'VADRINE' (S.131) ASSESSED BACTERIOLOGICALLY IN THE TREATMENT OF EXPERIMENTAL MURINE LEPROSY

H. BRODHAGE, M.D.

The Institute of Pathology and Bacteriology, Cantonal General Hospital, Lucerne, Switzerland.

The tuberculostatic action of the p-amino-salicylate of 2-pyridyl-(4)-1,3,4-oxdiazolone-(5) ('Vadrine'—Research Number of Edward Geistlich Sons Ltd., Wolhusen 'S. 131') has been described by Wilder Smith, (1954) and by Brodhage and Wilder Smith, (1955). The *in vitro* efficiency of 'Vadrine' against the Tb. strain H 37 Rv is within 1:2,560,000. In attempts to produce *in vitro* resistant variants it was observed after 18 sub-cultures that resistance of the tested strain H 37 Rv to 'Vadrine' had in no way increased (Brodhage and Wilder Smith, 1955; Brodhage, 1955). This fact, coupled with the slight toxicity, seemed sufficient justification to test the effect of 'Vadrine' on leprosy. To this end, using 4:4′diaminodiphenyl-sulphone (DDS) as a control, we examined the effect of 'Vadrine' in the treatment of experimental leprosy in the rat.

In view of the impossibility of transferring human leprosy to suitable test animals, murine leprosy is the best experimental infection on which new substances intended for the treatment of human leprosy can be tested (Carpenter, 1951). A final assessment on the antileprous effect of a substance can of course only be made on the results obtained in treating leprosy patients. Bearing in mind these limitations we give the following account of the bacteriological results of the animal tests we carried out.

Material and Method

Forty-five white rats (weight about 100 g.) were infected intra-testicularly with a suspension of *Myc. leprae murium*, strain 'Wells'. (This strain was kindly put at our disposal by Dr. G. R. F. Hilson, St. George's Hospital, London).

The infection dose was about 0.02 ml. (about 250,000,000 bacilli) in physiological saline solution + 5% bovine albumin fraction V (pH = 7.0). The animals were divided into three groups (each containing 15 rats) receiving either no treatment or treatment with DDS or 'Vadrine'. A further 15 animals were intratesticularly infected with the same dose of heat treated (5 mins. at 100° C.) murine leprosy bacilli.

The substances to be tested were mixed in their pure state with the rat food, in the amount of 0.2% DDS and 1% 'Vadrine' that is based on the relative dosage of DDS in leprosy and of 'Vadrine' in tuberculosis. Treatment commenced 21 days after infection. At the end of one year the test animals were killed.

The efficiency of the tested substances was judged on the basis of the following criteria:

1. Size of the testis (length, breadth and depth) and the spleen (length and breadth).
2. Bacterial index of tissue suspension of lung, liver and spleen according to the following schedule:

Bacterial Index	*Number of Bacteria*	*Number of Fields**
5	more than 100	1
4	10 —100	1
3	1 — 10	1
2	1 — 10	10
1	1 — 10	100
0	none	100

* Oil immersion and x 10 binocular.

3. Number of bacteria per ml. testis homogenate. The method chosen was the Hilson (1956) modification (Hilson and Elek) of the technique devised by Hobby et al (1954).

1. Preparation of stained slides.

(*a*) A suitable dilution of homogenate is required to provide, when the technique has been completed, an average of between 5 and 15 bacilli per counting area under the microscope. The diluting fluid must be proteinous in nature, and formalin-milk is used. A 25-ml. screw-cap universal container is filled with milk and spun in an ordinary laboratory centrifuge at 2,500–3,000 r.p.m. for 15 mins. to bring the cream to the top. A Pasteur pipette is introduced into the subnatant milk and the latter drawn off leaving the cream behind. 10 ml. of this defatted milk and 1.5 ml. formalin (40% HCHO in water) are brought to 100 ml. with distilled water and thoroughly mixed. This constitutes the diluting fluid: if one has no preliminary idea of the likely bacillary concentration, it may be convenient to make up 1:10, 1:100 and 1:1,000 dilutions of the homogenate. The dilutions are well shaken to produce even mixing.

(*b*) An 'Agla' micrometer syringe (Burroughs Wellcome) is fitted with a needle (Record fitting) slightly larger than that usually supplied with the syringe with the pointed end ground square. The larger bore avoids blocking with minute tissue fragments and the square end allows better control of the small drops which are formed at the tip.

(*c*) The syringe, detached from the micrometer holder, is filled and emptied twice with the dilution and finally filled with about 1cm. length of fluid. It is then fitted into the micrometer holder and the handle turned to expel a few drops and to set it to the zero mark, and the end of the needle is touched inside the fluid container to

detach the last drop. The black screw on the holder is tightened a little to provide a moderate braking action on the handle.

(*d*) Slides are cleaned and flamed in the usual way to render them grease-free. One slide is used for each dilution. The micrometer handle is turned so as to extrude 1 microlitre of fluid (one complete turn of the handle extrudes 0.01 ml.; turning it through only 5 of the smallest divisions on the handle delivers 1 microlitre); this is touched on to the corner of the slide to set up standard conditions at the needle tip and this drop is not considered further. Five more drops are then placed at the centre of the slide, in a row of 3 and a row of 2, being set just far enough apart to avoid their running into each other. Without delay the slide is placed on a piece of white paper carrying an ink outline of a square of side 0.5 cm. Each drop in turn is brought over the square and spread to the same area with the end of a straight platinum wire (the operations of placing the drops on the slide and spreading them must be done quickly to prevent irregular distribution of bacilli due to drying). The slide is then placed on the levelled lid of a boiling waterbath for about 1–2 mins. to dry and fix the films. The slide is taken off, allowed to cool and flooded with phenol-gelatin solution which consists of 0.5% gelatin and 0.5% phenol in distilled water. The solution is then poured off the slide which is allowed to drain for 2 or 3 seconds and then placed on the water-bath lid again for 2 minutes. The slide is then held in formalin vapour for 3–5 mins, placed on the lid again to drive off formaldehyde vapour and stained by Ziehl-Neelsen method. Naked-eye examination should confirm that the centre of each square film is thicker and more deeply counter-stained (counter-staining with Loeffler 1:5 for 30 seconds should be very light) than the periphery; if the thickest part is deviated away from the centre, there may be an incompletely homogenized particle causing distortion or the water-bath lid may not be level. Inaccuracy will follow. A deeply-stained ring within a square indicates that too much drying occurred before the drop was spread to its square form; a lightly-stained ring is often found on warm dry days and is not a serious cause of error.

(*e*) Ten fields are counted across the equator of each drop in the following way. The lower, or nearer, edge of the drop is found under the microscope (oil-immersion 2 mm. objective) and then, using the stage micrometer, the stage is moved down, or nearer by 2½ mm. which brings the field on to the equator of the drop. The slide is then moved right or left until the edge of the drop is again found and the moving stage adjusted if necessary so that the stage micrometer registering lateral movement is set at a division of exactly midway between two divisions and the microscope field is just inside the spot edge. The bacilli in this field are counted and then those in 9 other fields, found by traversing the stage by ½ mm. intervals and the average count per field calculated. The last field should be just

within the opposite edge of the drop; if the latter has been spread rather small, it may lie outside and the average of the 9 fields counted should be taken. The counts increase, of course, as the thicker centre is approached and diminish again towards the opposite edge. The "grand average" count per field for all 5 drops is determined and the numbers of bacilli per ml. of dilution are found by the calibration factors set out below.

2. Calibration of microscopes and calculations

Bacilli may be counted in the whole circular field (Hilson, 1956; Hilson and Elek) within the area of an eyepiece graticule or using a square eyepiece mask as in reticulocyte estimations. Whichever is chosen, it must be calibrated, most conveniently by examining the smallest square of a Neubauer (or other) haemacytometer chamber with the same oil-immersion lens as is to be used for the counting. To visualise the lines, the haemacytometer ruled area is not covered with a cover-slip, the lens is used dry and the microscope condenser is racked well down. Hilson found with his objective and × 5 binocular eyepieces that the field corresponded to 4 smallest squares (1/100 sq. mm.).

Calculation: 1/1000 ml. of material is spread over an area of 25 sq. mm. giving an average volume of 1/25,000 ml. per sq. mm. Each field has an area of 1/100 sq. mm. giving the volume per field as 1/2,500,000 ml. Therefore the numbers of bacilli per ml. of dilution examined (N) = average count per field (n) × 2.5 × 10^6. Obviously this must be multiplied by the dilution factor to arrive at the numbers of bacilli in the original homogenate.

(Note again, that for maximum accuracy n should be between 5 and 15).

Specimen: Counts: Drop 1: 4, 2, 4, 12, 13, 10, 15, 10, 8, 4; mean 8.2
Drop 2: 0, 3, 9, 17, 15, 13, 11, 12, 3, 1; mean 8.4
Drop 3: 1, 6, 2, 15, 14, 14, 14, 8, 2, 1; mean 7.7
Drop 4: 2, 3, 5, 14, 12, 12, 10, 3, 0, 1; mean 6.2
Drop 5: 5, 8, 7, 11, 5, 12, 12, 9, 5, 2; mean 7.6

mean 7.6

Dilution of homogenate: 1:30;

Bacilli/ml. of homogenate = 7.6 × 2.5 × 30 × 10^6
= 570 million.

It should be mentioned here that with the technique applied, no distinction is made between living and non-living bacilli. The number of bacteria found and calculated are derived from those micro-organisms that were stainable (Hanks, 1951; Hobby et al, 1954).

Test Group	No. of animals	Testis (cm.)			Spleen (cm.)		Bacterial Index				Bacteria per ml. testis homogenate	*Ratio
		Length	Breadth	Depth	Length	Breadth	Lung	Liver	Spleen	Mean		
(1) Controls untreated ...	15	2.2	1.2	0.8	4.6	1.1	1.3	1.3	1.4	1.33	1,304,885,000,000	0
(2) DDS 0.2 % in the food ...	13	2.0	1.1	0.8	5.5	1.3	0.6	0.1	0.4	0.37	19,939,980,800	65
(3) 'Vadrine' 1.0% in the food ...	10	1.6	0.8	0.5	3.9	0.8	0.2	0.2	0.3	0.23	1,631,000,000	800
(4) Controls infected with heat-treated bacilli (5 mins. at 100° C.) ...	7	1.7	0.9	0.6	4.1	0.7	0.4	0.8	0.4	0.53	2,257,643,000	578

$$* = \frac{\text{No. of Bacteria per ml. Testis Homogenate Control Group (1)}}{\text{No. of Bacteria per ml. Testis Homogenate Test Group 2, 3 or 4}}$$

Results

The results obtained in the animal tests are summarized in the table. These are expressed in simple average figures which were calculated for the individual test groups.

Fifteen rats died from non-specific infections during the course of the experiment.

It is shown in the summarized average figures that DDS and 'Vadrine', which were both mixed as pure substances with the food, exert a marked therapeutic effect on experimental leprosy in the rat. This is revealed in the average size of the testis and the spleen with the exception that the spleen in the animals under DDS is larger than that of the untreated controls. The bacterial index, calculated from the number of bacteria present in tissue suspensions of lung, liver and spleen, shows an average total degree of infection in the untreated controls of 1.33 as compared to 0.37 in the DDS, 0.23 in the 'Vadrine' and 0.53 in the group infected with heat treated bacilli.

A comparison of the average number of bacteria per ml. testis homogenate clearly reveals the effect of DDS and 'Vadrine'. With an infection dose of about 250,000,000 bacilli, the calculated number of bacteria per ml. testis homogenate indicates that neither DDS nor 'Vadrine' are able to destroy the murine leprosy bacilli. Both substances, however, inhibit the increase of the micro-organism to a marked degree as is demonstrated by a comparison with the untreated control group. With the test series as arranged by us, and the dosage chosen, 'Vadrine' has proved to be more efficient than DDS.

Hobby et al, (1954) also observed that in the tissue suspensions of animals (lung, liver and spleen), bacteria could be demonstrated in the animals which had been infected with heat treated suspensions *Myc. leprae murium*. The question of whether this is due to the transport of non-living micro-organisms of these organs or to the fact that heating for 5 mins. at 100° C. is inadequate to destroy all the bacteria is left open by the authors. According to the findings reported by Naguib et al, (1956), the latter possibility is the more probable. In tests carried out by these authors (intracorneal injection in the mouse) even suspensions of *Myc. leprae murium* heated to 60° C. for 120 mins. caused typical lesions although only after a prolonged latent period of up to eight months.

Elek and Hilson (1956) assume that non-living lepra bacilli are taken up by tissue phagocytes and remain demonstrable by staining for long periods. Four months after intraperitoneal infection of white rats these authors were unable to distinguish any difference (number of bacteria, histological changes) between the animals which had been infected with living murine leprosy bacilli and those infected with heat treated bacilli. Our own results, i.e., the number of bacilli per ml. testis homogenate in the group infected with heat treated organisms is significantly lower than that of the control group,

although it is larger than should be assumed on the basis of the amount injected. Provided the technique of our heat treatment is correct, it must be assumed that this treatment did not destroy all the bacilli. This may have permitted a certain proliferation of bacilli. It is expressed by a larger number of bacteria per ml. testis homogenate compared to the injection amount. It would appear therefore that our observations support the opinion of Naguib and his associates.

Summary

The treatment of experimental murine leprosy in the rat with 4:4 'diaminodiphenyl-sulphone (DDS) and p-amino-salicylate of 2-pyridyl (4)-1,3,4-oxdiazolone-(5) ('Vadrine') under our test conditions and with the chosen dosage reveals a marked therapeutic effect of both substances. 'Vadrine' would appear to be more efficient than DDS. The test results obtained in animals infected with heat-treated suspensions (5 mins. at 100° C.) of murine leprosy bacilli give rise to the suspicion that either our technique for the heat-treatment was incorrect or that this treatment is insufficient to destroy all the bacilli.

Acknowledgements

I should like to thank Dr. G. R. F. Hilson, London, for making available to me the murine leprosy strain "Wells" and for his most helpful suggestions; Dr. W. H. Jopling, Jordan Hospital, Earlswood, for his proof-reading and Miss Trudi Zumstein for her excellent technical assistance.

Also my grateful acknowledgement to the firm of Edward Geistlich Sons, Ltd., Wolhusen (Switzerland) for putting the necessary test substances at my disposal.

REFERENCES

1. BRODHAGE, H. and WILDER SMITH, A. E. (1955) Brit. J. Tuberc., **49,** 185.
2. BRODHAGE, H. (1955) Lancet, **1,** 570.
3. CARPENTER CH. M. (1951) Ann. New York Acad. Sci., **54,** 101.
4. ELEK, S. D. and HILSON, G. R. F. (1956) Brit. J. Path. Bact., **72,** 427.
5. HANKS, J. H. (1951) Ann. New York Acad. Sci., **54,** 12.
6. HILSON, G. R. F. (1956) personal communication.
7. HILSON, G. R. F. and ELEK, S. D. Internat. J. Leprosy (in press).
8. HOBBY, G. L., HANKS, J. H., DONIKIAN, M. A. and BACKERMANN, T. (1954) Amer. Rev. Tuberc., **69,** 173.
9. NAGUIB, M., REES, R. J. W. and ROBSON, J. M. (1956) Brit. J. Path. Bact. **71,** 409.
10. WILDER SMITH, A. E. (1954) Science, **119,** 514.