APPENDIX

A. Method: clotting time and prothrombin time.

B. Estimation of hydroxyproline and hexosamine

C. Criteria for histological evaluation.

D. Staining Technique.

E. Calculation of WC^ by Litchfield and Wilcoxon method - Working scheme.

F. Drug directory.
APPENDIX - A

PROTHROMBIN - TIME

METHOD: Brain thromboplastin Kit Method, (ortho).

(Quick's Method as described by Varley et al (130)

PROCEDURE:

1. Collect 1.6 ml blood in 0.4 cc 3.13% Sodium citrate and mix.
2. Centrifuge to separate plasma.
3. Prepare a thromboplastin suspension by adding 5 c.c. distilled water and keep at 37°C for 30 mts.
4. In water bath kept at 37°C place small glass tube add 0.1 c.c. plasma after 2 mts. Add 0.2 c.c. of thromboplastin suspension.
5. Start a stop watch after 10 seconds take out the tube from the bath and tilt the tube till the plasma clots. Note this time.

CLOTTING TIME: (Wright's Method) as described by Ranade et al. (124)

APPARATUS: Capillary tubes. Stop watch.

METHOD: Inflict a deep cutting wound to ensure flow of blood. Collect the blood into the capillary tube from the bleeding point. Note the time.

Break the capillary tube at the intervals of 10 seconds, time taken for the appearance of fibre between two ends of broken capillary is noted.
B. ESTIMATION OF HYDROXYPROLINE (NEUMAN & LOGAN) AND HEXOSMINE (ELSON & MORGAN)

(I). TISSUE HYDROLYSIS:

Granuloma were dried at 60°C for 24 hrs. and weighed. Tissues were hydrolysed with 6N HCl at 110°C for 24 hrs., kept in a sealed tubes.

Hydrolysate was neutralized: volume measured. From this solution aliquots, were taken for estimation of hydroxyproline as measure of total collagen of the tissue.

(II) STEPS INVOLVED IN ESTIMATION OF HYDROXYPROLINE:

(a) To test solution:

1 ml of 2NaOH, 0.01 m copper sulphate solution (shake on adding) and 6% Hydrogen peroxide (shake on adding) were added. Immediately kept at 80°C for 16 mts. Cooled for 5 minutes.

(b) Freshly prepared 2 ml 5% para-dimethyl benzaldehyde in N-propanol was added.

(c) 4 ml of 3 N-Sulphuric acid was added and kept at 80°C for 15 mts. Cooled for 5 minutes.

(III) With known strength of standard solution - Estimation of hydroxyproline was done at 540 nm. (Spectronic 21: Bush - Lamb).
ESTIMATION OF HEXOSAMINE:

1) Hydrolysate was neutralized with 3 NaOH: diluted in the ratio of 1:9

2) 1 ml of acetylacetone was mixed and kept in boiling water for 15 minutes.

3) Cooled: 95% ethanol was added.

4) 1 ml of paradimethyl bezaldehyde in conc. Hydrochloric acid and diluted with 95% Alcohol used as colorizing agent: kept for 30 minutes.

5) Hexosamine was estimated at 543 nm.
C. **HISTOLOGICAL FEATURES**:

Criteria followed for tissue cellular evaluation in 11 days old granulomas are:

1. **FIBROBLAST** - Young / matured, pattern of arrangement - compact / loose.

2. **COLLAGEN** - Plenty / Scanty: organised / disorganised.

3. **INFLAMMATORY CELL INFILTRATION** - Represented +/2+

4. **VASCULARITY** - Any specific feature observed.

5. **FIBROSIS** -
   - early phase
   - Advanced.
D. STAINING METHODS:

VAN-GIESEN'S STAIN FOR COLLAGEN FIBRES

Fixation : Formalin.

Technique: Paraffin, cut sections at 6 microns.

Solutions:

Weigert's Iron Hematoxylin.

**SOLUTION - A**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>HEMATOXYLIN</td>
<td>1.0gm</td>
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<tr>
<td>Absolute alcohol</td>
<td>100.0cc</td>
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</table>

**SOLUTION B**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>29% ferric chloride</td>
<td>4.0 cc</td>
</tr>
<tr>
<td>Distilled water</td>
<td>95.0 cc</td>
</tr>
<tr>
<td>Hydrochloric acid, concentrated</td>
<td>1.0 cc</td>
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</tbody>
</table>

Working solution

Equal parts of solution A and solution B

**VAN GIESEN'S SOLUTION**

Acid fuchsin, 1% aqueous solution 2.5 cc
Picric acid, saturated aqueous solution 97.5 cc

Staining Procedure:

1. Xylene
2. Absolute alcohol
3. 95% alcohol
4. Rinse in distilled water.
5. Stain in Weigert's hematoxylin solution for 10 minutes.
6. Wash in distilled water.
7. Counterstain in van Gieson's solution for 1 to 3 minutes.
8. 95% alcohol.
9. Absolute alcohol - 2 changes.
10. Xylene - 2 changes.
11. Mount in D.P.X. add 3 drops of saturated alcoholic picric acid to each 50 cc of xylene used in clearing. Mount from acidified xylene. This intensified the background and prevents sections from fading.

RESULTS:
Collagen - red
Muscle, cornified epithelium - yellow
Nuclei - blue to black
Running water will remove van Gieson's solution.
Solution B will remove Weigert's hematoxylin.

MASSON'S TRICHROME STAIN.
Fixation: Bouin's or formalin. Mordant section of formalin fixed material in Bouin's fluid for one hour at 56°C or overnight at room temperature.
Technique: Parafin, cut sections at 6 microns.
Solutions:
Bouins: Solution
Picric acid saturated aqueous solution 75.0 cc
Formaldehyde, 37-40% 25.0 cc
Glacial acetic acid 5.0 cc
WEIGER'S IRON HEMATOXYLIN:

Solution A and B and working solution as in Van Gieson's stain for Biebrich Scarlet - Acid Fuchsin solution

Biebrich Scarlet, aqueous 1% 90.0 cc
Acid fuchsin, aqueous 1% 10.0 cc
Glacial acetic acid 1.0 cc

Phosphomolybdate - Phosphotungstic Acid solution

Phosphomolybdate acid 5.0 gm
Phosphotungstic acid 5.0 gm
Distilled water 200.0 cc

ANILINE BLUE SOLUTION:

Aniline blue 2.5 gm
Acetic acid 2.0 cc
Distilled water 100.0 cc

Light Green Solution

Light green solution
Light green 5.0 cc
Distilled water 250.0 cc
Glacial acetic acid 2.0 cc

Heat water, dissolve light green, cool filter and add acid.

1% ACETIC WATER SOLUTION:

Glacial acetic acid 1.0 cc
Distilled water 100.0 cc

Staining Procedure:
1. Xylene.

2. Absolute alcohol

3. 95% alcohol

4. Rinse in distilled water

5. Mordant in Boun's fixative for 1 hour at 56°C, or overnight at room temperature.

6. Cool and wash in running water until yellow colour disappears.

7. Rinse in distilled water.

8. Weigrt's iron hemtoxylin solution for 10 minutes.

9. Rinse in distilled water.

10. Biebrich scarlet - acid fuchsin solution for 15 minutes save solution.

11. Rinse in distilled water.

12. Phosphomolybdic acid-phosphotungstic acid solution for 10 to 15 minutes before aniline blue solution. Aqueous phosphotungstic acid 5% for 15 minutes before light green counterstain. Discard solution.

13. Aniline blue solution for 5 to 10 minutes or light green solution for 1 minute. Save solution.

14. Rinse in distilled water.

15. Acetic water 1% for 3 to 5 minutes Discard solution.

16. Alcohol, 95%

17. Absolute alcohol-3 changes.

18. Xylene - 2 changes.

19. Mount in D.P.X.

RESULTS:

Nuclei - black.

Cytoplasm, keratin, muscle fibers, intercellular fibres - red.

Collagen - blue.
**E. J. T. LITCHFIELD JR. AND F. WILCOXON: METHOD FOR ESTIMATION**

We$_{50}$ - Working scheme: For control:

- $K =$ the number of days plotted
- $n = K - 2$ : degree of freedom . . . for $(\chi)_2^2$

<table>
<thead>
<tr>
<th>DAYS</th>
<th>EXPECTED VALUE</th>
<th>OBSERVED VALUE</th>
<th>DIFFERENCE</th>
<th>CONTRIBUTION TO $(\chi)_2^2$</th>
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<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>35</td>
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<td>0.045</td>
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<tr>
<td>7</td>
<td>54</td>
<td>32</td>
<td>2</td>
<td>0.20</td>
</tr>
<tr>
<td>9</td>
<td>69</td>
<td>61</td>
<td>8</td>
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<tr>
<td>11</td>
<td>77</td>
<td>81</td>
<td>4</td>
<td>0.010</td>
</tr>
<tr>
<td>13</td>
<td>87</td>
<td>90</td>
<td>3</td>
<td>0.007</td>
</tr>
<tr>
<td>15</td>
<td>91</td>
<td>93</td>
<td>2</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Total of the contributions to $(\chi)_2^2$ 0.297 x 1

multiplied by $n/k$ : to get $(\chi)_2^2$ of the line.

For degree of freedom tabled $X^2$ is higher than the value obtained, the best fit line. The points are not heterogenous.

We$_{16}$, We$_{50}$, We$_{84}$ are deduced to calculate the slope function-$S$

\[
S = \frac{\text{We}_{84}/\text{We}_{50} + \text{We}_{50}/\text{We}_{16}}{2}
\]

From 'S' value FED$_{50}$ is calculated. From which confidence limits for We$_{50}$ has been deduced as

- \[ \text{We}_{50} \times \text{FED}_{50} = \text{upper} \]
- \[ \text{We}_{50} / \text{FED}_{50} = \text{lower} \]

Limits for probability.

Mean of We$_{50}$ is calculated based on this formula:

\[
\text{We}_{50} = \frac{L \cdot \text{S} \cdot 13 - \sigma^2 \cdot 13}{2n}
\]
<table>
<thead>
<tr>
<th>NAME</th>
<th>MANUFACTURED BY</th>
</tr>
</thead>
</table>
| HEPARIN | Biological E. Ltd.  
| | Das Chambers, 25, Dalal Street. BOMBAY - 400 001. |
| WARFARIN | Unichem Laboratories Ltd., Unichem Bhavan, S.V. Road, BOMBAY - 400 026. |
| BOTROPASE (R) | Juggat Pharma Private Ltd., 7. Harris Road, BANGALORE - 560 046. |
| (Reptilase, Hemocoagulant) | |
| Batroxobin | |
| MENADIONE | Searle (India) Ltd., Ceat Mhal., 463, De. A.B. Road, BOMBAY - 400 025. |
| THROMBIN | Rat Plasma Thrombin T - 5772  
| | Lyophilized Powder SIGMA - U.S.A. |
| FIBRIN |  
| | F 5760  
| | Bovine Washed. SIGMA - U.S.A. |

W.H.O. approved name (R).