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2. MATERIALS AND METHODS

2.1 Isolation and characterisation of Penitrem A

2.1.1 Maintenance of *Penicillium crustosum* cultures

The fungus was maintained on Czapek-Dox agar medium having the following composition per litre:
- glucose - 50 g
- sodium nitrate - 2.5 g
- potassium dihydrogen phosphate - 1.0 g
- potassium chloride - 0.5 g
- magnesium sulphate \((\text{MgSO}_4 \cdot 7\text{H}_2\text{O})\) - 0.5 g
- ferrous sulphate \((\text{FeSO}_4 \cdot 7\text{H}_2\text{O})\) - 0.01 g
- agar - 20 g.

The pH was adjusted to 6.4. The medium was sterilised at 15 pounds per square inch pressure for 15 minutes and slants were prepared. The organism was subcultured at regular intervals of 14 days.

2.1.2 Composition of experimental liquid medium

Czapek-Dox medium having the following composition in g per litre was used:
- glucose - 50 g
- sodium nitrate - 2.5 g
- potassium dihydrogen phosphate - 1.0 g
- potassium chloride - 0.5 g
- magnesium sulphate \((\text{MgSO}_4 \cdot 7\text{H}_2\text{O})\) - 0.5 g
- ferrous sulphate \((\text{FeSO}_4 \cdot 7\text{H}_2\text{O})\) - 0.01 g.

The medium dispensed into 4-litre Hoffkin flasks, the pH was adjusted to 6.4 and sterilised at 15 pounds per square inch pressure for 15 minutes, when cold the medium was
inoculated with a spore suspension of *P. crustosum* containing approximately 2.0x10⁶ spores per ml (as counted using a haemocytometer) of saline and incubated at 25° to 27°C for 14 days.

2.1.3 Isolation, purification and characterisation of the isolated penitrem A

2.1.3.1 Isolation of penitrem A: Penitrem A was isolated from the mycelial mat according to the procedures described by Ciegler and Pitt (1970) and Richard and Arp (1979).

*P. crustosum* was allowed to grow on Czapek-Dox medium for 2 weeks at 25°-27°C and then at 4°C in a cold room for an additional week. At the end of these periods mycelial mat was removed and homogenized with chloroform in a waring blender for 5 minutes.

The homogenates were centrifuged to separate chloroform from mycelium and water, water was then removed by suction and the mycelia, by filtration through a Buchner funnel using Whatman No. 1 filter paper, dried with Na₂SO₄. The solvent was finally evaporated to dryness at room temperature or by flash evaporation at 40°C and the oily residue was used for tlc analysis.
2.1.3.2 Thin layer chromatography

5.4 g of silica gel G-HR (Merck) was mixed with 10 ml of distilled water and stirred vigorously. The slurry was used to coat the glass plate (20 x 15 cm) using a tlc applicator.

The silica gel coated glass plate was dried at room temperature for 15 minutes and then placed in an oven at 110°C for 60 minutes for activation. The chloroform extract containing penitrem A, was spotted on tlc plate along with an authentic sample of penitrem A which is used as a reference standard. The plate was first developed in a glass tank with hexane to remove the interferring lipids towards the top of the plate and then in a glass tank containing 93:7 (v/v) chloroform : acetone and finally removed from the tank, dried, sprayed with 1 per cent p-dimethylaminobenzaldehyde (in 95 per cent ethanol) and dried again. The blue colour of the penitrem A with a Rf value of 0.5 was visualized when the plate was placed in a tank containing HCl vapour for 15 minutes.

The developed plates could also be sprayed with 1 per cent FeCl₃ in butanol, and heated at 100°C for about 20 minutes when penitrem A appears as a dark blue spot with an Rf of 0.5.
2.1.3.3 Purification of penitrem A is achieved by adsorption chromatography. A chromatographic column of silica gel 60 (0.05-0.2 mm) in hexane was prepared. The crude extract containing penitrem A was redissolved in a few ml of acetone or chloroform and was allowed to pass through the column when it gets adsorbed and then eluted from the column using a mixture of chloroform : acetone (93:7) by volume. The flow rate was adjusted to about 5.0 ml/min and 10 ml fractions were collected. The eluent in every fifth tube was evaporated almost to dryness and dissolved in approximately 0.2 ml of acetone or chloroform and was subjected to tlc. The spots including the authentic sample were detected by exposure to UV illumination and also by the colour given when 1 per cent FeCl₃ in butanol was sprayed.

2.1.3.4 Further purification of penitrem A was carried out by preparative tlc. The tlc plates were prepared in such a way that silica gel G was about a mm thickness and activated by keeping in an oven at 120°C for one hour. The chloroform extract containing penitrem A was chromatographed using solvent system chloroform : acetone (93:7, v/v). After the run, it was removed, dried at room
temperature and examined under ultra violet light (when penitrem A appears as a light purple colour with a Rf value of 0.5). The spot corresponding to authentic penitrem A was scraped and again eluted with acetone or chloroform and evaporated to dryness and was further purified by repeated tlc separation on silica gel. The final eluate was treated with activated carbon until no yellow colour remained. The acetone solution after dilution with water (55:45, v/v) was permitted to evaporate slowly at room temperature. The crystals formed were filtered, washed with water dried and redissolved in chloroform (Ciegler, 1969), a sample of this isolated penitrem A was used for the determination of the melting point, UV absorption spectrum, IR and NMR spectrum which were carried along with an authentic sample.

2.1.4 Effect of temperature and moisture on production of toxin penitrem A using different solid substrates

2.1.4.1 To study the effect of temperature: 20 g of rice, wheat, Bengal gram dhal, barley and yellow corn were taken in different flasks and kept moist at 30 per cent level (except for the rice which was kept moist at 20 per cent) were sterilised and inoculated with a spore
suspension of *P. crustosum* and incubated at different temperatures (3° to 5°C, 18°C, 25° to 27°C, 28° to 30°C, 37°C, 40°C, 60°C) for 14 days. The toxin penitrem A was extracted and separated as described earlier in this section and the amount obtained were carefully weighed.

2.1.4.2 To study the effect of moisture, the substrate used (rice, wheat, barley, yellow corn, Bengal gram dhal) were dried at 50°C overnight and then enough water was added to give a moisture content of 10, 20, 30, 40, 50 per cent respectively and sterilised, inoculated with the respective organism and incubated at 25° to 27°C. After 14 days of incubation, penitrem A was separated as described earlier and weighed.

2.2 Toxicity studies on rats

2.2.1 Preparation of *P. crustosum* infected diet
50 grams of rice obtained from the market was taken in 500 ml conical flask, added sufficient water to make 30 per cent moisture and then sterilised at 15 pounds per square inch pressure for 10-15 minutes, then cooled and were inoculated with 0.5 ml spore suspension of
*P. crustosum* and incubated at 25° to 27°C for 14 days. The organism grown was destroyed using chloroform. Thus contaminated rice was completely freed from chloroform and powdered which was then mixed well with commercial feed used for rats in the ratio 40:60. This mixed food called as "contaminated feed" was fed to rats.

2.2.2 Preparation of crude toxin suspension

Crude extract suspension in dilute ethanol was prepared by dissolving 10 mg of the crude extract in 4.0 ml of alcohol and this was made upto 100 ml. This gives 0.1 mg of extract in dilute alcohol.

2.2.3 Preparation of pure toxin in vehicle

10 mg of pure penitrem A was dissolved in 2.0 ml of ethanol and was made upto 100 ml with sterilised distilled water. 0.1 ml of this solution was given to rats. This corresponds to about 0.3 mg/kg body weight of penitrem A in 0.1 ml of dilute ethanol as vehicle.
2.2.4 Effect of *P. crustosum* infected diet and crude extract in rats

Ninety young weanling albino rats of Wistar strain of both sexes weighing about 30-35 g were divided into 3 groups. One group was fed with uninfected rice mixed with commercial rat feed, while the second group was fed with *P. crustosum* contaminated diet. To a third group of animals 0.5 ml of crude extract of the toxin as suspension in dilute alcohol, was orally administered. Water was given *ad libitum*. The experimental animals along with the controls were sacrificed at the end of 2, 4 and 6 weeks. Liver, kidney, small intestine, heart and brain tissues were used for estimation of cellular constituents and assay of enzymes. Blood was collected by cutting the neck or by cardiac puncture and used for some haematological studies. A part of tissues were processed in ten per cent formalin for histopathological studies.

2.2.5 Effect of pure penitrem A on rats

Sixty albino rats of both sexes weighing 35-40 g were divided into two groups of thirty each. Experimental animals received 10 micrograms of penitrem A in 0.1 ml of vehicle intraperitoneally on alternate days. Control group received 0.1 ml of vehicle alone. After six weeks of experimental period the animals were fasted overnight and sacrificed. Biochemical and haematological studies were carried out in tissues and blood respectively of
both the groups and part of the tissues were used for histopathological studies.

2.2.6 Histopathological changes

Small portions of tissues were transferred immediately after sacrifice and fixed in formal saline for histopathological investigations. Then, tissues were processed for paraffin embedding and sections so obtained were stained with haematoxylin and eosin.

2.2.7 $LD_{50}$ value of isolated penitrem A

The method used was that of Weil (1952), 24 male weanling rats weighing about 30-35 g were divided into six groups and the penitrem A toxin at a concentration of 0.6, 1.2, 2.4, 4.8 mg/kg body weight in 0.5 ml of very dilute alcohol was injected intraperitoneally to each group respectively. Mortality rats were observed and $LD_{50}$ value was calculated.

2.3 Preparation of the tissue

To collect enough blood, the animals were sacrificed by cutting the neck. After collecting the blood
the liver, kidney, small intestine and brain tissues were immediately removed, washed well with ice-cold saline and placed in a beaker standing on cracked ice.

The organs were blotted on filter paper and cut into small bits before weighing. One per cent homogenate of the tissue was prepared in 0.1 M tris-hydrochloric acid buffer of pH 7.4 in a Potter-Elevehjem homogeniser using a teflon pestle at 600 rpm for three minutes. This homogenate was used for the assay of enzymes.

Separate portions of the tissues were weighed and used for the estimation of cellular constituents.

In case of brain tissue they were removed and chilled immediately, adhering membrane and blood clots, if any, were removed from the surface. The cerebral hemisphere, cerebellum and medulla oblongata were separated carefully and homogenised in sodium phosphate buffer (0.1 M, pH 8.0) in a cold room at 4°C.
2.4 Estimation of cellular constituents

2.4.1 Estimation of proteins

The protein contents of the tissues were estimated by the method of Lowry et al (1951).

Reagents:

1. Alkaline copper reagent: 50 ml of reagent A (2 per cent sodium carbonate in 0.1 N sodium hydroxide) was mixed with 1.0 ml of reagent B (0.5 per cent copper sulphate in one per cent sodium potassium tartrate) just before use.

2. Folin's-phenol reagent: This was prepared according to the method described by Polin and Ciocalteu (1927).

Into a two litre flask was added 100 g of sodium tungstate, 25 g sodium molybdate, 700 ml of water, 50 ml of ortho-phosphoric acid (85 per cent) and 100 ml of concentrated hydrochloric acid. The mixture was refluxed gently for 10 hours, 150 g of lithium sulphate, 50 ml of water and a few drops of bromine were added. The mixture was boiled for 15 minutes without a condenser to remove excess bromine. The contents were cooled, made upto a litre and filtered.
For the estimations, the above stock solution was diluted 1:2 with water prior to use.

3. **Standard**: 20 mg of bovine serum albumin was dissolved in water and made upto 100 ml. This gives a standard containing 200 mcg per ml.

**Procedure:**

0.1 ml of the tissue homogenate was taken and made upto 1.0 ml with water. To this, 4.5 ml of alkaline copper reagent was added, mixed and allowed to stand at room temperature for 10 minutes. 0.5 ml of Folin's-phenol reagent was added and standards were treated in a similar manner. The blue complex formed was measured at 640 nm after 20 minutes in a Klett-Summerson photoelectric colorimeter against a water blank.

2.4.2 **Estimation of nucleic acids**

The nucleic acids in the tissues were first extracted. The tissues from experimental animals as well as normal controls were washed with ice-cold saline, blotted dry and weighed accurately and finally homogenised in 5.0 ml of distilled water. To this, 5.0 ml of 10 percent trichloroacetic acid was added and kept in ice for
30 minutes to allow complete precipitation of proteins and nucleic acids. The mixture was centrifuged and the precipitate obtained was washed thrice with ice-cold 10 per cent trichloroacetic acid. The precipitate was treated with 95 per cent ethanol to remove lipid materials, centrifuged and the lipid free precipitate was resuspended in 5.0 ml of 5 per cent trichloroacetic acid, heated in a hot water bath maintained at 90°C for 15 minutes with occasional stirring. This treatment quantitatively separated both DNA and RNA from the tissue proteins. After centrifugation the supernatant was used for the estimation of DNA and RNA.

2.4.2.1 Estimation of DNA

DNA was estimated according to the method of Burton (1956).

Reagents:

1. Diphenylamine reagent: 1.5 per cent crystalline diphenylamine in redistilled acetic acid containing 1.5 ml of concentrated sulphuric acid was prepared just before use.
2. Standard DNA: 10 mg of DNA was dissolved in 100 ml of 1 N perchloric acid.

Procedure:

A known volume of the nucleic acid extract was mixed with 2.0 ml of freshly prepared diphenylamine reagent. The total volume was made up to 5.0 ml with 0.6 N perchloric acid. The tubes were stoppered and heated in a boiling water bath for 10 minutes. Aliquots of standard DNA and blank containing perchloric acid alone were also treated similarly. The blue colour developed and read at 640 nm in a Klett-Summerson photoelectric colorimeter.

The values were expressed as mg/g of fresh tissue.

2.4.2.2 Estimation of RNA

RNA was estimated according to the method of Schneider (1957).

Reagents:

1. Orcinol - ferric chloride reagent: One per cent orcinol in concentrated hydrochloric acid containing
0.5 per cent ferric chloride was prepared freshly every time.

2. Standard RNA: This was prepared by dissolving yeast RNA in hot 5 per cent trichloroacetic acid to give a concentration of 200 mcg/ml.

Procedure:

Aliquots of nucleic acid extract were made up to 2.0 ml with 5 per cent trichloroacetic acid. 3.0 ml orcinol reagent was added and mixed well. The tubes were heated in a boiling water bath for 20 minutes. A series of standard RNA solutions were also treated in a similar manner along with a blank containing 5 per cent trichloroacetic acid. After 20 minutes, the tubes were cooled and the green colour developed was read at 640 nm in Klett-Summerson photoelectric colorimeter.

The values were expressed as mg/g of fresh tissue.

2.4.3 Estimation of total alkali extractable carbohydrates and glycogen

The estimation of alkali extractable carbohydrates and glycogen were carried out by the method of Seifter et al (1950).
2.4.3.1 Estimation of alkali extractable carbohydrate

50 mg of the tissue was accurately weighed in a test tube, treated with 3.0 ml of 30 per cent potassium hydroxide. The tubes were kept in a boiling water bath for 15 minutes, cooled and centrifuged. From the supernatant, aliquots were taken for the estimation of total carbohydrates.

2.4.3.2 Extraction of glycogen

The extraction was carried out by the method described by Morales et al (1973). Glycogen was precipitated from the alkali extract of the tissues by adding 1:3 volumes of 95 per cent ethanol and a drop of 1 M ammonium acetate. The tubes were kept in a boiling water bath for 10 minutes. After cooling, they were transferred to a freezer for 24 hours to facilitate complete precipitation. The precipitated material was collected by centrifugation and dissolved in one ml of water with the aid of heating. The precipitation procedure was repeated once more. The final precipitate was dissolved in one ml of saturated ammonium chloride solution and heated for 5 minutes in a boiling water bath. Aliquots
from this were used for the estimation of carbohydrate by the anthrone reaction.

Reagents:

1. Anthrone reagent: 200 mg of anthrone was dissolved in 100 ml of analar sulphuric acid.

2. Standard glucose solution: 10 mg of glucose was dissolved in 100 ml of distilled water, saturated with benzoic acid.

Procedure:

Suitable aliquots of the alkali extractable carbohydrates and glycogen were made upto one ml with water. A set of standard glucose solution and a blank containing water alone were set up. All the tubes were cooled in an ice-bath and 4.0 ml of anthrone reagent was added. The contents of the tubes were well mixed. All the tubes were covered with glass marbles and heated for 20 minutes in a boiling water bath. The tubes were cooled and the green colour developed was read at 600 nm in a Klett-Summerson photoelectric colorimeter. The glycogen and alkali extractable carbohydrates were expressed in terms of glucose.
2.4.4 Estimation of total lipids

Total lipids were extracted by the method of Folch et al (1957), using chloroform : methanol in the ratio of 2:1. A weighed amount of tissue was extracted by homogenisation with chloroform : methanol mixture in a Teflon homogeniser. The extraction was repeated thrice with fresh aliquots of chloroform : methanol. The lipid extracts were transferred to separating funnels containing 2.0 ml of physiological saline and left overnight, after which the lipid extracts were drained into weighed beakers and allowed to dry to constant weight. The total lipid contents were calculated and expressed as mg/g fresh tissue.

The lipid contents were redissolved in 2.0 ml of chloroform : methanol mixture and aliquots (0.2 ml) were used for the estimation of total cholesterol, free cholesterol, esterified cholesterol and phospholipids.

2.4.4.1 Estimation of cholesterol

Cholesterol was estimated according to the method described by Leffler and McDougald (1963). Isopropanol was used to extract the total cholesterol.
Reagents:

1. Digitonin solution: 100 mg of digitonin was solubilised in 50 per cent ethanol by heating gently and agitating for about 5-10 minutes.

2. Analar grade concentrated sulphuric acid.

3. Ferric chloride solution: 500 mg of crystalline dry ferric chloride was dissolved in 500 ml of redistilled glacial acetic acid.

4. Cholesterol stock standard: Reagent grade cholesterol was recrystallised and 100 mg of the recrystallised cholesterol was dissolved in 100 ml isopropanol and stored at room temperature.

5. Cholesterol working standard: 0.04 mg of cholesterol/ml was prepared by diluting 4.0 ml of the stock solution to 100 with isopropanol.

Procedure:

0.2 ml of the total lipid extract was evaporated to dryness and the residue was dissolved in 2.0 ml of isopropanol. This extract was used for cholesterol assay.
2.4.4.2 Free cholesterol estimation

To one ml of the isopropanol extract was added 2.0 ml of acetone and 1.0 ml of digitonin. The mixture was shaken well and kept in ice-bath for 30 minutes to allow complete precipitation of free cholesterol which was removed. To the precipitate was added 5.0 ml of acetone, stirred, recentrifuged and the acetone alone was removed. To the precipitate was again added 5.0 ml of acetone recentrifuged and the acetone was discarded leaving the precipitate to which 1.0 ml of isopropanol was added. The cholesterol was estimated using ferric chloride and sulphuric acid.

2.4.4.3 Total cholesterol estimation

To 1.0 ml of isopropanol extract (and also to the digitonin precipitate in isopropanol) was added 2.0 ml of ferric chloride solution and mixed thoroughly with 2.0 ml of concentrated sulphuric acid with constant shaking. The reddish brown colour developed was measured at 540 nm against a blank in a Klett-Summerson photo-electric colorimeter. Standard solutions containing 25-100 mcg of cholesterol were also treated in a similar manner.
2.4.4.4 Estimation of esterified cholesterol

The difference between the total cholesterol and free cholesterol gives the esterified cholesterol values.

The values were expressed as mg/g of fresh tissue.

2.4.4.5 Estimation of phospholipids

Phospholipids were estimated by the method of Fiske and Subbarow (1925) as inorganic phosphorus after digesting with perchloric acid.

Reagents:

1. Ammonium molybdate: 2.5 g of ammonium molybdate was dissolved in 100 ml of 3 N H₂SO₄.

2. Aminonaphthol sulphonic acid (ANSA): 50 mg of 1,2,4-aminonaphthol sulphonic acid was dissolved in 195 ml of 15 per cent sodium bisulphite and 5.0 ml of 20 per cent sodium sulphite was added for complete solubilisation. The solution was filtered and stored in a brown bottle.
3. Perchloric acid A.R.

Procedure:

An aliquot of the total lipid extract in chloroform - methanol was evaporated to dryness in a water bath. The residue obtained was evaporated to dryness in a water bath and finally digested with 0.1 ml of perchloric acid over a sand bath. The clear solution was used for the estimation of inorganic phosphorus. The phospholipid content of the extracted tissue lipid was arrived at by multiplying the value of phosphorus by 25.

The values were expressed as mg/g of fresh tissue.

2.4.5 Estimation of lactic acid

Tissue lactate was estimated by the method of Barker and Summerson (1941).

Reagents:

1. 10 per cent trichloroacetic acid solution.

2. Calcium hydroxide powder.
3. 4 per cent copper sulphate solution.

4. 20 per cent copper sulphate solution.

5. p-Hydroxydiphenyl reagent: 1.5 per cent p-hydroxydiphenyl solution in 0.5 per cent sodium hydroxide.

6. Concentrated sulphuric acid (Analar).

7. Standard lactate solution: 21.3 mg of lithium lactate was dissolved in small amount of water. 0.1 ml of concentrated sulphuric acid was added and the volume was made upto 100 ml with water.

Procedure:

The tissues were weighed accurately and homogenised in 0.01 M Tris-HCl buffer (pH 7.5). An aliquot of the homogenate was treated with ice-cold 10 per cent trichloroacetic acid. After 30 minutes, it was centrifuged at 2000 x g for 20 minutes and an aliquot of the supernatant was mixed with 1.0 ml of 20 per cent copper sulphate and made upto 10 ml with water. To this was added about 1.0 g of calcium hydroxide powder with occasional shaking. It was centrifuged after 30 minutes. An aliquot of the supernatant was treated with 0.05 ml of 4 per cent copper sulphate followed by 6.0 ml of concentrated sulphuric acid.
The contents were mixed well and the tubes were placed in a boiling water bath for 5 minutes, cooled and 0.1 ml of p-hydroxydiphenyl reagent was added. The tubes were left at room temperature for 30 minutes, the precipitate formed during this period was dissolved and finally kept in a boiling water bath for 90 seconds, cooled and the violet colour formed was read at 540 nm in a Klett-Summerson photoelectric colorimeter against a water blank treated in a similar manner. Aliquots of standard lithium lactate solution were also treated in a similar manner.

2.4.6 Estimation of pyruvic acid

Tissue pyruvate was estimated by the method of Friedemann and Haugen (1943) with slight modifications.

Reagents:

1. 2,4-Dinitrophenyl hydrazine (DNPH) reagent: 0.1 per cent DNPH solution in 2 N hydrochloric acid.

2. 1.5 N sodium hydroxide solution.

3. Standard pyruvate solution: 12.5 mg of sodium pyruvate was dissolved in 100 ml of water.
Procedure:

Tissues were weighed accurately and homogenised in 0.01 M Tris-HCl buffer (pH 7.5). An aliquot of this homogenate was treated with ice-cold 10 per cent trichloroacetic acid. After 30 minutes, it was centrifuged at 2000 x g for 20 minutes. An aliquot of the supernatant was treated with 1.0 ml of DNPH. The tubes were left at room temperature for 15 minutes. Then 6.0 ml of 1.5N sodium hydroxide solution was added and the red colour formed was read at 540 nm in a Klett-Summerson photoelectric colorimeter against a water blank treated in a similar manner as well as aliquots of standard sodium pyruvate solution.

2.4.7 Estimation of acetylcholine

Estimation of acetylcholine was carried out by the method of Hestrin (1949) with slight modifications.

Reagents:

1. Hydroxylamine: Hydroxylamine hydrochloride, 2.0 M.
   The solution was stored in the cold.

2. Alkali: Sodium hydroxide, 3.5 N.
3. Acid: Concentrated hydrochloric acid, diluted with two parts by volume of water.

4. Ferric chloride - 0.37 M in 0.1 N hydrochloric acid.

5. Standard solution: Acetylcholine chloride, 0.004 M in sodium acetate buffer (0.001 N of pH 4.5).

Procedure:

Alkaline hydroxylamine reagent was prepared freshly before use by mixing equal volumes of reagents 1 and 2. The mixture can be kept for about 3 hours at room temperature. Two ml of the alkaline hydroxylamine reagent was added to 1.0 ml of the brain homogenate in a test tube. The pH was brought to 1.2±0.2 with 1.0 ml of acid and 1.0 ml of ferric chloride solution was added and centrifuged. The purple-brown colour of the supernatant was measured at 540 nm.

The amount of acetylcholine present was expressed as n moles per gram tissue.

2.5 Assay of some important enzymes in normal and experimental animals

Liver, kidney and intestine from control and experimental animals were rinsed with ice-cold saline,
blotted dry and weighed. The tissues were homogenised in Tris-HCl buffer, pH 7.5 (0.01 M) at 4°C using a Teflon homogeniser. The homogenate was kept cooled in ice while in use and the following enzyme estimations were done as quickly as possible.

2.5.1 Alkaline phosphatase (ortho-phosphoric monoester phosphohydrolase, E.C. 3.1.3.1)

Alkaline phosphatase was assayed by the method of Moog (1946) modified by King (1965) using disodium phenyl phosphate as the substrate.

The incubation mixture contained the following components in a final volume of 3.0 ml. 150 micromoles of carbonate - bicarbonate buffer, pH 10.0, 10 micromoles of disodium phenyl phosphate, 10 micromoles of magnesium chloride and requisite amount of the enzyme. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of Folin's phenol reagent. If turbidity appeared, the tubes were centrifuged. Controls without enzyme solution were also incubated and the enzyme was added after the addition of Folin's phenol reagent. Then 1.0 ml of 15 per cent
sodium carbonate was added and incubated for further 10 minutes at 37°C. The blue colour developed was read at 640 nm against a blank. Standard solution of phenol containing 120 - 480 millimicromoles were also treated similarly. The activity of the enzyme was expressed as micromoles of phenol liberated per milligram protein under the incubation conditions.

2.5.2 Acid phosphatase (ortho-phosphoric monoester phosphohydrolase, E.C. 3.1.3.2)

The procedure adopted for the assay of tissue acid phosphatase was the same as described above for alkaline phosphatase, excepting that a citrate buffer (0.1 M, pH 4.9) was used and the magnesium ions were omitted from incubation mixture. The enzyme activity was expressed as micromoles of phenol liberated per milligram protein under the incubation conditions.

2.5.3 5'-adenosine monophosphatase/or 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, E.C. 3.1.3.5)

5'-nucleotidase activity was estimated by the method of Campbell (1962) with some modifications.
This enzyme hydrolyses nucleotides with a phosphate group on carbon atom 5' of the ribose for example adenosine 5' phosphate which is hydrolysed to adenosine and inorganic phosphate. These nucleotides are also hydrolysed by non-specific phosphatases such as alkaline phosphatase. However 5'-nucleotidase is inactivated by nickel so that if the hydrolysis of the substrate is carried out with and without added nickel, the difference gives 5'-nucleotidase activity.

Reagents:
1. Buffer: Tris-Hydrochloric acid buffer, pH 7.5, 0.1 M.
2. Substrate: 0.01 M adenosine monophosphate solution.
3. 0.1 M nickel chloride.
4. 0.02 M manganous sulphate.
5. 10 per cent trichloroacetic acid.
6. 2.5 per cent ammonium molybdate in 3.0 N sulphuric acid.
7. 0.25 per cent ANSA in 15 per cent sodium metabisulphite.

Procedure:
A. Total activity:
   To 1.0 ml of buffer added 0.1 ml of manganous sulphate and 0.2 ml of substrate. To the tubes labelled
in test, 0.5 ml of serum was added and the total volume made up to 2.0 ml with water. The tubes were incubated at 37°C for 30 minutes. At the end of the incubation period, 1.5 ml of 10 per cent trichloroacetic acid was added to each tube to arrest the enzyme reaction. Enzyme was added to the 'control' tubes after the addition of trichloroacetic acid. The solutions were centrifuged. In a suitable aliquot of the supernatant, phosphorus was estimated according to the method of Fiske and Subbarow (1925) as described earlier.

B. Non-specific alkaline phosphatase activity:

This was estimated as given for total activity except that 0.2 ml of nickel chloride was added, in addition, to the incubation mixture.

The liberated phosphorus was estimated by the method of Fiske and Subbarow (1925) as described earlier.

The difference between the total activity and non-specific alkaline phosphatase activity gave the specific 5'-nucleotidase activity.
The specific activity was defined as the number of millimicro moles of phosphorus liberated per mg of protein under incubation conditions.

2.5.4 Aspartate transaminase (L-aspartate: 2-oxoglutarate aminotransferase, E.C. 2.6.1.1)

Tissue aspartate transaminase was assayed by the method of Hansen (1959).

Reagents:

1. Buffered substrate: 1.33 g of L-aspartic acid and 30 mg of 2-oxoglutarate were dissolved in 20.5 ml of 0.4 N sodium hydroxide and made up to 100 ml with 0.1 M phosphate buffer, pH 7.5.

2. 2,4-Dinitrophenyl hydrazine (DNPH) reagent: 0.02 percent DNPH in 1 N hydrochloric acid.

3. 0.4 N sodium hydroxide.

4. Standard pyruvate solution: 22 mg of sodium pyruvate was dissolved in 100 ml of buffer.
Procedure:

The incubation mixture contained 1.0 ml of the buffered substrate (preincubated at 37°C) and requisite amount of enzyme solution. The reaction mixture was incubated at 37°C for 60 minutes and the reaction was terminated by the addition of DNP H. Control tubes received the enzyme after the addition of DNP H. The tubes were incubated for further 15 minutes at 37°C and the colour developed by the addition of 10 ml of 0.4 N sodium hydroxide was read at 540 nm along with standard pyruvate solutions.

The enzyme activity was expressed as micromoles of pyruvate formed per milligram protein under the incubation conditions.

2.5.5 Lactate dehydrogenase (L-lactate: NAD oxido-reductase, E.C. 1.1.1.27)

Lactate dehydrogenase was assayed by the colorimetric method described by King (1965).
Reagents:

1. Buffered substrate: 4.0 gm of lactate was dissolved in 125 ml of glycine - sodium chloride buffer (pH 10.0) and 75 ml of 0.1 N sodium hydroxide. The pH was adjusted to 10.0.

2. Coenzyme solution: 5.0 mg of NAD\(^+\) was dissolved in 1.0 ml of water and was prepared just before use.

3. 0.4 N sodium hydroxide.

4. 0.02 per cent 2,4-dinitrophenyl hydrazine in 1 N hydrochloric acid.

5. Standard solution: 11 mg of sodium pyruvate was dissolved in 100 ml of buffered substrate.

6. Reduced coenzyme solution: 8.5 mg of NADH was dissolved in 10 ml of water.

Procedure:

The incubation mixture contained 1.0 ml of buffered substrate, 0.2 ml of NAD\(^+\) solution (pre-incubated at 37°C) and requisite amount of enzyme solution. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of DNPH. The tubes were incubated further for 20 minutes.
The colour developed after the addition of 10 ml 0.4 N sodium hydroxide was read at 540 nm in a Klett-Summerson photoelectric colorimeter. Control tube did not receive the coenzyme solution. Standard curve was prepared with aliquots of pyruvate in buffered substrate, NAD$^+$ and NADH in a similar manner as given above. The activity of the enzyme was expressed as micro moles of pyruvate formed per milligram protein under incubation conditions.

2.5.6 Aldolase (Ketose-1-phosphate aldehyde lyase, E.C. 4.1.2.7)

Tissue aldolase activity was assayed by the method of King (1965).

Reagents:

1. Tris-HCl buffer (0.1 M, pH 8.6): 1.21 gm of Tris was dissolved in water, the pH was adjusted to 8.6 with known volume of 0.1 N hydrochloric acid and made upto 100 ml with water.

2. Substrate: 0.05 M fructose-1,6-diphosphate in buffer, prepared fresh.

3. 0.56 N hydrazine sulphate.
4. Colour reagent: 0.1 per cent 2,4-dinitrophenyl hydrazine in 2 N hydrochloric acid.

5. 0.75 N sodium hydroxide.

Procedure:

The incubation mixture in a total volume of 2.0 ml contained the following: 0.25 ml of substrate, 0.25 ml of hydrazine sulphate; 1.0 ml of buffer and requisite amount of enzyme preparation. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of 10 per cent trichloroacetic acid and the tubes were centrifuged. An aliquot of the supernatant was transferred to tubes containing 1.0 ml of 0.75 N sodium hydroxide. The tubes were left at room temperature for 10 minutes. Then 1.0 ml of dinitrophenyl hydrazine reagent was added and incubated at 37°C for 60 minutes. The colour developed after the addition of 7.0 ml of 0.75 N sodium hydroxide was read at 540 nm. DL-glyceraldehyde was used as the standard. The enzyme activity was expressed as micro moles of glyceraldehyde formed per milligram protein under the incubation conditions.
2.5.7 Fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate-1-phosphohydrolase, E.C. 3.1.3.11)

FDPase was assayed by the method of Gancedo and Gancedo (1971) with slight modifications.

The incubation mixture in a final volume of 2.5 ml contained the following: 150 micro moles of Tris-HCl buffer (pH 7.0). 250 micro moles of potassium chloride, 25 micro moles of magnesium chloride, 2.5 micro moles of EDTA, 25 micro moles of fructose-1,6-diphosphate and requisite amount of enzyme preparation.

The reaction mixture was incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of 10 per cent trichloroacetic acid. The supernatant after the centrifugation was used for the estimation of inorganic phosphorous by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as micro moles of Pi liberated per milligram protein under incubation conditions.
2.5.8 Glucose-6-phosphatase (D-Glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9)

G6Pase was assayed by the method of Koide and Oda (1959).

The incubation mixture in a final volume of 1.0 ml contained 0.5 ml of 0.01 M glucose-6-phosphate, 0.4 ml of 0.1 M citrate buffer (pH 6.5) and 0.1 ml of the enzyme preparation. The mixture was incubated at 37°C for 60 minutes and the reaction was terminated by the addition of 1.0 ml of 10 per cent trichloroacetic acid. The tubes were centrifuged and supernatant was used for the estimation of inorganic phosphorous by the method of Fiske and Subbarow (1925). The enzyme activity was expressed as micro moles of Pi liberated per milligram protein under incubation conditions.

2.5.9 Glycogen phosphorylase (α-1,4-glucan: Ortho-phosphate glucosyl transferase, E.C. 2.4.1.1)

The enzyme was assayed by the method of Cornblath (1963).
The incubation mixture in a final volume of 0.5 ml contained 6.4 micro moles of glucose-1-phosphate, pH 6.1, 4.0 mg of glycogen and requisite amount of the enzyme solution. The incubation was carried out at room temperature for 10 minutes. The reaction was terminated by the addition of 1.0 ml of 10 per cent trichloroacetic acid. The liberated Pi in the supernatant was estimated by the method of Fiske and Subbarow (1925). Since the preparation showed glucose-1-phosphatase activity, the assay was done in the presence, as well as in the absence of glycogen. The difference between the two gives the true phosphorylase activity.

The enzyme activity was expressed as micro moles of Pi liberated per milligram protein under the incubation conditions.

2.5.10 Hexokinase (ATP: D-hexose-6-phospho transferase, E.C. 2.7.1.1)

Hexokinase was assayed by the method of Brandstrup et al (1957) by determining the rate of disappearance of glucose at 37°C in a reaction mixture with the following composition in a total volume of 5.0 ml: Glucose - 1 mM, ATP - 36 mM, magnesium chloride -
1.0 mM, dipotassium hydrogen phosphate - 4.0 mM, potassium chloride - 30 mM, sodium fluoride - 10 mM, and tris-HCl buffer (pH 8.0) - 12 mM. The reaction mixture was initiated by the addition of 2.0 ml of the tissue homogenate. One ml of aliquot of the reaction mixture was removed immediately (0 time) to tubes containing 1.0 ml of 10 per cent trichloroacetic acid. A second aliquot was removed after 30 minutes of incubation at 37°C. Both the samples were estimated for glucose using o-toluidine according to the method of Sasaki et al (1972). A reagent blank was run with each test.

The difference between the two values gives the amount of glucose-6-phosphate formed. The enzyme activity was expressed as millimicro moles of glucose-6-phosphate formed per milligram protein under the incubation conditions.

2.5.11 Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxido reductase, E.C. 1.1.1.49)

Tissue glucose-6-phosphate dehydrogenase was assayed by the method of Eels and Kirkman (1961).
Reagents:

1. Tris-HCl buffer - 0.05 M (pH 7.5).
2. Magnesium chloride (1 M solution).
3. NADP⁺ (1 M solution).
4. Phenazine methosulphate: 0.005 per cent in water.
5. 2,6-dichlorophenol indophenol dye: 0.01 per cent in water.
6. Glucose-6-phosphate (0.02 M).

Procedure:

The incubation mixture in a total volume of 5.5 ml contained 1.0 ml of Tris-HCl buffer, 0.1 ml of magnesium chloride, 0.1 ml of NADP⁺, 0.5 ml of phenazine-methosulphate, 0.4 ml of 2,6-dichlorophenolindophenol dye and requisite amount of enzyme solution. The mixture was allowed to stand at room temperature for 10 minutes to permit the oxidation of endogenous materials. The reaction was initiated by the addition of 0.5 ml of glucose-6-phosphate. The optical density was read, at one minute intervals for 3-5 minutes at 640 nm against a water blank in a spectrophotometer. The activity of the enzyme was calculated in units by multiplying the change in optical
density per minute by factor $6/17.6$ which is the molar extinction coefficient.

The enzyme activity was expressed as units/mg protein under the incubation conditions.

2.5.12 Adenosine triphosphatase (ATP-phosphohydrolase, E.C. 3.6.1.3)

Adenosine triphosphatase was assayed according to the method described by Evans (1969) with slight modifications.

Reagents:

1. Tris-HCl buffer: 0.1 M, pH 7.5.

2. Substrate - 0.0075 M adenosine triphosphate.

3. Activators:
   a) Magnesium chloride - 0.1 M solution
   b) Sodium chloride  - 0.1 M solution
   c) Potassium chloride - 0.1 M solution
   d) Calcium chloride  - 0.1 M solution

4. 10 per cent trichloroacetic acid solution.
5. Reagents according to Fiske and Subbarow (1925) for the estimation of phosphorous.

Procedure:

For the assay of total ATPase activity, the incubation mixture in a total volume of 2.0 ml contained the following: 100 micro moles of buffer, 75 millimicro moles of substrate, 10 micro moles each of magnesium chloride, sodium chloride and potassium chloride and 0.1 ml of the enzyme extract. The incubation was carried out at 37°C for 20 minutes. The reaction was terminated by the addition of 1.0 ml of 10 per cent TCA. The suspension was centrifuged and the phosphorous content of the supernatant was assayed according to the method of Fiske and Subbarow (1925).

For the assay of sodium, potassium dependent ATPase activity, magnesium was omitted from the assay medium.

The calcium dependent ATPase activity was assayed in the presence of 10 micro moles of calcium chloride and in the absence of other activators.
The enzyme activities were expressed as micro moles of phosphorus liberated per milligram protein under the incubation conditions.

2.5.13 Cholinesterase (E.C. 3.1.1.8)

Cholinesterase was assayed by the method of de la Huerga et al (1952) with slight modifications.

Reagents:

1. Buffer solution: 10.3 g of sodium barbitone was dissolved in 300 ml of water. 60 ml of normal hydrochloric acid was added slowly. To this 5.3 g of anhydrous sodium carbonate was added and warmed gently. Cooled to room temperature and made upto 500 ml.

2. Salt mixture: 4.2 g of anhydrous magnesium chloride and 0.2 g of potassium chloride were dissolved in water and made upto 100 ml.

3. Substrate: 45.4 mg of acetylcholine chloride was dissolved in 100 ml of buffer.

4. 14 per cent hydroxylamine hydrochloride solution.

5. 3.5 N sodium hydroxide solution.
6. Alkaline hydroxylamine: Equal volumes of (4) and (5) were mixed just before use.

7. Ferric chloride solution: 6.0 g of ferric chloride was dissolved in 100 ml of 0.1 N hydrochloric acid.

8. 4 N hydrochloric acid.

9. Standard acetylcholine chloride solution: 0.5 M acetylcholine chloride solution was diluted 1 in 10. This contains 50 micro moles per ml.

Procedure:

The incubation mixture contained the following components in a final volume of 4.5 ml: 2.0 of buffered substrate, 1.0 ml of buffer, 0.5 ml of salt mixture and 1.0 ml of enzyme. The reaction mixture was terminated by the addition of 2.0 ml of alkaline hydroxylamine solution and centrifuged after 10 minutes. Controls without enzyme solution were also incubated and the enzyme was added after the addition of alkaline hydroxylamine. Then 1.5 ml of 4 N hydrochloric acid and 1.0 ml of ferric chloride solution were added. The brown colour developed was read at 540 nm in a Klett-Summerson photoelectric colorimeter. Standard solutions were treated in a similar manner. The activity of cholinesterase was expressed as micro moles
of acetylcholine liberated/mg of protein under incubation conditions.

2.5.14 Assay of acetylcholine esterase (E.C. 3.1.1.7)

AChE activity was determined spectrophotometrically using acetylthiocholine iodide as substrate by the method of Ellman et al (1961).

Reagent:

1. Sodium phosphate buffer (0.1 M, pH 8.0).
2. Dithio-bis-nitrobenzoic acid (DTNB), 0.01 M.
3. Acetylthiocholine iodide, 158.5 mM.

Procedure:

For assay, 0.1 ml of the enzyme was mixed with 3.0 ml of sodium phosphate buffer and incubated at 30°C for 10 minutes. Then, 0.1 ml of DTNB was added to the mixture followed by 0.2 ml of acetylthiocholine iodide (158.5 mM) to give a final substrate concentration of 1 mM. The hydrolysis of acetylthiocholine produces thiocholine which reacts with DTNB to give 5-thio-2-nitrobenzoate, a yellow chromophore that has an absorbance
maximum at 412 nm. The enzyme activity was measured by following the increase in absorbance at 412 nm in a Cecil linear read out spectrophotometer. The activity was determined in international units by calculating the extinction change (ΔE) with time (min.) and allowing for the molar extinction coefficient of the chromophore - 1.36x10⁴ mole⁻¹cm⁻¹, volume of the enzyme (0.1 ml), the total volume of the reaction mixture (3.4 ml) and a factor of 1000 to obtain micro moles of thiocholine released by the enzyme.

2.6 Constituents in blood and serum

2.6.1 Blood sugar estimation

Blood sugar was estimated using o-toluidine according to the method of Ceriotti and Frank (1969) as modified by Sasaki et al (1972).

Reagents:

1. 10 per cent trichloroacetic acid (TCA).

2. ortho-Toluidine - boric acid reagent: This contained 2.4 g boric acid and 2.5 g thiourea in 100 ml of a solution containing water glacial acetic acid and freshly distilled ortho-toluidine in the ratio 10 : 75 : 15.
3. Standard glucose:

Stock standard glucose was prepared by dissolving 100 mg of D-glucose in 100 ml of 0.1 per cent benzoic acid.

10 ml of this solution was diluted to 100 ml to get a working standard solution containing 100 mcg of glucose per ml.

Procedure:

0.1 ml of blood was mixed with 3.0 ml of 10 per cent TCA to precipitate the proteins. The suspension was centrifuged to remove the precipitated proteins. To one ml of the supernatant 4.0 ml of the o-toluidine reagent was added and kept in a boiling water bath for 15 minutes.

Standard solutions containing 25 - 100 mcg of glucose in 1.0 ml volume and a blank containing 1.0 ml water were also treated in a similar way. The blue colour developed was read after cooling at 640 nm.

2.6.2 Estimation of serum total protein

Aliquots of diluted serum was taken for the estimation of total serum protein by the method of Lowry et al (1951) as described earlier.
2.6.3 Serum lipid constituents

The estimation of total cholesterol, free cholesterol, esterified cholesterol and phospholipids were carried out by the same procedure described for each under tissue lipid constituents.

2.6.3.1 Serum total cholesterol

0.1 ml of serum was mixed with 3.9 ml of isopropanol. The precipitated proteins were removed by centrifugation. Aliquots of the supernatant was used for the estimation of total cholesterol according to the method of Leffler and McDougald (1963) as described under tissue cholesterol.

2.6.3.2 Serum free cholesterol

The free cholesterol was precipitated and separated according to the method of Sperry and Webb (1950) as modified by Mookerjee and Sadhu (1955) using digitonin.

Procedure:

0.1 ml of serum was treated with 3.0 ml of acetone - ethanol mixture (1:1, v/v) and heated gently on a
water bath. The contents in the tube were mixed well and the precipitated proteins were separated by centrifugation, the supernatant was collected. The precipitate was treated again with 3.0 ml of acetone - ethanol mixture. The combined supernatant was used for the estimation of free cholesterol as described earlier (Section 2.4.4.1).

2.6.3.3 Esterified cholesterol

The esterified cholesterol content was arrived at by subtracting the free cholesterol level from the total cholesterol level.

2.6.3.4 Serum phospholipids

Phospholipids in serum were estimated according to the method of Youngberg and Youngberg (1930).

To 0.1 ml of serum, 0.9 ml of ethanol - ether (3:1, v/v) mixture was added, mixed well and centrifuged. A known aliquot of the supernatant was transferred to a test tube and evaporated to dryness. The procedure was continued same as described for tissue phospholipid.
2.6.4 White blood cell count, differential counting and hemoglobin estimations were carried out according to the method found in "Bray's Clinical Laboratory Methods" (1968).

2.6.4.1 White blood cell count

Reagents:

Turk diluting fluid:

1. Glacial acetic acid, 2.0 ml.
2. Gentian violet (1 per cent aqueous), 1.0 ml.
3. Distilled water, 97 ml.

Procedure:

Blood was drawn to 0.5 mark on white cell diluting pipette, care being taken to avoid air bubbles. After wiping the tip of the pipette, the diluting fluid was sucked upto the mark and mixed well.

After discarding a third of the fluid and wiping the tip of the pipette, a drop of fluid was placed on the side of the double chamber and the next drop on the other side of the chamber and kept for three minutes for settling.
Cells in the 4 corner squares (1 sq. mm areas) were counted. From that, the average number of cells in one square was calculated.

2.6.4.2 Differential counting

Procedure:

A medium sized drop of blood was taken near one end of the clean microscope slide, another slide to spread the blood was placed in front of drop at an angle of about 45 degrees and the blood was allowed to be spread in the form of a smear. This smear was dried immediately in air and the slides were suitably labelled.

The satisfactory staining was done using Leishman stain for three minutes. The stain was diluted with double the volume of distilled water and kept for ten minutes after which the stain was washed off, with distilled water until a pinkish coloured smear was seen. The different cells were counted by using a microscope.

The counting was started at the upper edge of the smear, moved straight down to the lower edge, then moved from left to right and proceeded from the lower edge
upward and continued in the same manner, until a minimum of 100 cells were classified.

2.6.4.3 Haemoglobin estimation

Haemoglobin content was estimated by the conventional Sahlis Adam's acid haematin method.

2.7 In vivo absorption of $^{14}$C-amino acids (lysine, glycine, alanine, methionine and aspartic acid) and glucose by the intestine

2.7.1 Sixty male albino rats were divided into two groups of thirty animals each, one group served as the control and the other served as the experimental. This group was fed with *P. crustosum* infected diet, another sixty animals were divided into two equal groups, one group was treated with crude extract given orally while the other was given pure penitrem A injections intraperitoneally. This was compared with the same controls used above. At the end of two months of experimental period intestinal perfusion of labelled amino acids and glucose was performed on them.
Perfusion fluid was 0.15 M sodium phosphate buffer (pH 7.5) containing sodium chloride 135 mM, potassium chloride 5 mM, the respective amino acids at 1 mM and 14C-labelled amino acids.

The scintillating fluid was a mixture of dioxane and ethylene glycol (50 : 1, v/v) containing PPO (4 g/litre), POPOP (200 mg/litre) and naphthalene (50 g/litre).

Rats were fasted for 48 hours before the experiment. This ensures an intestinal 'steady state' and allows the emptying of most of the chyme and other intestinal residues. The animal was anaesthetized by intraperitoneal injection of sodium phenobarbitone (50 mg/kg). One such anaesthetized rat was placed on its back lying on the table so that the ventral surface was accessible. The limbs were gently stretched. The abdomen was opened by a mid line incision, the common bile duct was ligated and an inlet cannula was inserted at the pylorus and an outlet cannula was inserted at the terminal ileum. The entire small intestine was perfused at a constant rate with 135 mM sodium chloride solution containing 5 mM amino acid with known concentration of 14C-labelled amino acid. The perfusion rate was 1.0 ml/minute. After initial equilibration with buffer for a period of fifteen minutes,
six 10 min. samples were collected for analysis. 0.1 ml of the perfusate was taken immediately after perfusion and the radioactivity was measured in a liquid scintillation counter with 10 ml of scintillating fluid. Radioactivity of the perfusate was also measured separately.