CHAPTER II

MATERIALS AND METHODS
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**General:**

Preparation of media, maintenance of stock cultures of bacteria and fungi, staining and microscopic examination of cultures and other routine microbiological operations were carried out as described by Mackie and McCartney\(^{178}\) and Levine\(^{179}\).

Recordings of pH of media were made with a Beckman instrument using glass and calomel electrodes, ultraviolet absorption spectra with Beckman spectrophotometer DU model using quartz cells 1 cm. thickness, infrared spectra with a Perkin-Elmer Infra-ord using cells of 1 mm. thickness and optical rotations using 0.5 dm. tubes in a Hilger and Watts polarimeter.

**Radioactivity measurements:**

These were made using a thin-window Geiger-Müller tube, type E.H.M.I/3 - General Electric Company (operating voltage 1520 v), coupled to a Panax scaler (type 1000, Redhill, Surrey). The counts were corrected for self-absorption, decay and coincidence (when necessary).
Materials:

Apart from reagent grade chemicals, Difco, Oxo and B.D.H. preparations (except corn-steep liquor which was of Indian manufacture) were used for preparation of bacteriological media.

(a) Side-chain precursors: Phenoxacetic acid was prepared from chloracetic acid and sodium phenolate according to the method of Giocessa\textsuperscript{180} and the requisite quantity of its potassium salt (5% stock solution) was incorporated into fermentation broths. Similarly, potassium phenylacetate (5% stock solution) was used.

(b) Sulphurylcholine\textsuperscript{181}, benzylpenicilloic acid\textsuperscript{182}, benzylpenilloic acid\textsuperscript{182}, desthiopenicillin\textsuperscript{183} and DNP-derivatives of \(\alpha\)-amino adipic acid and peptides\textsuperscript{184} were prepared as described in literature.

(c) Penicillinase: The enzyme was used to destroy penicillin in culture filtrates of \textit{N. pulchella} before malbranchin was assayed. It was prepared by harvesting cultures of \textit{Bacillus cereus} AB.2 grown on casein-hydrolysate-citrate medium as recommended by Pollock\textsuperscript{185}, but the enzyme was only partly purified in that, the final steps involving column chromatography were omitted. After precipitation with ethanol at 40% concentration at \(-20^\circ\text{C}\), the separated enzyme
was dissolved in phosphate buffer at pH 7.0 to give a concentration of 1000 units of enzyme activity per ml. (one unit inactivates 1 µmole of penicillin in 1 hour at pH 7.0 and 30°C). The stock solution was distributed in tubes (5 ml) and stored in deep freeze.

Sterilisation:

Media were sterilised, unless explicitly referred to, at 15 lbs. steam pressure for 15 mins. All glassware was sterilised by dry heat at 120°C for 3 hours.

d) Media:

I. **Nutrient agar**¹⁷⁸: Stock cultures were maintained by monthly transfers and stored at 2°C.

II. **Honey-peptone-agar**: bacterial

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honey</td>
<td>15.0 g.</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0 g.</td>
</tr>
<tr>
<td>Agar</td>
<td>25.0 g.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml.</td>
</tr>
<tr>
<td>pH</td>
<td>6.9 (before sterilisation).</td>
</tr>
</tbody>
</table>

Like *P. notatum* 832, *P. chrysogenum* Q-176 and Wls.51-2073 (which were incubated at 24°C), *N. pulchella* sporulates profusely on this medium when incubated at 45°C. Stock cultures of all fungi in soils were stored at 2°C.

III. **Yeast extract-glucose-agar**¹⁸⁶: Test actinomycetes and yeasts employed as test organisms in the determination of
the antimicrobial spectrum of malbranchin were sub-cultured on this medium.

IV. Sabouraud medium: Fungi, which were used as test organisms for antimicrobial assays of malbranchin, were sub-cultured on this medium.

V. Germinating medium: Spores of P. chrysogenum and P. notatum strains were inoculated into this medium and after 48 hrs. incubation at 24°C in shake cultures, the fermentation medium (Medium No. VI) was inoculated with the vegetative growth obtained.

VI. Corn-steep liquor—lactose: Corn-steep liquor (solids) 15.0 g.
Sodium sulphate 0.5 g.
Lactose 25.0 g.
Glucose 10.0 g.
Calcium carbonate 1.0 g.
Distilled water 1000 ml.
ph — 4.2 before sterilization.

VII. Modified Soltero-Johnson medium: Salt mixture 10 ml.
Ammonium acetate 0.25 g.
Ammonium lactate 2.7 g.
Sodium acetate (anhydrous) 0.1 g.
Urea 0.25 g.
Lactose 2.0 g.
Glucose 0.6 g.
Calcium carbonate 0.3 g.
Distilled water 100 ml.
ph — 6.5
The salt mixture consisted of the following:

Dihydrogen potassium phosphate 6.0 g/litre
Magnesium sulphate (7H₂O) 0.25 g/litre
Zinc sulphate (7H₂O) 0.02 
Ferrous sulphate 0.02 
Manganese sulphate 0.02 
Sodium sulphate 0.50 

VIII. "Minimal sulphur medium":

Salt mixture 10 ml.
Ammonium acetate 0.25 g.
Ammonium lactate 2.70 g.
Sodium acetate (anhydrous) 0.10 g.
Urea 0.25 g.
Calcium carbonate 0.3 g.
Glucose 4.0 g.
Distilled water 100 ml.
pH: 6.5

The salt mixture contained:

Dihydrogen potassium phosphate 6.0 g/litre
Magnesium acetate 0.25 
Zinc chloride 0.02 
Ferric chloride (6H₂O) 0.02 
Manganese chloride (4H₂O) 0.02 

Standard sodium sulphate (4.433 g/100 ml) solution (1 ml) was added to the medium to give the required concentration of sulphur in the medium (100 µg/ml).
e) Bioassays:

(i) **Antimicrobial spectrum of malbranchin** was determined by serial dilution method.

Bacteriostatic activity of malbranchin against various test organisms was determined by serial dilution technique\(^1\), using nutrient broth (medium I without agar) containing 1% potassium phosphate buffer pH 7.6. 0.05 ml (containing \(5 \times 10^6\) organisms) of acclimatized, 18 hr. old culture of the test organism served as an inoculum for 5 ml of broth.

*Mycobacterium phlei* and *M. smegmatis* inocula involved transfer of small pellicles of growth into the assay medium.

Antifungal assays were carried out by the "agar dilution technique" of Waksman and Reilly\(^2\). The inhibition of growth of the test organism was recorded after incubation for 24 hours or longer, at 24\(\degree\)C (fungi - 72 hrs - medium IV), 28\(\degree\)C (actinomycetes - 72 hrs - medium III) and 37\(\degree\)C (yeasts - 24 hrs - medium I containing 1% glucose).

(ii) **Estimation of penicillin**. Penicillin present in culture filtrates of *M. pulchella* was assayed by the method of Schmidt and Moyer\(^3\) since very small amounts of malbranchin in the broths did not materially affect the penicillin values. However, to ensure that the values obtained truly represent penicillin, controls were invariably, simultaneously run after treatment with penicillinase. Malbranchin is unaffected by the enzyme.
(iii) Estimation of malbranchnin: Since malbranchnin occurs in very small quantities, broth filtrates were first concentrated and then assayed. One hundred millilitres of culture filtrate adjusted to pH 7.0 were incubated with penicillinase (1000 units) for 1 hr at 37°C. The solution was cooled to 0°C, acidified to pH 2.0 with 10% phosphoric acid and extracted with ethyl acetate (3 x 20). The ethyl acetate extracts were dried (Na₂SO₄), and evaporated to dryness in vacuo. The residue was dissolved in a known volume (1 to 5 ml) of acetone and the solution assayed by the serial dilution technique using Medium I (without agar) containing 1% potassium phosphate buffer pH 7.6 and Micrococcus pyogenes var. aureus N-15 as the test organism.

(f) Iodometric assay of penicillin:

Alcino's procedure with a slight modification, using sodium phosphate buffer pH 6.3 to ensure maximum stability of penicillin, was adopted. Very dilute solutions of penicillin were concentrated below 0°C in vacuo and assayed. The procedure is as follows: Aliquots (2 ml) were pipetted into glass-stoppered conical flasks (125 ml) and treated with normal sodium hydroxide (2 ml). After exactly six minutes, the excess of alkali was neutralised with phosphoric acid (10%; 1.5 ml) while the flask was cooled. After mixing with iodine (0.01 M; 10 ml), the flask was quickly stoppered and with a water-seal kept at 25°C in the dark for exactly
10 minutes. The stopper was washed and the product along with washings titrated against sodium thiosulphate (0.005N freshly prepared from 0.1N stock solution) using starch indicator (1%). Blanks were run with 2 ml of the solution mixed with sodium phosphate buffer (3.5 ml; pH 6.3, prepared by mixing 2 ml of N sodium hydroxide and 1.5 ml of 10% phosphoric acid) and the iodine solution. After 10 minutes the mixture was titrated as before. The difference in the two titres indicates the consumption of iodine due to penicillin in the test solution. 1 ml of 0.005N thiosulphate = 350 units of benzylpenicillin. Substituting penicillinase (1000 units/ml) for the alkali to inactivate penicillin in the above procedure made no material difference in the penicillin assay values.

g) Fermentations:

(1) *P. notatum* and *P. chrysogenum* strains: Fermentations were carried out as described by Johnson using corn-steep liquor lactose and Soltero and Johnson media.

(ii) *M. pulchella* AB.22: Rode, Foster and Schuhardt used shake cultures of *M. pulchella* ATCC 9989 in corn-steep liquor medium. However fermentations with the strain AB22 under forced aerated conditions at 45° were subject to great variations inspite of every effort made to maintain optimum humidity and other operative conditions. For these reasons the organism was grown in stationary cultures using media.
Nos. VI, VII and VIII. These media were chosen after a study of the fermentative characteristics of the strain (vide p. 54-56). The broths and mycelia of the organism grown in the first two media were examined for formation of penicillin, malbranchin and "iodine reacting compounds". The last medium was used in hot fermentations incorporating labelled sulphur. The routine procedure used during these fermentations is described below.

One hundred millilitres of medium (No. VI or VII or VIII) were distributed in Erlenmeyer flasks (500 ml) and sterilised. The sugars and calcium carbonate were sterilised separately and added to the flasks which were then inoculated with spores of M. pulchella AB 22 (5 ml of spore suspension containing ca. 20,000 spores/ml) harvested from honey-peptone-agar slants. The flasks in triplicate were incubated at 45°C. After the germination was complete (about 48 hours) and the pH was between 6.7 and 6.9, potassium phenylacetate solution (1 ml) was aseptically added at 24 hours intervals (total 4 ml) and the fermentation continued at 45°C. Samples were withdrawn at intervals of 24 hours for determination of pH, mycelial weight, penicillin and malbranchin.

During hot fermentations with medium VIII, carrier-free $^{35}$-sodium sulphate (0.1 to 0.2 mc) was added before sterilisation of the medium. Samples withdrawn in this case
were analysed for pH, penicillin, total and organic sulphur in broths and mycelia. In all six sets of hot fermentations were run and the results of a typical one alone are presented. Although, numerically the values differed somewhat in each run, the general pattern of sulphur distribution in the various fractions remained same.

h) Analytical procedures:

(1) Mycelial weight: The mycelial mat was filtered and washed free from medium and dried over phosphorus pentoxide in a desiccator (in vacuo) for 20 hours and weighed.

(ii) Sulphur:

Broth samples: The clear culture filtrate (2 ml) acidified with hydrochloric acid (2N; 0.25 ml) was mixed with a saturated solution of barium chloride (0.25 ml) followed by sodium sulphate solution (0.02N; 0.5 ml). The mixture was boiled for 5 minutes and centrifuged after cooling. The supernatant was transferred to a standard flask (100 ml). The residue was washed with hydrochloric acid (2N; 2 x 0.5 ml) and the washings separated by centrifugation, added to the previous supernatant. After neutralising the combined supernatant with sodium hydroxide and making up the volume to 100 ml, aliquots (0.1 to 0.2 ml) were plated on aluminium planchets dried for half an hour under an infra-red lamp and counted at infinite thickness to obtain the organic sulphur counts in the broth. Similarly the total counts in the medium were
determined at the start of the fermentation and at the time of sampling but before precipitation with barium chloride. The difference between the two counts obtained before and after precipitation with barium chloride is considered to represent inorganic sulphur present in the broth. Since the specific activity of sulphur present in the medium at the start of fermentation is known (1 ml contains 100 µg of sulphur), the amount of sulphur in different fractions was calculated from the counts obtained. Further, the difference between the sulphur counts added to the medium at the start of fermentation and the total counts present at any given time of fermentation represents the sulphur incorporated into the mycelium and the decrease in the inorganic sulphur in the broth corresponds to the sulphur utilised by the mould.

(iii) Extractable sulphur from mycelium:

(a) The procedure described by Tardrew and Johnson was adopted for this purpose. The separated mycelium was washed with distilled water, pressed dry between folds of filter paper and weighed. It was then extracted with aqueous ethanol (70%) by keeping at -20°C for 24 hours. The mycelium was centrifuged, washed with cold ethanol and the combined supernatant and washings made up to a known volume. The sulphur counts were determined as before. This procedure was adopted when no further fractionation of the constituents of the extracts was carried out.
(b) For fractionation of the different constituents present in the mycelium, the following procedure after Ballio et al.\textsuperscript{158} was employed. The mycelium collected by filtration was washed with cold phosphate buffer (1%, pH 7.0) and stored at -20°C till required. A weighed portion was suspended in ten times its weight of ethanol (75%) and gently boiled on a water bath for 10 minutes with continuous stirring. After cooling, the suspension was centrifuged and the residue extracted twice with 20 ml portions of the ethanol as before. The total ethanolic extracts were concentrated \textit{in vacuo} to remove the solvent and the aqueous solution made up to known volume (5 ml per gram of wet weight of mycelium) and used for fractionation on ion-exchange columns.

(i) \textit{Fractionation of sulphur-containing constituents on ion-exchange resins:}

Culture filtrates and mycelial extracts from hot fermentations were prepared and the sulphur-containing constituents fractionated as described by Ballio et al. for \textit{P. chrysogenum} fermentations\textsuperscript{158}. However, columns (25 x 1.5 cms) packed with Dowex-1 x 8 and Dowex-50 x 8 (200-400 mesh) were employed in the formate\textsuperscript{194} and hydrogen \textsuperscript{195} forms respectively.

(j) \textit{Isolation of Malbrancheins A and B:}

Malbranchein titres of culture filtrates were uniformly
very low and hence mycelium was used for its isolation. The mycelium was separated from fermentation liquor (Medium VI with added phenylacetic acid) after 120 hours and pressed between folds of filter paper. The mat (25 gms) was ground with silica under a layer of acetone (100 ml) at 5°C and the solvent separated by centrifugation. The residue was further extracted with acetone (3 x 30 ml). The clear combined acetone extracts were evaporated in vacuo, the residue washed twice with cold water and dried in vacuo (CaCl₂) for 20 hours. The dry powder thus obtained was stirred with ether (3 x 25 ml), the insoluble material dissolved in ethylacetate (100 ml) and extracted with sodium carbonate (0.2M; 3 x 10 ml). After acidification (pH 2.0) of the aqueous extract with cold phosphoric acid (10%), it was re-extracted with ethylacetate (3 x 5 ml), washed with a little water and dried (Na₂SO₄) overnight at 5°C. On removal of the solvent in vacuo, crude malbranchin (15 mgs) was left behind as a brown amorphous powder.

In preliminary experiments, attempts to purify crude malbranchin by chromatography on charcoal, alumina, Deacidite PF and Dowex-1 columns prepared as described by Abraham⁹⁶ and Batchelor et al.¹²⁰ were unsuccessful.

A slurry of cellulose (Carl Schleicher and Schull – No.123 – chromatography grade) in ether saturated with
k) Isolation of compound B from culture filtrates of M. pulchella:

Culture filtrates (500 ml) of M. pulchella harvested after 120 hours fermentation (Medium VIII with added phenyl-acetic acid) were adjusted to pH 7.0, concentrated in vacuo to 25 ml and loaded on a Dowex-1 x 8 column (formate form, 2 x 30 cms). The substances not retained on the column were washed down with water (200 ml). Fractions eluted with 0.2N formic acid (250 ml) were concentrated to 50 ml in vacuo in a desiccator (over NaOH pellets) and the residual solution extracted with ether (5 x 10 ml). The ethereal extract was dried (Na$_2$SO$_4$) and evaporated under reduced pressure, when compound B was obtained as a brown viscous mass (12.5 mgs). This was dissolved in ether (10 ml) and extracted with 0.2M sodium bicarbonate. The aqueous phase was cooled to 5°C, acidified (pH 2.0) and re-extracted with ether. After drying...
(Na₂SO₄) and removal of the solvent in vacuo, compound B was obtained as a colourless waxy solid (8.75 mgs) melting around 40°C. Although it was homogeneous on paper chromatograms, an analytically pure sample was not obtained.

1) Isolation of Malbranchesterol from mycelium of M. pulchella:

The ethereal extracts (p. 102) from the mycelium were evaporated under reduced pressure. The residue (139.7 mgs) was stirred with a few drops of cold petrol (b.p. 64-68°C) in which the pigments were readily soluble, and the solvent removed by centrifugation. The residue (77.1 mgs) was dissolved in hot methanol. On cooling, the sterol crystallised in colourless plates. On repeated recrystallisation from aqueous acetone the sterol separated in long colourless needles (26.5 mgs).

Sterol acetate: A mixture of the sterol (50 mg), pyridine (1 ml) and acetic anhydride (2 ml) was left at 45°C for 20 hours. The product was poured into ice-water (10 ml), the precipitated acetate filtered and washed with cold, very dilute hydrochloric acid followed by water. It recrystallised from ethanol-water in colourless glistening leaflets (44.2 mgs).

m) Thin layer and paper chromatography:

(i) Resolution of penicillin and malbrachin:

The procedure
of Brewer and Johnson\textsuperscript{197} for separation of the different penicillins was used. However, the paper strips were impregnated with 1\% potassium citrate (pH 5.8). This modification was found necessary to resolve malbranchein from penicillin, both of which were detected by bioautographic technique\textsuperscript{198} on agar plates seeded with \textit{Micrococcus pyogenes} var. \textit{aureus} \textsc{N}-15. No resolution of the two substances occurred on unimpregnated paper chromatograms developed with the following solvent systems:

2. \textit{n}-propanol:water (7:3).

Penicillin and allied substances were detected on paper chromatograms by spraying with iodinated penicillinase and starch–iodide solution as described by Sneath and Collins\textsuperscript{199}.

(ii) \textit{Fractions eluted from ion-exchange columns:} The solvent systems used for separation of the different constituents are given in Table V. Penicillin and allied substances were detected by bioautographic technique\textsuperscript{198} or by spraying with iodinated-penicillinase\textsuperscript{199} starch–iodide solution.

Substances which do not react with penicillinase were detected as described by Thomas using sodium hydroxide–acetic acid–starch–iodine solution\textsuperscript{207}. Other chromogenic reagents used for spraying paper chromatograms are given in Table VI.
### Table V

**Solvent systems used for paper chromatographic separation of different fractions eluted from ion-exchange columns.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Solvent system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sec.butanol:formic acid:water (75:15.5:14.5)</td>
<td>158</td>
</tr>
<tr>
<td>2</td>
<td>n-butanol:ethanol:water (4:1:5 - top layer)</td>
<td>158</td>
</tr>
<tr>
<td>3</td>
<td>n-propanol:water (7:3)</td>
<td>158</td>
</tr>
<tr>
<td>4</td>
<td>n-butanol:acetic acid:water (12:3:5)</td>
<td>130</td>
</tr>
<tr>
<td>5</td>
<td>Ethanol (95%) and n-butanol (90:10), equilibrated with 50 ml of 28% aqueous ammonium sulphate; paper buffered with 10% citrate buffer, pH 5.0.</td>
<td>131</td>
</tr>
<tr>
<td>6</td>
<td>Ether saturated with 28% ammonium sulphate; paper buffered at pH 6.5 (1% citrate).</td>
<td>197</td>
</tr>
<tr>
<td>7</td>
<td>Phenol:water (5:2; v/v) (Ammonia in the tank)</td>
<td>134</td>
</tr>
<tr>
<td>8</td>
<td>Pyridine:water (4:1)</td>
<td>134</td>
</tr>
<tr>
<td>9</td>
<td>M. ammonium acetate, pH 5.8-ethanol (30:75)</td>
<td>158</td>
</tr>
<tr>
<td>10</td>
<td>M. ammonium acetate, pH 7.5 - ethanol (30:75)</td>
<td>158</td>
</tr>
<tr>
<td>11</td>
<td>n-butanol:ethanol:H hydrochloric acid (40:11:19)</td>
<td>158</td>
</tr>
<tr>
<td>12</td>
<td>n-butanol:pyridine:water (1:1:1)</td>
<td>156</td>
</tr>
<tr>
<td>13</td>
<td>Phenol:water (4:1)</td>
<td>200</td>
</tr>
<tr>
<td>14</td>
<td>n-butanol:acetic acid:water (63:10:27)</td>
<td>201</td>
</tr>
<tr>
<td>15</td>
<td>n-butanol:acetic acid:water (100:24:100)</td>
<td>202</td>
</tr>
<tr>
<td>16</td>
<td>amylacetate:acetic acid:water (5:2:5)</td>
<td>203</td>
</tr>
<tr>
<td>17</td>
<td>sec.butanol equilibrated with 0.05 phthalate buffer; pH 6 - organic phase used; paper impregnated with the buffer.</td>
<td>204</td>
</tr>
</tbody>
</table>

... contd.
Table V (continued)

<table>
<thead>
<tr>
<th>No.</th>
<th>Solvent system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.</td>
<td>1.5M sodium phosphate buffer, pH 6, paper impregnated with the same buffer.</td>
<td>205</td>
</tr>
<tr>
<td>19.</td>
<td>phenol:water (5:1; 0.3% ammonia in tank)</td>
<td>206</td>
</tr>
</tbody>
</table>

Table VI

Chromogenic reagents used for spraying paper chromatograms of the fractions eluted from ion exchange columns.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Compound(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Alkaline hydroxylamine-ferric chloride</td>
<td>β-lactams, esters and ester lactones</td>
<td>206</td>
</tr>
<tr>
<td>2. Iodine-potassium iodide</td>
<td>Sulphuryl cholines</td>
<td>209</td>
</tr>
<tr>
<td>3. Ferrocyanide-cobalt chloride</td>
<td>-do-</td>
<td>210</td>
</tr>
<tr>
<td>4. Barium chloride-sodium rhodinate</td>
<td>Inorganic sulphate</td>
<td>211</td>
</tr>
<tr>
<td>5. Chloroplatinic-iodide</td>
<td>Sulphur containing amino acids.</td>
<td>212</td>
</tr>
<tr>
<td>6. Iodo-palladate</td>
<td>-do-</td>
<td>213</td>
</tr>
<tr>
<td>7. Ninhydrin</td>
<td>Amino acids</td>
<td>214,215</td>
</tr>
</tbody>
</table>
(iii) **Chromatography of sterol fractions:**

The crude ether extracts and the isolated sterol were examined by paper chromatography using Saffarardi solvent systems.\(^{216}\) Paper strips (Whatman No.1) were impregnated with propylene glycol as described by Baker et al.\(^ {217}\) Chloroform solutions of the samples along with ergosterol for reference were spotted and the chromatograms developed by the descending technique in the dark. The sterols were detected by spraying with a solution (10%) of antimony trichloride in chloroform.\(^ {218}\)

Thin layer chromatographic technique was used to check the homogeneity of the sterol isolated from *M. pulchella* mycelium as well as to detect accompanying sterols in the crude ether extracts. Plates (20 x 20 cms) mechanically coated with silica gel \(^ {9}\) (E. Merck, Darmstadt) were prepared as described by Stahl.\(^ {219}\) The crude ether extracts and solution of sterol fractions in chloroform were spotted and the plates developed in the dark with the solvent systems mentioned in Table XXXVI. Ergosterol was used as a reference standard. The sterols were detected by spraying with anisaldehyde-sulphuric acid reagent and heating at 90° for 5 minutes.\(^ {218}\)

n) **Electrophoresis:**

1. **Dinitrophenyl-peptides (DNP-peptides) and dinitrophenyl-amino acids (DNP-amino acids):**

DNP-peptides and DNP-amino acids (vide p. 81) along with
reference compounds (DNP-glutamic and DNP-DL-\(\alpha\)-aminoadipic acids) were resolved electrophoretically on Whatman No.3 paper in 0.08M pyridine-acetic acid buffer, pH 5.0, at 250 volts/cm during 5 hours.

(ii) Inorganic sulphate:

To confirm the presence of inorganic labelled sulphate, the fractions along with [reference standard (\(3^{35}\) labelled sodium sulphate) were subjected to electrophoresis at 25\(^{\circ}\)C in 0.1M citrate buffer, pH 5.5 at 250 volts/cm for 5 hours. The spot corresponding to the sulphate was eluted and radioactivity counts determined. Further, the spot was detected on paper by spraying with barium chloride-sodium rhodizonate solutions\(^{211}\).

c) Toxicity of malbrachin A:

Freshly prepared sterile solutions of malbrachins A in 0.1M sodium carbonate (pH 9.0), were given intraperitoneally to experimental animals.

Experimental animals: Albino mice of Haffkine strain reared in these laboratories were fed the stock diet\(^{221}\) (supplemented with cod-liver oil and salt mixture\(^{222}\) ad \(\textit{libitum}\) along with water.

The general design of the experiment was in accordance with the suggestions made by Allmark\(^{223}\) and Swoap\(^{224}\) for carrying out toxicity tests. Healthy unstarved mice, six to eight weeks
old were grouped into sets of six animals of nearly the same age and drawn as far as practicable from the same litter. They were housed in independent cages after segregating the sexes. Test substance was administered intraperitoneally by injection with a tuberculin syringe of 1 ml capacity graduated into 0.01 ml and fitted with a 1/2 - 5/8 inch 24-26 gauge needle. Apart from the effect of the diluent medium, other factors such as volume-concentration and volume-time relationship have been found to influence toxicological data. These were kept constant as far as possible. Each animal was injected with the required dose of the drug solution (calculated separately on the basis of its body weight) or equivalent volume of the vehicle in case of the control animal. Four hours before the test, food was withdrawn and after the treatment the animals were placed in respective groups. They were kept under observation for twenty days for any possible toxic reactions and a record of their weights was maintained. Autopsy was performed after a week to ascertain any tissue damage.

Summary

Chemical, microbiological techniques, radio-tracer technique, chromatography, electrophoresis and other procedures used in these studies have been described.