rat liver. The amount of methyl umbelliferone sulphate (MUS) formed was estimated.

The tissue was homogenised in isotonic KCl solution at 0°C (1:5 w/v) and centrifuged for 15 minutes at 2000xg at 0°C and the supernatant collected (A).

Normal rat liver, washed once with isotonic KCl (ice cold), was homogenised with ice cold isotonic KCl at 0°C (1:5 w/v) and centrifuged at 2000 g at 0°C (15 minutes) and the supernatant collected (B).

An aliquot of A (1.5 ml) was heat inactivated by placing in a boiling water bath for 60 seconds. It was then incubated with 0.4 M tris-HCl buffer, pH 7.4 (1.0 ml) containing 0.5 mM EDTA, 1.5 mM MU, 0.2 mM of KH$_2$PO$_4$ (pH adjusted to 7.4 with 8.0 N KOH) and B (0.5 ml).

Two controls were also run. In control 1, isotonic KCl (1.5 ml), 0.4 M tris-HCl buffer [(pH 7.4) (1.0 ml)] containing 0.5 mM EDTA, 1.5 mM MU, 0.2 mM KH$_2$PO$_4$ and B (0.5 ml) were taken. In control 2, A 1.5 ml (heat inactivated by placing in a water-bath for 60 seconds), 0.4 M tris-HCl buffer [(pH 7.4) (1.0 ml)] containing 0.5 mM EDTA, 1.5 mM MU, 0.2 mM KH$_2$PO$_4$ and distilled water (0.5 ml) were taken. All the tubes were then incubated for 30 minutes.
at 37°C and the reaction stopped by placing the tubes in a boiling waterbath for 60 seconds. The tubes were cooled to room temperature, centrifuged and 2.0 ml of the clear supernatant was passed through Dowex-50 (H+ form, 3×1 cm). The column was washed thrice with 1.5 ml distilled water and drained. The effluent and washings were collected and the volume was made up to 7.0 ml. 5.0 ml was pipetted out and hydrolysed with 1.0 ml of 2 N HCl at 80°C for 30 minutes. After cooling, the fluorescence intensity of the mixture was measured (Ex Max 324 nm and Em Max 484 nm). A standard curve of MU (0.5 mM) and 0.33 N HCl was prepared to quantitate the methyl umbelliferone sulfate formed.

b. Sulphate activating System.

[Sulphate adenylyl transerase (EC 2.7.7.4 and adenylyl sulphate Kinase (EC 2.7.1.25)].

The sulphate activating system present in the tissue extract was allowed to catalyse the formation of PAPS from ATP and inorganic sulphate. The PAPS formed was estimated as before.

To the enzyme solution A (1.5 ml), 0.4 M tris-HCl buffer pH 7.4 (4.0 ml) containing 2.2 mM MgCl₂, 50 mM K₂SO₄ and 4.4 mM ATP was added and incubated for one hour at 37°C.
The reaction was arrested by placing the tubes in a boiling waterbath for 60 seconds. In the control, the heat inactivated A (1.5 ml) was used in place of the enzyme, the other component of the reaction mixture being the same. The tubes were centrifuged and to the supernatant (1.5 ml), 250 mM EDTA (0.5 ml) in tris-HCl buffer, pH 7.4, 0.75 mM KH$_2$PO$_4$ (0.5 ml), 7.5 mM MU (0.5 ml) and B (0.5 ml) were added. After incubating the tubes for 30 minutes, the reaction was stopped by placing the tubes in a boiling water bath for 60 seconds, cooled and centrifuged. MUS in the supernatent (2.0 ml) was estimated as before.

c. Aryl Sulfotransferase activity.

PAPS was first generated by using the normal rat liver extract as the sulphate activating system in the presence of ATP and inorganic sulphate. The sulfotransferase in the tissue extract from the experimental animal was used to catalyse transfer of sulphate from PAPS to MU.

To 1.5 ml of B, 0.4 M tris-HCl buffer (4.0 ml) containing 2.2 mM MgCl$_2$, 50 mM K$_2$SO$_4$ and 4.4 mM ATP were added and incubated for 1 hour. The reaction was stopped by placing the tubes in a boiling water bath for 60 seconds, cooled and centrifuged. To an aliquot (1.5 ml) of the
supernatant, 0.4 M tris HCl buffer, pH 7.4 (1.0 ml) containing 0.05 M EDTA, 1.5 mM MU, 0.2 mM KH₂PO₄ and A (0.5 ml) were added and incubated for 30 minutes at 37°C.

In the control, heat inactivated A was used, the other components being the same. After incubation, the tubes were placed in a boiling waterbath for 60 seconds to arrest the reaction, cooled and centrifuged. The MUS was estimated in an aliquot (2.0 ml) as described before. Protein in the enzyme extract was estimated by the procedure of Lowry et al.⁶⁹

GLYCOPROTEINS AND GLYCOHYDROLASES

1. Extraction of glycoproteins from the tissues

Acetone dry powder was prepared by keeping the minced tissue in acetone at 0°C for 72 hr. The acetone was changed every 24 hr. The tissue was then extracted with ether : acetone (3:1, v/v) at 37°C for 1 hr, followed by ether for 1 hr. The defatted tissue was then dried under vaccum to constant weight.

The dry defatted tissue was digested with papain (crystalline papain, one third the dry weight of the tissue) for 72 hr at 65°C in 0.2 M acetate buffer (pH 7.0) containing 2.0 mg cysteine hydrochloride/ml. Fresh papain
was added every 24 hr. The digest was then cooled to room temperature and 4-5 volumes of ethanol was added at 0°C and kept at this temperature for 24 hr. It was centrifuged and the supernatant was evaporated to dryness in the cold in vacum. The residue was dissolved in a known volume of water and aliquots were used for the analysis of carbohydrate components. The procedure used is similar to that described by Wagh et al.\textsuperscript{89} except that ethanol was used instead of TCA to deproteinise the digest, since TCA keeps the tissue glycogen and GAG in solution.

2. Estimation of carbohydrate components of glycoproteins.
   a. Estimation of total hexose

   Total hexose was estimated by phenol-sulphuric acid method.\textsuperscript{90}

   b. Estimation of fucose.

   Fucose was estimated by the method of Dische and Shettles.\textsuperscript{91}

   c. Estimation of sialic acid.

   Sialic acid was estimated by the thiobarbituric acid method of Warren.\textsuperscript{92}

   d. Estimation of total protein

   Total protein was estimated in the dry defatted
tissue by microkjeldhal digestion followed by nesslerisation.

3. Assay of glycohydrolases.

The tissue was homogenised at 0°C in 0.1% Brij-35 solution (1-3, w/v). After centrifugation at 4°C for 10 min at 2000 x g, the supernatant was diluted suitably with appropriate double strength buffer. The final concentration of Brij-35 was less than 0.025% in every case.

The enzyme activities were determined by the method of Kawai and Anno. P-nitrophenyl-β-D-galactoside in 0.02 M citrate phosphate buffer (pH 3.7) was used as substrate for β-galactosidase (EC 3.2.1.23), p-nitrophenyl-β-D-fucoside in 0.02 M citrate buffer (pH 5.0) for β-fucosidase (EC 3.2.1.38), p-nitrophenyl β-D-glucoside in 0.02 citrate buffer (pH 5.0) for β-glucosidase (EC 3.2.1.21) and p-nitrophenyl β-D-N-acetyl glucosaminide in 0.1 M acetate buffer (pH 4.5) for β-N-acetyl glucosaminidase (EC 3.2.1.30).

The substrate in each case (100 µg/0.4 ml) in buffer solution mentioned above was incubated with the enzyme solution (0.1 ml) at 37°C for 30 min (1 hr for aorta). Controls were run in which enzyme and substrate were separately incubated. After the incubation period, 2.5 ml of 0.2 N Na₂CO₃ was added and the absorbance was measured at 400 nm. A standard of p-nitrophenol (20 µg/0.5 ml) was
treated similarly. The enzyme activity in each case is expressed in terms of micrograms of p-nitrophenol liberated per hour per milligram protein. Protein in the enzyme extract was estimated by the procedure of Lowry et al. 69

**ISOLATION AND ESTIMATION OF COLLAGEN**

Total salt and acid extractable collagen from dry defatted tissue were prepared by the procedure of Jackson and Cleary. 94 Collagen was hydrolysed with 6 N HCl at 118°C for 8 hrs in a sealed tube. The hydrolysate was evaporated to dryness in a dessicator over NaOH and HCl was removed by repeated addition of small quantities of water and evaporation. The residue was dissolved in a known volume of water. Collagen was estimated by determining the hydroxy proline content of the hydrolysate by the method of Neuman and Logan. 95

The insoluble collagen was calculated as the difference between the total and the soluble collagen.

**Reagents**

1. 0.01 M copper sulphate
2. 2.5 N NaOH
3. 6% H₂O₂
4. 5% p-dimethylaminobenzaldehyde in n-propanol (p-dmab)
5. 3.0 N H₂SO₄
0.5 ml of the sample was taken in a test tube. 0.5 ml of 0.01 M copper sulphate, 0.5 ml of 2.5 N NaOH and 0.5 ml of 6% H$_2$O$_2$ were added and kept for 5 minutes at room temperature with intermittent shaking. It was heated at 80°C for 5 minutes and then chilled in ice bath for 10 minutes. 1.0 ml of p-dmab was added to this along with 2 ml of 3N H$_2$SO$_4$ and heated at 70°C for 10 minutes. The optical density was read at 550 nm.

**ESTIMATION OF ELASTIN**

Nucleic acids were removed from the acetone dry tissue by successive extraction with 5% TCA at 90°C for 15 minutes. It was then extracted with 0.1 N NaOH thrice to remove soluble proteins. The contents were stirred with 0.1 N NaOH for two hours at room temperature and then kept overnight at 0°C. It was then digested with collagenase in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.36 mM CaCl$_2$ for 24 hours twice. (100 units of enzyme/gram equivalent of dry defatted tissue). The residue was repeatedly washed with water, dried and elastin estimated by nitrogen estimation by the microkjeldahl method.

**STATISTICAL ANALYSIS**

The data given in the tables are the average values indicated in each case ± SEM, Statistical significance was calculated using Student's 't' test.