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EXPERIMENTAL TUBERCULOUS INFECTIONS OF THE CORNEA OF THE MOUSE: A SCREENING TEST FOR ANTI-TUBERCULOUS SUBSTANCES

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The need for a reliable and rapid *in vivo* test for screening compounds for anti-tuberculous activity remains as great as ever. It was in the hope of developing such a test that the investigation into the possibility of producing experimental tuberculous corneal lesions in the rabbit (Robson, 1944; Gardiner, Rees, and Robson, 1949) was started. The corneal method has now been extended to the mouse. While still retaining the advantage of allowing direct observation of the lesions throughout the experiment, this newer method has the advantage that is offered by a smaller animal—economy of drug, or for the same amount of drug a larger number of test animals. The results obtained by the method with streptomycin, *p*-aminosalicylic acid, a diaminodiphenylsulphone derivative, and combined streptomycin and *p*-aminosalicylic acid are also described. They are sufficiently encouraging to suggest that the method may have considerable value for the preliminary testing *in vivo* of compounds with promising *in vitro* activity.

METHOD

Female albino mice, 18–25 g. in weight, were used. Although tuberculous corneal lesions were produced in mice both with a bovine and a human strain (H37Rv) of *Mycobact. tuberculosis* (Table I) the bovine strain was used throughout the present experiments. The strain was the same as that used in the earlier work on intracorneal infections in rabbits (Robson, 1944, and Gardiner *et al.*, 1949). The strain is as sensitive both to streptomycin and to sodium *p*-aminosalicylate as the standard strain, H37Rv.

A seven-day culture in a 'Tween'-80-Albumen medium was centrifuged, resuspended in Tween-saline, and adjusted by means of a photoelectric absorptiometer to contain 0.1 mg. (dry weight) of organisms per ml. Inocula of various sizes, containing approximately 100 to 10,000 tubercle bacilli, were tried (Table I) before finally choosing an inoculum containing 1,000 organisms which was found to be the minimum dose capable of producing consistent tuberculous lesions. It is of interest that the mouse requires a larger inoculum than the rabbit (1,000 as against 300 organisms) to produce an active tuberculous corneal infection in 100 per cent of animals. As in the rabbit the intracorneal injections were made with a tuberculin type syringe and a fine short bevelled needle (0.30 or 0.35 mm. × $\frac{1}{2}$ in; obtainable from the Holborn Surgical Instrument Co.). Deep anaesthesia is essential, but it was found that a sufficiently

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TABLE I

EFFECT OF STRAIN AND SIZE OF INOCULUM ON INCUBATION PERIODS OF TUBERCULOUS LESIONS IN THE CORNEA OF MICE

Inoculum		Number of mice	Mean incubation period (days)
Strain of *M. tuberculosis*	Number of organisms		
Bovine	1,000	56	11.8
Bovine	10,000	14	8.0
Human (H37Rv) ..	1,000	10	11.1

deep anaesthesia with ether alone resulted in a high mortality, and for this reason the mice were given a preliminary dose of α-bromo*iso*valerylurea; this was given about ¼ hour before the inoculation as an aqueous suspension in 6 per cent gum acacia by stomach tube in a dose of 0.4 g. per kg. body weight. A relatively short exposure to ether then gave a safe and deep anaesthesia. Immediately before injection all whiskers were clipped short round the eye. The mouse was held firmly by an assistant and then, by means of fine eye forceps, the eye was fixed and the needle introduced obliquely into the periphery of the cornea by a gentle rotating movement (Plate I). It was found possible, with a good point source of light and a lens placed at a suitable distance between the mouse and the operator, to perfect this intracorneal technique so that some forty animals could be injected in about two hours. One eye only in each animal was inoculated. The injection immediately produced a readily visible opaque bleb (Plate II). The volume of the inoculum was estimated to be about 0.01 ml. Throughout this work all observations on the cornea were made with a binocular dissecting microscope (× 10).

RESULTS

By this technique tuberculous corneal lesions developed in all untreated eyes inoculated. Unlike the rabbit (Gardiner *et al.*, 1949) in which secondary infection of the cornea was never observed, about 5 per cent of mouse corneae showed early acute infections which healed rapidly and very rarely interfered with the subsequent development of tuberculous lesions.

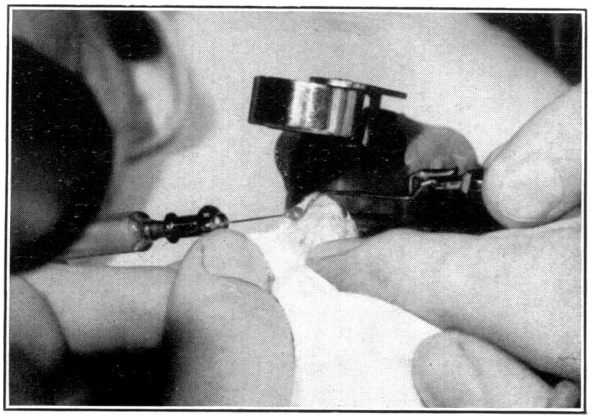
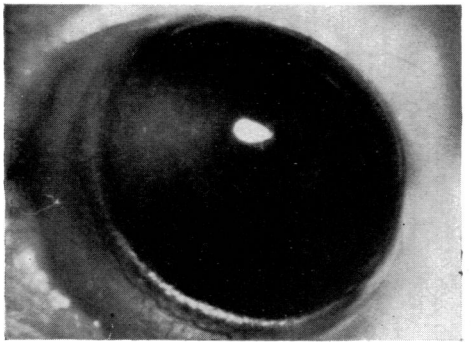


PLATE I.—Technique of inoculation. The mouse, held by an assistant, is shown in the foreground. On the left side is seen the end of the pointer-light, which illuminates the eye. The operator, sitting opposite the assistant, views the eye through the magnifying lens, placed above the eye.



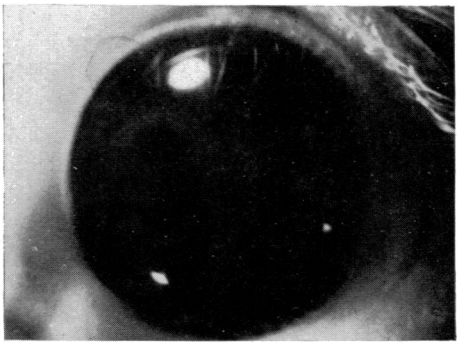


PLATE II.—Opacity produced in the cornea of a mouse, photographed immediately after an intracorneal injection. A normal eye is shown at the same time, for comparison. (× 20.)

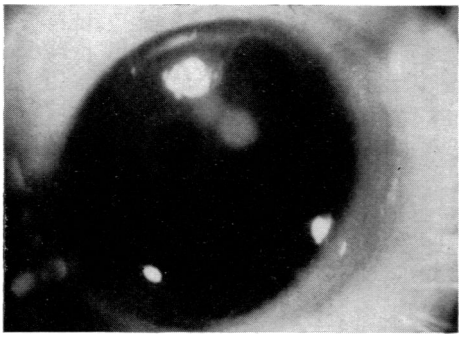


PLATE III.—Appearance of an early, five-day-old, tuberculous corneal lesion (16 days after inoculation). × 20.

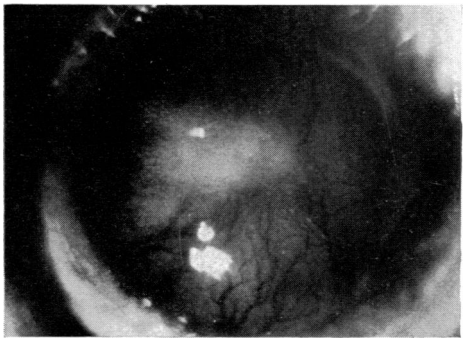


PLATE IV.—A more advanced lesion showing marked vascularization (29 days after inoculation). × 20.

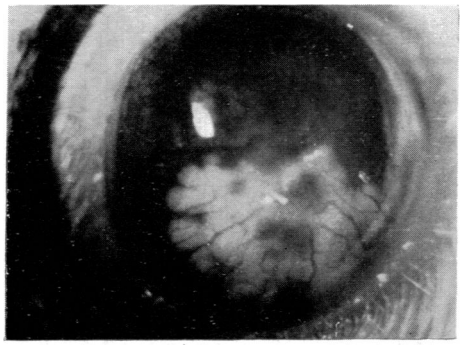


PLATE V.—A chronic corneal lesion, undergoing partial reduction in size, showing characteristic irregularity (65 days after inoculation). × 20.

After an incubation period of about twelve days, the primary lesion, usually single but sometimes multiple, first appears as a minute opaque focus just visible under the dissecting microscope (Plate III) and rapidly increases in size in a few days to become visible to the naked eye. By the 30th day the lesion has still further increased in size, become much denser and obviously caseous in appearance with vessels streaming in from the limbus to form, quite frequently, a well marked pannus (Plate IV). Occasionally the corneal lesion is associated with a hypopyon, but, in contrast to the rabbit, ulceration is very rare. From about the 30th to the 50th day the majority of lesions undergo slow regression involving disappearance of the peripheral oedema, decrease in vascularization, and decrease in size; during this period the lesion characteristically becomes broken up into smaller foci (Plate V). This process of healing is very slow and was never complete even in lesions followed for periods of 100 days; usually a reduction in the size of the lesion by about half occurs.

It is generally recognized that the mouse is more resistant to experimental tuberculosis than the rabbit, and this is very well demonstrated by comparing the corneal tuberculosis in the two species. In the rabbit (Robson, 1944; Gardiner *et al.*, 1949) a smaller inoculum of tubercle bacilli produces a much more acute and rapidly progressive lesion which always terminates in ulceration and ultimately in death of the animal from generalized tuberculosis. In the mouse the ocular lesion is less acutely destructive; it finally undergoes partial regression and the animal never dies from tuberculosis within a period of 100 days. The difference between the development of lesions in the mouse and in the rabbit, as well as the relatively chronic nature of the established lesions in the mouse, can be gauged by comparing Fig. 2 (below) with Fig. 1 in the paper by Gardiner *et al.* (1949).

At an early period in the work it was considered possible that some of the ocular lesions were due, in part at least, to infection of the anterior chamber, particularly as it seemed possible in such a thin tissue as the mouse cornea that some of the inoculum might well reach the anterior chamber directly. To test this, mice were deliberately inoculated directly into the anterior chamber with the same dose of tubercle bacilli. This resulted in early opacity of the anterior chamber followed later by clearing, leaving a characteristic tuberculous iritis—i.e., an entirely different picture from that obtained in mice infected by intracorneal injection.

Quantitative analysis of tuberculous corneal lesions

The development of a lesion is preceded by a latent or incubation period which has proved to be reasonably constant for any particular size of inoculum (Fig. 1) and can therefore be used as a comparative measurement. As in the rabbit (Gardiner *et al.*, 1949) an arbitrary numerical scoring method, denoting size of the corneal lesions, has proved suitable for studying their natural development and serves as another measure (Fig. 2).

Having once established a reliable technique for producing a standard tuberculous corneal lesion in the mouse, characterized by an incubation period and followed by a progressive period, both of which are reasonably constant and readily assessed, it remained to be seen whether such an infection could be used to detect the activity of tuberculostatic compounds.

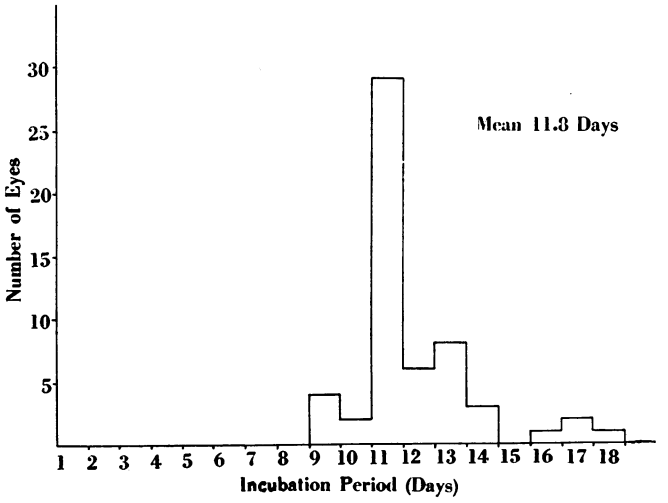


FIG. 1.—Frequency distribution curve of the incubation periods after inoculation of the cornea with approximately 1,000 tubercle bacilli (analysis of 56 eyes).

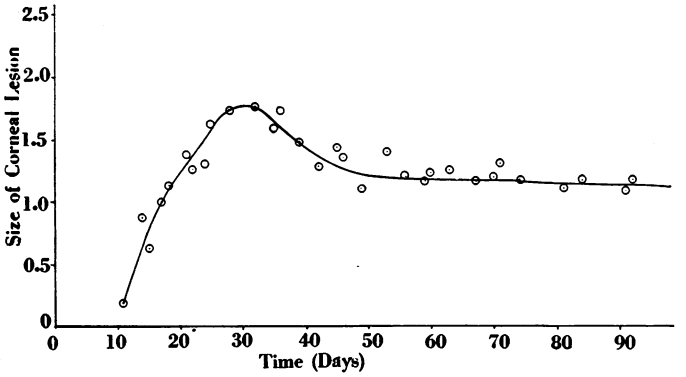


FIG. 2.—The natural development of tuberculous corneal lesions produced by an inoculum of approximately 1,000 tubercle bacilli in the mouse. Each point on the curve represents the mean of a number of observations.

Chemotherapeutic tests

Mice were infected in one eye only, divided into a control and treated group, the latter being given the drug either incorporated in the food or by subcutaneous injection.

1. *Experiments with streptomycin*

In one set of experiments treatment was continued for 28 days and in another for 56 days. In both the total daily dose was 8 mg. given in equally divided doses in 0.2 ml. of sterile distilled water and injected morning and evening. The first injection was always given within an hour of infection.

In the experiment in which streptomycin was given daily for 28 days, the five control mice all developed lesions between the 8th and 13th days whereas only three out of thirteen treated mice had developed lesions by the 28th day—i.e., one each on the 16th, 19th, and 24th days respectively. Five more of the treated group developed lesions between the 28th and 40th days and the remaining five animals were free from infection when the experiment was terminated on the 65th day (Fig. 3 A). Of the eight treated animals developing lesions, four remained

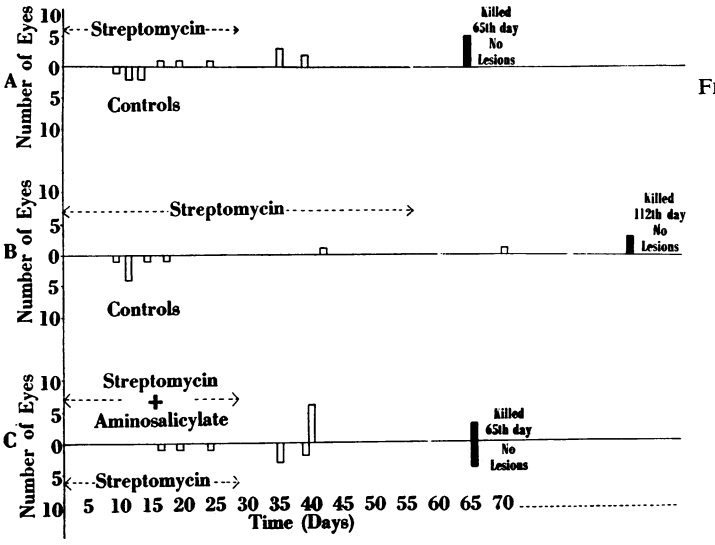


FIG. 3.—The effect of streptomycin therapy, (A) continued for 28 days, (B) continued for 56 days, (C) combined with *p*-aminosalicylic acid for 28 days, on the incubation period of the corneal lesions. The number of animals developing lesions on any day of the experiment is shown along the ordinate. The black squares at the end show the number of animals which never developed lesions.

minimal in size; the other four were progressive in type and consisted of two appearing during treatment and two after treatment was stopped.

In the experiment in which streptomycin treatment was continued for 56 days, the seven control animals all developed lesions between the 9th and 17th day whereas only one of the five treated animals developed a lesion (42nd day) while on streptomycin. One further animal developed a lesion on the 71st day, while the remaining three were free from lesions when the experiment was terminated on the 112th day. Both the lesions which developed in treated animals remained minimal in size (Fig. 3 B).

2. *Experiments with sodium* p-*aminosalicylate*

The treated animals were given sodium *p*-aminosalicylate thoroughly mixed into a powdered diet (M.R.C. Mouse diet No. 41; see Bruce and Parkes, 1949)

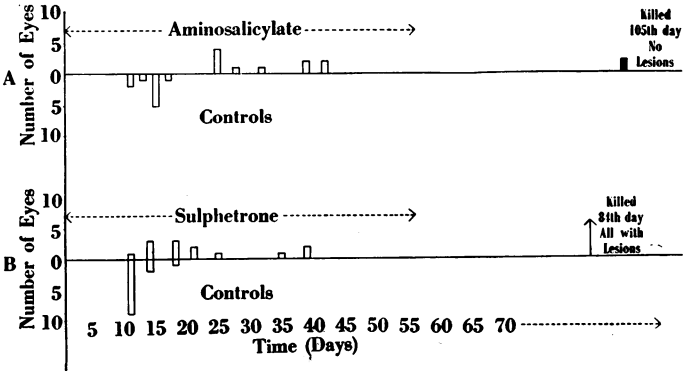


FIG. 4.—The effect of (A) *p*-aminosalicylic acid and (B) sulphetrone therapy on the incubation period of corneal tuberculous lesions.

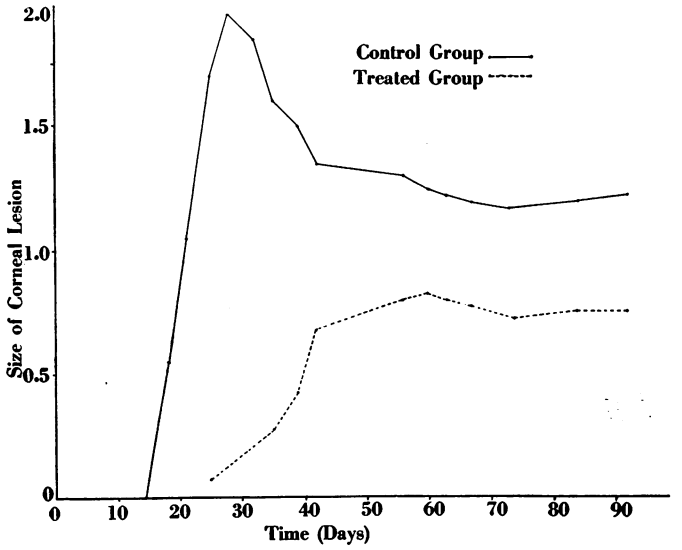


FIG. 5.—Showing the effect of *p*-aminosalicylic acid therapy, started one day before inoculation of the cornea and continued for 56 days.

to give a final mixture of 2 per cent; this would give an approximate daily dose of 5 g./kg. body weight, which is similar to that used by other workers. The drug was started 24 hours before infection and maintained for 56 days. The results of an experiment, in which there were ten controls and twelve treated animals, are shown in Fig. 4 A and Fig. 5. The ten controls all developed lesions between the 11th and 17th days, whereas in the treated group the incubation period was prolonged to between the 25th and 32nd day in ten and the remaining two were free from infection when the experiment was terminated on the 105th day. By plotting the mean size of lesions (taking into account all treated animals) against the time of observation, it was possible to demonstrate that the corneal lesions in the treated group were smaller than in the control group (Fig. 5).

3. *Experiment with sulphetrone*

Sulphetrone was given as a 3 per cent mixture in the diet—i.e., a dose similar to that used by Brownlee and Kennedy (1948) in guinea-pigs. The treated animals were started on sulphetrone 24 hours before infection and continued on the drug for 56 days. In the experiment there were twelve control and thirteen treated animals. All the controls and seven of the treated animals had developed lesions between the 11th and 18th days. The remaining six treated animals all developed corneal lesions, the last two appearing on the 39th day (Fig. 4 B). Although the incubation period of the treated group was only prolonged in six out of thirteen animals the lesions themselves, as a group, were smaller and less active than their controls (Fig. 6).

4. *Experiment with combined sodium* p-*aminosalicylate and streptomycin*

It has been shown by Swedberg and Widström (1948) in the mouse and guinea-pig (intravenous technique) and by Rees and Robson (1949), using the rabbit corneal method, that an enhanced effect is given by combining *p*-aminosalicylic

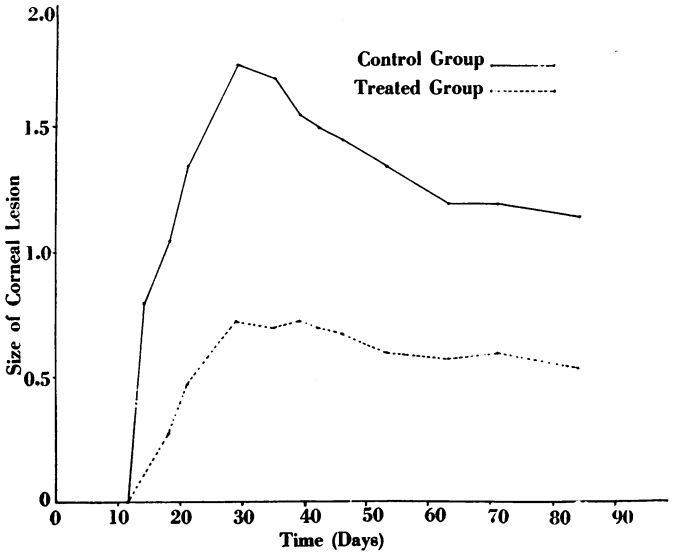


FIG. 6.—Showing the effect of sulphetrone therapy, started one day before inoculation of the cornea and continued for 56 days.

acid with streptomycin in experimental tuberculosis. In a similar experiment with the present technique three groups of animals were used—one a control, one on streptomycin, and a third on streptomycin and *p*-aminosalicylic acid. Streptomycin (8 mg. per day) was given by subcutaneous injection in two daily doses, the first injection being given within one hour of injection ; *p*-aminosalicylic acid administration (2 per cent in the diet) was started 24 hours before inoculation. Treatment was maintained for 28 days. The results are shown in Fig. 3 C. The five control animals all developed lesions between the 8th and 13th days. Three out of twelve animals on streptomycin and none of the ten on combined therapy had developed lesions by the 28th day. Within 12 days of stopping treatment all but three on combined treatment and four on streptomycin developed corneal lesions. Under the conditions of these experiments there had been a significant enhanced effect with the two drugs while on treatment, but the relapse rate after cessation of treatment was identical.

DISCUSSION

It has been found that, by intracorneal injection of a standard suspension of a bovine strain of tubercle bacilli, the mouse cornea responds in a regular and predictable manner. Moreover the natural history of such standard corneal infections can be significantly modified by treating the animals with drugs known to have anti-tuberculous activity (i.e., streptomycin, *p*-aminosalicylic acid, and sulphetrone). Not only does the method show these compounds to be active but it is possible to compare their relative activities quantitatively and place them in the following order: (1) streptomycin ; (2) *p*-aminosalicylic acid ; (3) sulphetrone (Fig. 3 B and Fig. 4 A and B). This agrees with other laboratory tests and with clinical observation. It is also possible to demonstrate, as has already been shown by means of the rabbit cornea method (Rees and Robson,

1949), that a combination of streptomycin and *p*-aminosalicylic acid is more effective than either drug alone.

The activity of a chemotherapeutic agent can be assessed by analysing its effect on (*a*) the incubation period and (*b*) the subsequent development of the lesion. The former method of analysis is very simple, requires a relatively short time, and is capable of giving at least a preliminary indication of activity in some twenty days.

In the untreated mouse cornea the lesions reach a maximum by about the 30th day, then slowly decrease in size, and finally become stabilized at about the 50th day. This natural regression of tuberculous corneal lesions in the mouse must be taken into consideration when assessing the activity of drugs after the 30th day. By applying these two methods of analysis to the three compounds described in this paper, it has been found (1) that streptomycin markedly prolongs the incubation period; up to half the inoculated eyes show no lesions throughout the period of the experiment, and where lesions develop they remain minimal in activity and size; (2) that *p*-aminosalicylic acid always prolongs the incubation period, but the effect is less striking than with streptomycin and only a few eyes remain inactive throughout the period of the experiment. Although those lesions which do develop are rather smaller than the controls, the effect is far less striking than with streptomycin. (3) Lastly with sulphetrone, the incubation is not prolonged beyond the normal in half the animals, and none of the inoculated eyes are inactive at the end of the experiment, but the lesions themselves tend to remain smaller than the controls. With both *p*-aminosalicylic acid and sulphetrone the difference between the size of the lesions in the treated and control animals is most marked during the first 30–40 days, and once the period of natural regression is reached any differences become less marked. The activity of a drug should probably be assessed by its ability to prolong the incubation period and inhibit the progress of the lesion during the phase of natural progress—i.e., up to 30 days. This implies that all compounds for assay should be given either at the time of, or 24 hours before, the inoculation of the eyes; this will give the drug its maximum opportunity for effective action and the observer the earliest opportunity of judging its activity.

The cornea is admittedly a specialized tissue, normally isolated from any direct blood supply, and might therefore be considered to be at a relative disadvantage so far as the normal host response to infection is concerned. But it is a living tissue, dependent ultimately on a normal blood supply to the surrounding areas: hence the effect of systematically introduced chemotherapeutic substances is dependent on their ability to diffuse into the cornea, while still retaining their activity. In the earlier experiments on the rabbit cornea, all drugs were given by intravitreous injection, and, although it is convenient to have in one and the same animal a control and a treated eye, the treated eye acted as a rather artificially boosted depot. In the mouse corneal test all drugs were given either by mouth or subcutaneous injection in quantities similar to those used by other workers, and although compounds of larger molecular size may diffuse less readily into the cornea than for example into the lung, yet with all of them it has been possible to demonstrate anti-tuberculous activity.

SUMMARY

1. Details of a method for producing experimental tuberculous corneal lesions in the mouse are given, together with a description of the natural history of the infection and a quantitative analysis of the resulting lesions.

2. The results obtained with streptomycin, sodium *p*-aminosalicylate, and sulphetrone suggest that this method may prove of value in the screening of new substances for anti-tuberculous activity *in vivo*.

3. With the use of this method, an enhanced effect was demonstrated with combined streptomycin and *p*-aminosalicylic acid treatment.

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The photographs were taken by Mr. K. Didcock.

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