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INTRACORNEAL INFECTION AS A METHOD FOR TESTING ANTITUBERCULOUS SUBSTANCES

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Since 1935 several synthetic and antibiotic substances with marked antituberculous activity have been discovered. Many more such substances will be produced in the future, all of which will require screening and comparing with those already available. The *in vitro* screening of these compounds for their antituberculous activity is relatively simple. If these tests give a positive result and the substance is of low toxicity then the vital stage of testing the substance *in vivo* against experimentally produced tuberculous infections in animals is reached. At present guinea-pigs or mice are the experimental animals generally used, and infection is established by the intravenous, intraperitoneal, or subcutaneous route. The efficiency of the substance is measured by comparing the survival times, weight changes, and detailed histopathological findings of the treated group with those of the control group. Feldman and Hinshaw (1945), summarizing their extensive experience with guinea-pigs as the test animal, conclude that at least sixty days are needed for a screening test. Youmans and Raleigh (1948), using mice, sacrificed all survivors at thirty-five days and compared average survival times, weight changes, and detailed histopathology with the controls. Martin (1946) and Hoggarth and Martin (1948) also used mice but in larger groups, and inoculated them with a fairly acute type of infection: they determined the antituberculous activity by a statistical analysis of differences in mean survival time. One other method worth mentioning makes use of the chorio-allantoic membrane of the developing chick embryo; this was originally suggested as a method for screening compounds by Emmart and Smith (1941). Here the chorio-allantoic membrane is inoculated with tubercle bacilli and the drug for screening is introduced into the amniotic cavity or yolk sac. Although this is an *in vivo* method by definition, the natural progress of the tuberculous lesion is so rapid and the environment so specialized that it adds little information to that obtained from an *in vitro* test.

At present it is impossible to decide which of the methods in guinea-pigs and mice is the best as there are so many variables. The guinea-pig is uniformly a highly susceptible animal to tuberculosis and therefore the lesions are of the acute type and unaffected by any natural host resistance. Mice generally have a greater and more variable natural resistance with a marked strain variation and therefore every grade of activity may be met. Whatever the final choice, all the methods at present available for satisfactory *in vivo* screening tests are laborious and time consuming. The assessment is frequently delayed till the animal is sacrificed after many weeks and not till then is it possible to say whether the drug is of value or otherwise. The *in vivo* methods at present are formidable enough to curtail very seriously the screening of potentially active antituberculous substances.

The present paper describes an entirely new *in vivo* method which is relatively simple to perform and gives unique opportunity for observation of the tuberculous lesion, and comparison with a control lesion, throughout the chemotherapeutic trials. The method was tried with proved antituberculous substances—viz., streptomycin and the sodium salt of *para*-aminosalicylic acid, and the results so obtained are also described.

METHODS

All experiments were performed on mature rabbits of various breeds and both sexes. Throughout the experiments a bovine strain of *Mycobacterium tuberculosis* was used to inoculate the cornea because rabbits have a well-developed natural immunity to human strains. The strain, which was the same as that used in earlier similar work (Robson, 1944), was kindly supplied by Dr. W. M. Levinthal of the Royal College of Physicians, Edinburgh. The strain was sensitive to between 0.2 and 0.4 μg. streptomycin per ml. of culture medium —i.e., similar in sensitivity to the standard virulent human strain, H37Rv.

A synthetic medium containing Tween 80 and bovine albumin (Dubos and Davis, 1946), as modified by the Medical Research Council (1948), was used for culturing

the organism. Seven-day actively growing cultures gave a homogeneous suspension which was adjusted, by means of turbidimetric estimation with a photoelectric absorptiometer, to contain 0.1 mg. (dried weight) of organisms per ml. (equivalent to between 10^8 and 10^9 viable organisms per ml.). After many trials it was found that an inoculum containing approximately 300 tubercle bacilli consistently produced a suitable progressive tuberculous lesion of the cornea.

As in earlier work (Robson, 1944), the lesions were produced by intracorneal injection, with a tuberculin syringe with a fine and short bevelled needle, into rabbits deeply anaesthetized with ether. A white bleb was produced in the cornea and, by restricting the bleb to about 5 mm. in diameter (which gives an inoculum volume of about 0.03 ml. of a suitably diluted standardized suspension of organisms), uniformity in size of inoculum and lesion was obtained.

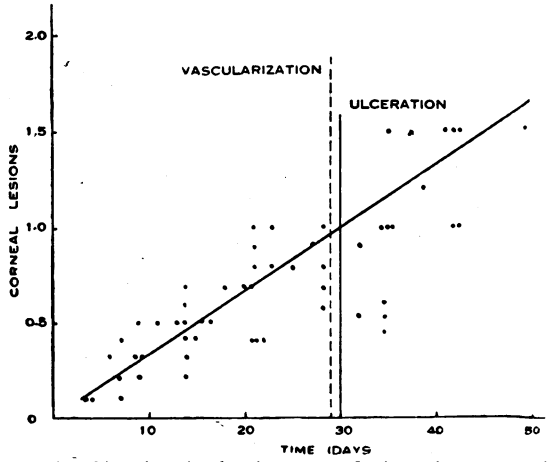

FIG. 1.—Showing the development of tuberculous corneal lesions produced by an inoculum of approximately 300 tubercle bacilli. The average times at which vascularization and ulceration occur are also shown.

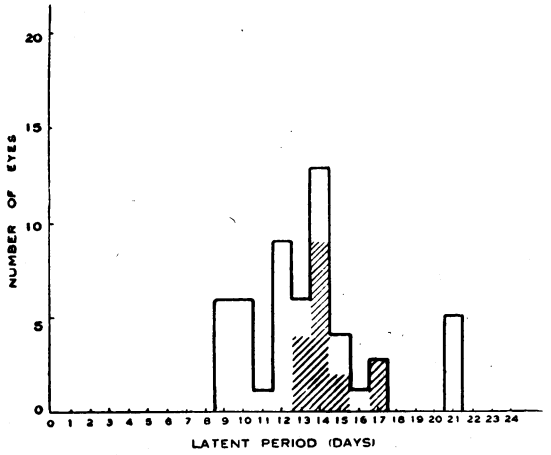

FIG. 2.—Frequency distribution of the latent periods after inoculation of the cornea with tubercle bacilli (analysis of 55 eyes). The shaded area shows the results in one particular experiment.

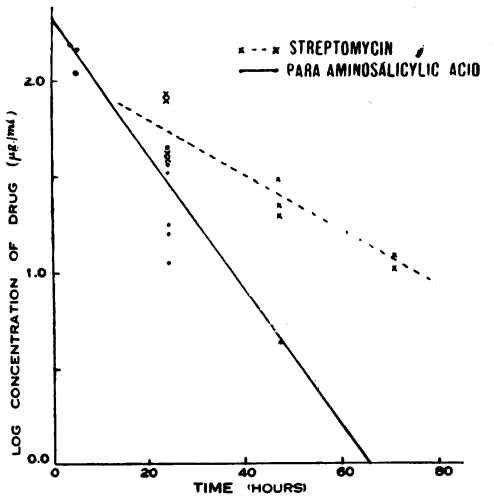

FIG. 3.—Comparison of the concentrations of streptomycin and the sodium salt of *p*-aminosalicylic acid in the aqueous after intravitreous injection of 2 mg. streptomycin and 10 mg. sodium *p*-aminosalicylate.

With this technique lesions are consistently produced. Single or multiple minute white primary lesions appear in the needle track and rapidly progress in size by direct spread and by peripheral satellite tubercle formation. At the same time caseation occurs at the centre of the lesion which later, at about the thirtieth day, breaks down to form an ulcer, and vessels grow in from the limbus (Plates I and II). By an arbitrary numerical method it is possible to study quantitatively the natural development of these corneal lesions and by this method it is found that they progress at a remarkably constant rate (Fig. 1).

The development of a primary lesion is preceded by a latent or incubation period which again has proved to be reasonably constant. About 50 per cent of all the lesions so far observed have appeared within a three-day period—i.e., between the 12th and 14th days inclusive after inoculation. The latent period is even more constant in any one particular experiment, and in one such experiment the lesions appeared within a three-day period in 85 per cent of animals (Fig. 2).

It has been previously shown (Gardiner *et al.*, 1948) that after a single intravitreous injection of 2,000 μg. streptomycin an effective chemotherapeutic concentration is maintained in the ocular fluids for at least three days. (Fig. 3.) The posterior chamber acts as a reservoir for the streptomycin which slowly diffuses into the anterior chamber. No detectable concentration has been found in the opposite untreated eye. It has also been shown that streptomycin produces local toxic effects on the retina and vitreous, but this is not sufficient to interfere with successful antituberculosis therapy. Consideration of the diffusion curve led to the conclusion

that twice-weekly intravitreous injections of 10,000 μg. streptomycin would ensure adequate chemotherapeutic levels throughout the course of therapy. Streptomycin, dissolved in 0.1 ml. of sterile normal saline, was introduced into the vitreous by the method previously described (Duguid *et al.*, 1947).

Experiments with Streptomycin

The experiments with streptomycin in tuberculous corneal infections can be divided into three groups:

A. " Umbrella " type of experiment.

B. Treatment shortly after inoculation.

C. Treatment of developed tuberculous lesions.

A. Streptomycin " umbrella " experiment

In this experiment streptomycin was injected into the vitreous in order to produce a chemotherapeutic level in the cornea, which was then inoculated with tubercle bacilli. Six rabbits were used; on the first day 10,000 μg. streptomycin were injected into the vitreous of the right eyes; 24 hours later the corneae of both eyes of all six rabbits were inoculated with about 300 tubercle bacilli. Twice-weekly intravitreous streptomycin therapy of the right eyes was continued for a period of 28 days only. The six left eyes acted as controls, and typical progressive tuberculous lesions appeared in them after a latent period of between 13 and 15 days.

In four out of the six animals the treated eyes were still clear from lesions after periods of 77, 82, 82, and 99 days, when the animals were finally destroyed owing to generalized infections with some loss of weight. Of the other two right eyes, one remained clear till the 55th day and the other till the 60th day, when definite lesions appeared for the first time and subsequently progressed and were confirmed to be tuberculous in origin by histological examination.

B. Streptomycin therapy after inoculation

In this experiment streptomycin therapy was started three days after inoculation and maintained twice weekly throughout. Seven animals were used and both eyes were inoculated with tubercle bacilli. Three days later the right eyes were treated with 10,000 μg. streptomycin and maintained twice weekly on this therapy. The left eyes acted as controls. In this experiment different doses of tubercle bacilli were used, ranging from 30 to 3,000 organisms per inoculum, and therefore the latent period varied more, as shown in Fig. 4. One of the seven treated eyes had developed no lesion when the animal was killed on the 94th day; the other six eyes all developed lesions after a prolonged latent period but only one progressed; the others remained minute and later healed. Histological examination showed healed lesions free from acid-fast bacilli. Streptomycin therapy was maintained for periods of 91 to 143 days and in two animals terminated six weeks before they were killed. Reactivation did not occur in either animal.

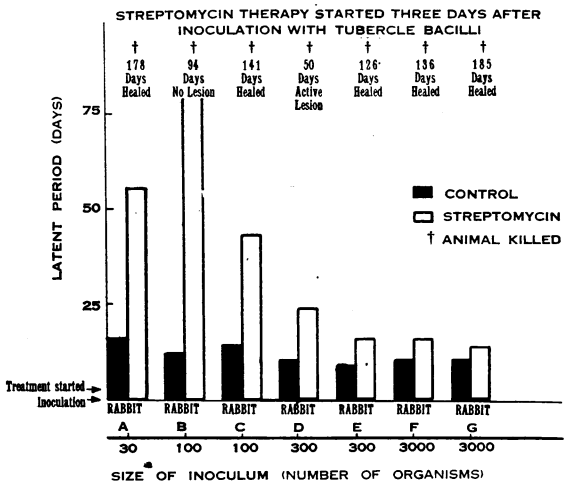

FIG. 4.—Showing effect of continued streptomycin therapy started three days after inoculation of the cornea with different inocula of tubercle bacilli, on the latent period and subsequent lesions.

C. Treatment of developed tuberculous lesions

In this group lesions were allowed to develop before streptomycin therapy was started. Therapy was started at variable times, from the first day of the appearance of a lesion (called L) up to 12 days (L + 12), and continued twice weekly throughout.

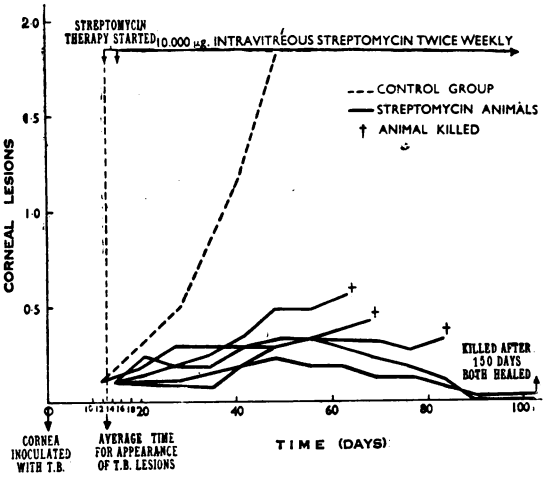

FIG. 5.—Showing the effect of continued streptomycin therapy of developed tuberculous corneal lesions started not more than two days after the first appearance of the lesion.

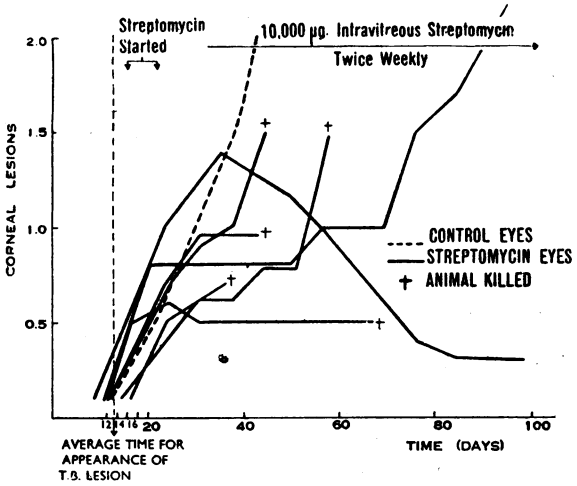

FIG. 6.—Showing the effect of continued streptomycin therapy of developed tuberculous corneal lesions. Treatment was started between the third and twelfth day after the first appearance of the lesions.

This group falls naturally into two parts. In the first, both eyes of five animals were inoculated with about 300 tubercle bacilli, and watched for the development of lesions. Therapy was started on day L to L + 2 in one eye of each animal, the other eye acting as the control. The natural development of the treated lesions was markedly slowed and two out of five showed clinical healing which was confirmed histologically (Fig. 5 and Plates III A and B).

In the second part both eyes of seven animals were inoculated with about 300 tubercle bacilli, and lesions allowed to develop. Treatment was started on day L + 3 to L + 12, the other eye acting as the control. In all treated eyes there was slowing of the natural progress of the lesion as compared with the control eyes, but none showed clinical healing (Fig. 6 and Plates IV A and B). In three eyes treated for 60 to 100 days with streptomycin, cultures of *M. tuberculosis* were obtained from lesions at death. Streptomycin sensitivity tests performed on these showed no increase in resistance to the drug.

EXPERIMENTS WITH *para*-AMINOSALICYLIC ACID

In 1940 Bernheim showed that the oxygen uptake of the tubercle bacillus was stimulated by benzoic and salicylic acid; later Lehmann (1946) discovered that *p*-aminosalicylic acid had considerable bacteriostatic action against the tubercle bacillus *in vitro*. *In vivo* tests with guinea-pigs (Feldman *et al.*, 1947) and clinical trials in man (Erdei, 1948) have given encouraging results. The first step, before proceeding with the rabbit eye *in vivo* test, was to investigate the drug concentration in the ocular fluids after intravitreous injection.

METHOD

Throughout these experiments a sterile solution of 10 g. sodium *p*-aminosalicylate per 100 ml., adjusted to *p*H 6.8, was used in 0.1 ml. quantities for intravitreous therapy.

p-Aminosalicylic acid was estimated on samples of aqueous and vitreous removed from eyes 6, 24 and 48 hours after an intravitreous dose of 10 mg. of the sodium salt. Measured quantities of aqueous and vitreous were treated with 10 per cent (w/v) trichloroacetic acid in order to precipitate proteins and after standing 10 minutes filtered, and the precipitate thoroughly washed with 5 per cent (w/v) trichloroacetic acid. The filtrate was made alkaline and with the addition of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) a yellow-green colour developed which was compared with standard solutions of *p*-aminosalicylic acid, made up in trichloroacetic acid, by means of a Spekker photoelectric colorimeter.

It was shown that after a single intravitreous injection of 10 mg. sodium aminosalicylate a chemotherapeutic level was maintained in the aqueous for at least 48 hours. These results are given in the Table and shown graphically in Fig. 3. Toxic effects were produced in the eye with aminosalicylic acid of similar or even greater severity than with streptomycin but not sufficient to interfere with successful therapy.

The bovine strain of *M. tuberculosis* was sensitive *in vitro* to between 0.4 and 0.8 µg. sodium aminosalicylate per ml. To maintain a chemotherapeutic level throughout the experiment 10 mg. sodium aminosalicylate was injected into the vitreous three times a week.

TABLE

CONCENTRATION OF *p*-AMINOSALICYLIC ACID AFTER INTRAVITREOUS INJECTION OF 10 MG. OF THE SODIUM SALT

Time after injection hours	Rabbit number	Concentration of aminosalicylic acid (µg. per ml.)	
		Aqueous	Vitreous
6	R. 1 R. eye	144	923
	R. 2 R. eye	232	1233
	R. 3 R. eye	113	1180
24	R. 4 L. eye	16	26
	R. eye	11	45
	R. 5 L. eye	19	—
	R. eye	33	—
	R. 6 L. eye	46	—
	R. eye	37	—
48	R. 7 R. eye	4	6
	R. 8 R. eye	4	6

It was anticipated from previous work (Lehmann, 1946) that the effect with aminosalicylic acid would be less definite than that of streptomycin, and therefore an " umbrella " type of experiment was planned to obtain the best possible effect.

para-*Aminosalicylic acid " umbrella " experiment*

In this experiment six rabbits were used; on the first day 10 mg. sodium aminosalicylate were injected into the vitreous of the right eyes; 12 hours later the corneae of both eyes of all rabbits were inoculated with about 300 tubercle bacilli; 10 mg. of sodium aminosalicylate were then injected into the vitreous of the right eyes three times a week for a preliminary period of 28 days. At the end of that period all eyes had developed tuberculous lesions (Fig. 7), but in the treated right eyes the latent

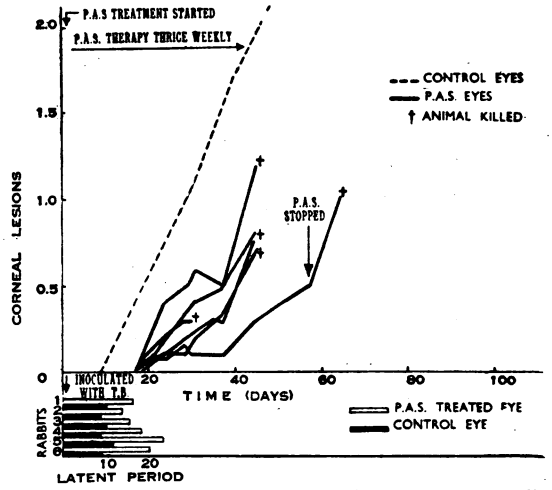

FIG. 7.—Showing the effect of continued aminosalicylic acid (P.A.S.) therapy, started one day before inoculation of the cornea with tubercle bacilli, on the latent period and development of lesions.

period was prolonged by an average of eight days over the left control eyes. Not only was the latent period prolonged in the treated group but the lesions themselves were slightly smaller and less active than the controls. It was decided to continue treatment, and, although at the end of the 38th day there was the same qualitative difference, by the 45th day the treated eyes showed marked deterioration in all but one animal. The remaining one still showed a marked difference and aminosalicylate was continued till the 58th day and the same effect maintained. At that stage the drug was discontinued and the lesion rapidly regressed. Tubercle bacilli isolated from the treated lesions showed no increase in resistance to aminosalicylic acid.

DISCUSSION

The present paper describes a simple method of producing consistently a tuberculous lesion of the cornea in the rabbit. It was found that with a standard inoculum the lesion is preceded by a reasonably constant latent period and, when the development of the lesions is studied quantitatively, a remarkably equal rate of progress is observed. The standard lesion is therefore constant and reliable enough to enable the effect of antituberculous substances to be studied. The results with streptomycin were similar to those reported by other workers—i.e., (1) the natural progress of a developed tuberculous lesion is markedly slowed; (2) the earlier the developed lesion is treated, the greater the chance of complete healing; (3) used as a " screen " or " umbrella," streptomycin is tuberculocidal. The last point was particularly well demonstrated. With *p*-aminosalicylic acid it is possible to demonstrate a favourable effect, such as has been shown in guinea-pigs (Feldman *et al.*, 1947) and in mice (Youmans, 1948), but which has not been confirmed by all workers. The effect is very much less than with streptomycin and confined to slowing of the natural progress rather than actual healing of established lesions.

Streptomycin and *p*-aminosalicylic acid, two substances of known antituberculous activity, both give with this method results comparable with those obtained with other *in vivo* tests, which in general are much more laborious and time consuming. The method has several unique advantages over other methods: the progress of the lesion can be readily observed, thus enabling a lesion of any particular age to be treated or ineffective treatment to be abandoned without any further loss of time. Photographic records of the lesion can be taken as required. The treated and control lesions can be situated in opposite eyes of the same animal, thus allowing for differences in individual animals. In addition, the cornea is suitable for histological or bacteriological examination. Primarily the method is offered as one suitable for preliminary *in vivo* screening of antituberculous compounds with favourable *in vitro* activity—i.e., a method that is capable of giving a more rapid, yet a sufficiently reliable, indication of the *in vivo* activity of some new compound. At the same time a reasonable comparison can be made with other substances of known activity. For such tests few animals and only small quantities of drug (with streptomycin 20 mg. per week per rabbit) are required. Thirty days would be sufficient to give a preliminary indication as to whether a drug was active or inactive against a developed lesion. With *p*-aminosalicylic

acid the " umbrella " type of test was used which requires even less time. It may well be that the " umbrella " type of screening is the method of choice for the earliest possible indication of antituberculous activity. To what extent the method can be used for more critical evaluation of the effectiveness of drugs depends on further work with other drugs, but present results suggest that it compares not unfavourably with the acute type of screening test in guinea-pigs or mice.

SUMMARY

1. *In vivo* screening tests for antituberculous substances are briefly discussed.

2. Details of a new *in vivo* method making use of the rabbit's eye are given, together with results obtained with streptomycin and *p*-aminosalicylic acid.

3. The advantages of the method are discussed. It is suggested that it may prove of value in the screening of new substances for antituberculous activity *in vivo*.

We are greatly indebted to the Antibiotics Study Section of the U.S. Public Health Service (through Dr. Seger) for the supply of streptomycin; to Herts Pharmaceuticals (through Mr. Seymour) for the supply of *p*-aminosalicylic acid; and to the W. H. Ross Foundation (Scotland) for the Prevention of Blindness, who have defrayed part of the expenses of this work.

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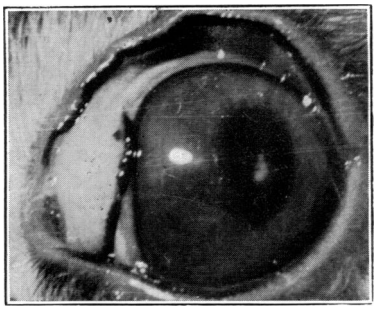
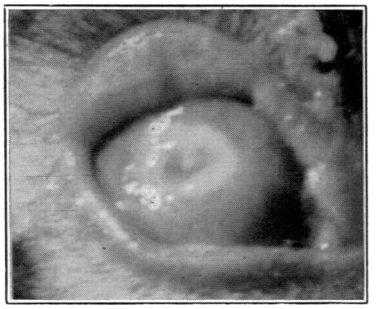
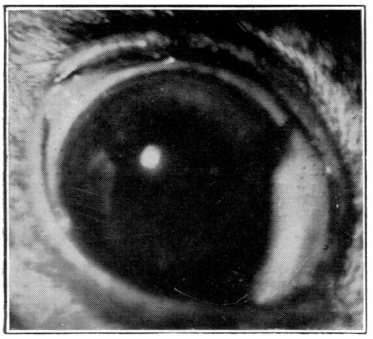


I.—Early tuberculous lesion of cornea (17 days after inoculation).



II.—Advanced tuberculous lesion of cornea, showing caseation with central ulceration (65 days after inoculation).



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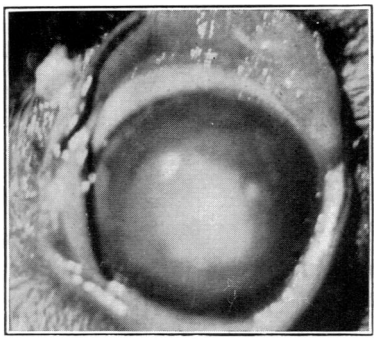
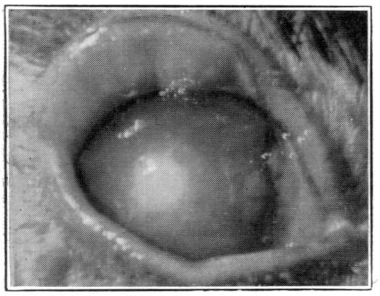


B

III.—Effect of streptomycin therapy started 3 days after first appearance of lesion. Condition of eyes 42 days after inoculation (i.e., 28 days after start of treatment). *A*. Right eye—treated. No lesion visible. *B*. Left eye—control.



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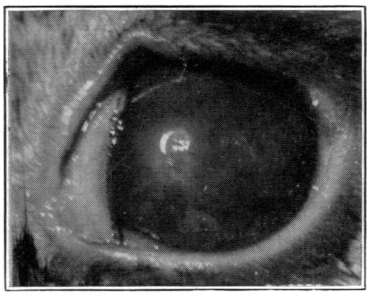

B

IV.—Effect of streptomycin therapy started 6 days after first appearance of lesions. Condition of eyes 38 days after inoculation (i.e., 21 days after start of treatment). *A*. Left eye—treated. Note that lesion has not become dense, especially at the centre where there is probably some attempt at healing. *B*. Right eye—control.