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THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS WITH SULPHETRONE*

BY

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"Sulphetrone" is the registered name for bis(γ-phenyl-*n*-propylamino) - di - phenylsulphone-tetrasodium sulphonate, the pharmacology and chemotherapy of which have been recently reported (Brownlee, Green, and Woodbine, 1948). Sulphetrone has a low toxicity and an antituberculous efficiency approaching that of its parent compound, diaminodiphenylsulphone; it is also curative in infections due to β-haemolytic streptococci. Moreover it satisfies the requirements of an *in vivo-in vitro* comparison with diaminodiphenylsulphone; the concentrations of these substances in guinea-pig blood after massive intraperitoneal injections are capable of inhibiting the growth of virulent strains of *Mycobacterium tuberculosis*. The present report is concerned with the treatment of experimental tuberculosis of both human and bovine types in the guinea-pig.

Methods and Materials

Animal management

In carrying out screening tests of chemotherapeutic agents against *M. tuberculosis* there are two essentials: protection of staff from the risks of infection, and prevention of intercurrent infection in the animals under experiment; these can be achieved only by continuous and vigilant cleanliness on the part of a co-operative and dependable staff of helpers. It is an advantage to adopt a rigid daily routine which is on record available to and approved by all concerned. The animal room is 8 ft. by 30 ft., with transom windows along the long side and with a single entrance set at the opposite end of the long side. In order to assist ventilation it consists of a frame filled with fine copper gauze, with which the windows are also screened. The door opens into a vestibule "lock" enclosed by frame-work and door of wire gauze. This arrangement reduces the potential passage of all possible vectors and serves in addition to emphasize the "isolation" nature of the work. Walls are of breeze-block and plywood, filled, and glossy enamelled, and the ceiling is of "cellotex," and is similarly treated. Switches, cable, and light fittings are waterproof. The floor, of concrete, is treated with spindle-oil at two-monthly intervals, and is scrubbed weekly. The whole is washed down at two-monthly intervals. Temperature is held approximately at 20° C. and is controlled by hand-controlled steam-heated radiators and an extractor fan.

Guinea-pigs are housed six to a galvanized-wire cage fitting in a sheet-metal tray. The floor space per animal is 48 sq. in. The animals are identified by a combination code of natural colouring and artificially applied dye. White-enamelled metal hook-on labels have been found the most satisfactory after trials of many other types and of other devices. Food containers of tin-plate are non-spill and consist of an annular moat of 8-in. diameter with a central hollow projecting pillar to prevent soiling, and hold 250 g. of dry diet. Glazed non-spill water-pots holding 120 c.c. of water, are refilled three times daily. Absorbent litter is large wood shavings which scatter less than does sawdust. Weights are recorded weekly with a spring-pan balance weighing 1,000 ± 10 g. Changing to clean, previously autoclaved cages is done daily, except Sunday. Soiled cages, together with feeding-troughs and water-pots, are steamed before cleaning, and for this and similar purposes a double-door autoclave connects the animal room to the cage-cleaning room.

Guinea-pigs

In recent years guinea-pigs bred and reared on our own farm have been used. This has created a further problem since these animals, in addition to being much less prone to intercurrent infection, are more resistant to artificially induced tubercle infections than are the guinea-pigs of commerce.

* The development of sulphetrone is part of the programme of work on antituberculous compounds carried out by the Therapeutic Research Corporation of Great Britain, Limited. The product was made and will be issued by the Wellcome Foundation, Ltd.

Diet

The dry, finely ground diet is based on that of Coward (1937) adapted to guinea-pig requirements. It is made up as follows:

Tubercle Diet No. 2

Yellow maize, whole, ground fine ...	700
Wheat, whole, ground fine	250
Milk, winter, dried (Glaxo)	50
Casein (B.D.H.)	90
Yeast, dried (Pharmaco-Chem. Prod.) ...	150
Salt mixture	12

The salt mixture has the following composition: manganese sulphate 0.1, potassium iodide 1.0, ferric citrate 1.0, sodium chloride 60.0, and calcium carbonate 1,000.0. The ingredients are mixed mechanically, and drug, when added, is mixed separately. The rate of consumption of this diet is 30–40 g. daily in guinea-pigs weighing 450–500 g., and their diet is supplemented with 100 g. of greenstuff.

Mycobacterium tuberculosis strains

The human strain CN. 271 (H. 37) is the virulent Saranac strain obtained from the Trudeau Sanatorium, N.Y., in 1931 and maintained on synthetic fluid medium by implant transfer at 21 days (Steenken and Gardner, 1946). In our hands this strain is moderately virulent. The bovine strain CN. 858 (A.N. 5 Weybridge) has proved to be apparently stable, by virulence titration, since its acquisition in 1943. It is highly virulent. In recent years both strains have been kept by Mr. H. Proom in a dried form which may prove to be the method of choice for maintaining virulence.

Grown on a solid egg-medium (Petragnani) for 14 days, suspensions are prepared by mechanically shaking with distilled water in a stainless steel bottle with steel balls and adjusted to 0.5 mg. per c.c. by reference to a previously standardized vaccine.

The estimation of sulphetrone in body fluids

The estimation of sulphetrone in body fluids has been described (Brownlee *et al.*, 1948) and is based upon diazotization and coupling to N(1-naphthyl)-ethylenediamine hydrochloride (Bratton and Marshall, 1939). The essential differences lie in the dilution of 1 in 15 and the concentration of acid, both of which are critical. Blood is obtained from the ear of the guinea-pig, from which, with patience and practice, up to 0.5 c.c. may be drawn into a clean heparinized capillary tube.

In Fig. 1 are given the blood-concentration time curves obtained with 1, 2, and 4 per cent of sulphetrone in the powder diet, and also the curve corresponding to a 2 per cent diet when greenstuff is fed in addition.

FIRST EXPERIMENT. *Bovine Strain CN. 858*

Forty young adult guinea-pigs of mixed sexes, weighing 450–500 g., were inoculated deeply into the right thigh muscles with 0.25 mg. of a fourteen-day-old sub-culture of bovine tubercle bacillus, CN. 858. The course of the infection was rapid, so that by the end of the second week glands were much enlarged and several animals had open abscesses. On the fifteenth day the animals were divided into two groups each of 20 animals, one to serve as control and the other to be the drug-treated group. Thereafter each animal in the latter group received in its food an estimated dose of 600 mg. of sulphetrone for the duration of the experiment. The blood sulphetrone concentration corresponding to this daily dose gave a mean value of 5 mg.±4 S.E. for weekly estimates during the course of the experiment.

RESULTS

Survival time.—A record of the weights and survival times of the animals is given in Fig. 2, from which it will be seen that the first animal in the untreated group died at 35 days and the last at 77 days after infection. About three-quarters of the animals were dead by 50 days. At a time when 50 per cent of animals in the control group were dead, 25 per cent were dead in the treated group; when all controls were dead, 25 per cent of the treated animals were still alive. The last animal in the drug treated group died 237 days after infection and 160 days after the last of the untreated animals had died. The average survival time after infection was 45 days in the untreated group and 77 in the treated group.

Necropsy studies.—Post mortem examination showed gross tuberculosis in 19 out of 20 animals in the untreated group, and one animal with severe tuberculosis. The nature of the infiltration was confirmed by histological examination and the organism was recovered by culture on solid egg-medium (Petragnani). In the drug-treated group of 20 animals, 9 showed gross, 5 severe, and 5 moderate tuberculosis; in one the only sign was a minute abscess at the site of injection from which the organism was recovered. The nature of the disease was confirmed by histological examination and the organism was recovered in 19 out of 20 animals.

The extent of the disease in the animals of both groups is shown pictorially in Fig. 3.

SECOND EXPERIMENT. *Human Strain CN. 271 (H. 37)*

Forty-five young adult guinea-pigs of mixed sex, weighing 450–500 g. were inoculated intramuscularly deep into the right thigh with 0.25 mg. of a 21-day-old culture of CN. 271 (H. 37). Fourteen days after infection the animals were divided

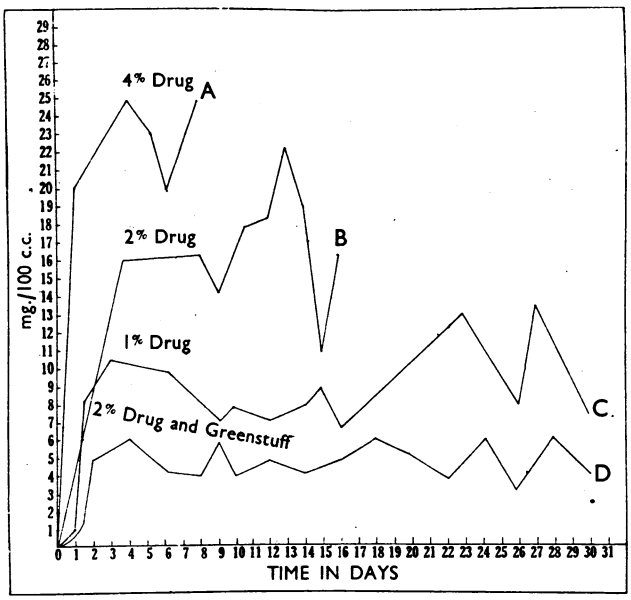

FIG. 1.—Blood concentration-time curves after administration of sulphetrone in the diet. Each curve is the average of estimates on a group of five guinea-pigs weighing 450–500 g. A, sulphetrone 4 per cent, equivalent to 1.4 g. daily; B, 2 per cent, or 0.7 g. daily; and C, 1 per cent, or 0.35 g. daily. In addition to 2 per cent drug in diet group D received 100 g. of greenstuff daily.

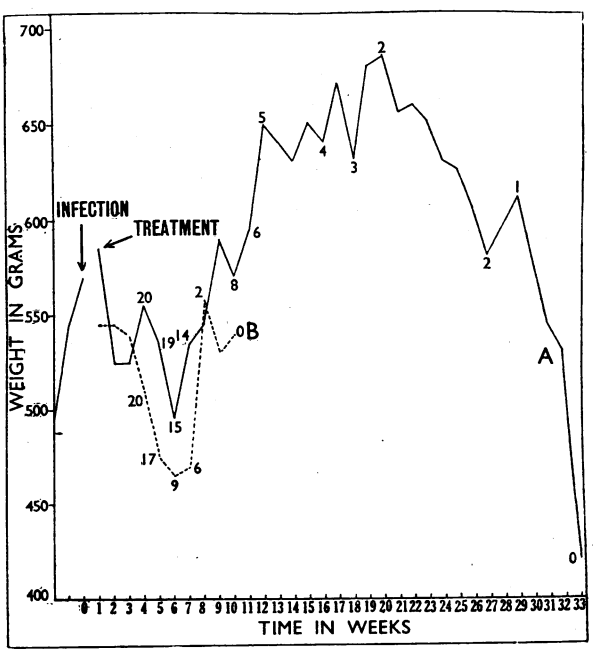

FIG. 2.—Weights and survival times of guinea-pigs after intramuscular infection with a bovine strain of tubercle bacillus. A, treated with sulphetrone; B, untreated. The figures on the graphs refer to survivors.

into two groups, one of 21 animals as a control, and the second of 24 animals as the drug-treated group; the latter group received 2 per cent of sulphetrone in the diet. The blood sulphetrone concentration corresponding to this daily dose, derived from weekly estimates made during the course of the experiment, was 4.8 mg. ± 2.8 S.E.

Results

Survival time.—There was a considerable difference in the survival times of the untreated and treated groups (Fig. 4). When the last of the controls had died at 154 days only 25 per cent of the group that had received sulphetrone had died. In only one guinea-pig of the untreated group was the cause of death possibly not due to tuberculosis. At the 154th day the 18 survivors in the drug-treated group were divided into two groups, one called drug-continued group, which continued to receive drug until the experiment terminated at 61 weeks when the last survivor was killed, and the other a drug-discontinued group from which drug was withdrawn. These two groups afford an interesting parallel. Within the drug-discontinued group deaths occurred regularly until the last animal died on the 280th day, or 126 days after the last animal in the control group had died. At this stage 50 per cent (4/9) of the animals in the drug-continued group were still alive.

Necropsy studies.—The extent of the disease in both groups of animals is shown pictorially in Fig. 5. Post-mortem examination showed gross tuberculosis in 13 out of 21 animals in the control group, five animals had severe tuberculosis, two moderate, and one in which the infection was minimal. The gall bladder and the small intestines of this animal showed the appearance characteristic of salmonella infection and there seems little doubt that this was the cause of death. The recovered organism was identified by Mr. Proom as *Salmonella bovis-morbificans.* The extent of the tuberculosis in all the drug-treated animals was very much less than in the untreated group. In the same period of 154 days in which all the controls died, three drug-treated guinea-pigs died with gross tuberculosis, one with severe and two with moderate lesions. Within the drug-discontinued group of nine animals, three showed gross tuberculosis, three severe, and three moderate. Within the drug-continued group two had gross, and four moderate tuberculosis, and in one animal the disease was minimal. The organism was recovered in all cases by culture methods.

Histology.—Histological studies confirmed the extent and nature of the disease. Not only was its distribution and extent in the treated group very much less but the numbers of acid-fast organisms were noticeably less. In addition there was much evidence of healing in lung, liver, spleen, and glands. The numerous regressive lesions with fibroblastic changes, many of them calcified, were impressive (Figs. 6 and 7). This change was observed also in lymph nodes in a number of animals. There was more tuberculosis of a progressive nature in the drug-discontinued group than in the drug-continued group, but here again there was impressive microscopic evidence of healing. The picture was that of a resurgence of tuberculosis which had been repressed by the drug; in the lungs this had the appearance of miliary tuberculosis (Fig. 7).

Discussion

The design of an animal experiment calculated to assess the therapeutic worth of a potential anti-tuberculous agent for man is a critical exercise fraught with difficulty. Tuberculosis in the experimental animal is a very different disease from tuberculosis in man in whom it is frequently characterized by an insidious onset, a chronic course, and a liability to relapse. Yet, the ultimate goal may be simply stated as the elimination of virulent tubercle bacilli, a point on which there is universal agreement (Feldman, Hinshaw, and Mann, 1944). The absence, to date, of a substance with this ideal requirement has dictated the evolution of other standards of comparison. These have been the comparison between survival-times of drug-treated and untreated groups of animals, a comparison of the nature and extent of tuberculous lesions in the organs of predilection, and a comparison between pieces of liver obtained by biopsy and at the final post-mortem examination. Ideally, a standard of comparison such as diaminodiphenylsulphone is included in the test. In a series of classical reports, Feldman and his collaborators (1940, 1942, 1943, 1944, 1945) have evaluated the status of promin and diasone, derivatives of diaminodiphenylsulphone, and promizole. Probably by reason of the exploratory nature of their first experiments these workers adopted the use of what may be called a minimal lethal infection, using the virulent Saranac strain of H. 37, and examined the treated animals at the time when the last control animal had died. This kind of experiment presents an effective anti-tuberculous agent in its most favourable light. Nevertheless, this is an entirely satisfactory practice when comparisons are made, especially when taken together with the biopsy control technique, and it has enabled Feldman and

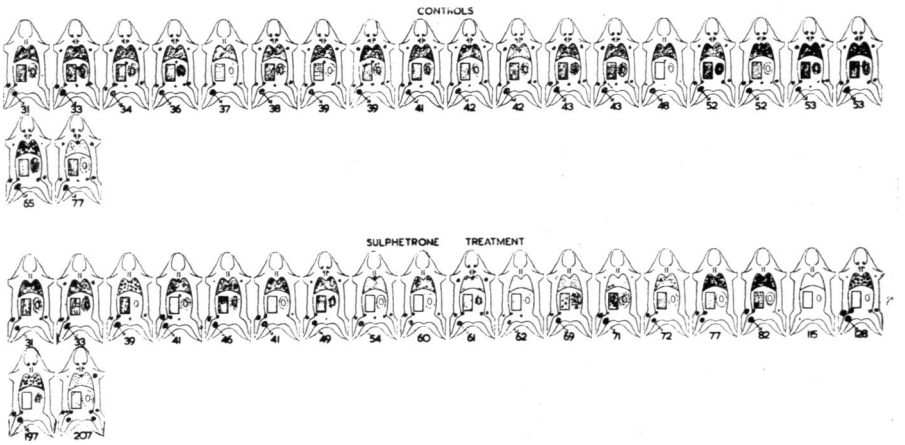

FIG. 3.—*First experiment.* Guinea-pigs infected with 0.25 mg. of the virulent bovine strain of tubercle bacilli CN. 858. A pictorial record of the gross amount and distribution of tuberculosis seen at death in untreated and sulphetrone-treated animals. The number beneath an animal shows the days of life after infection. Although observed until death from tuberculosis, there is less gross disease in the treated group.

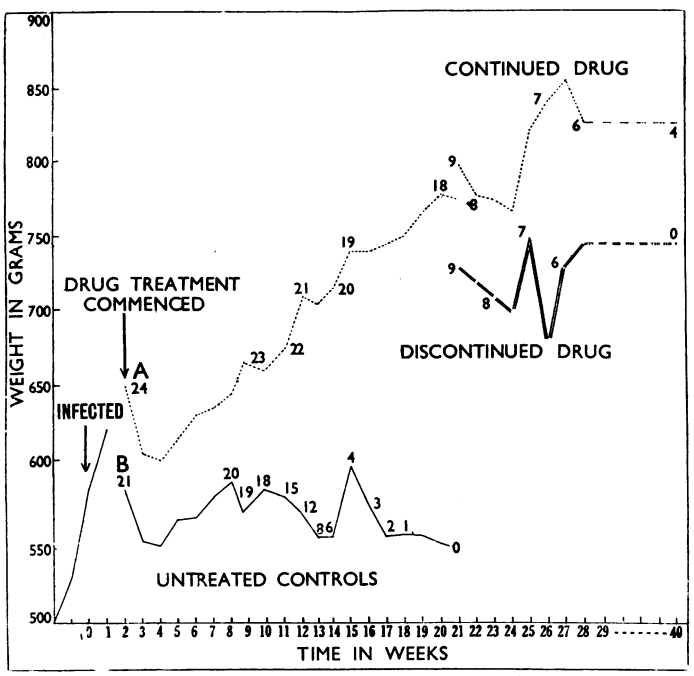

FIG. 4.—*Second experiment.* Weights and survival times of guinea-pigs after intramuscular infection with a human strain of tubercle bacillus. A, treated with sulphetrone; B, untreated. When all controls had died, the 18 survivors in group A were subdivided to give a drug-continued group and a drug-discontinued group. The figures on the graphs refer to survivors.

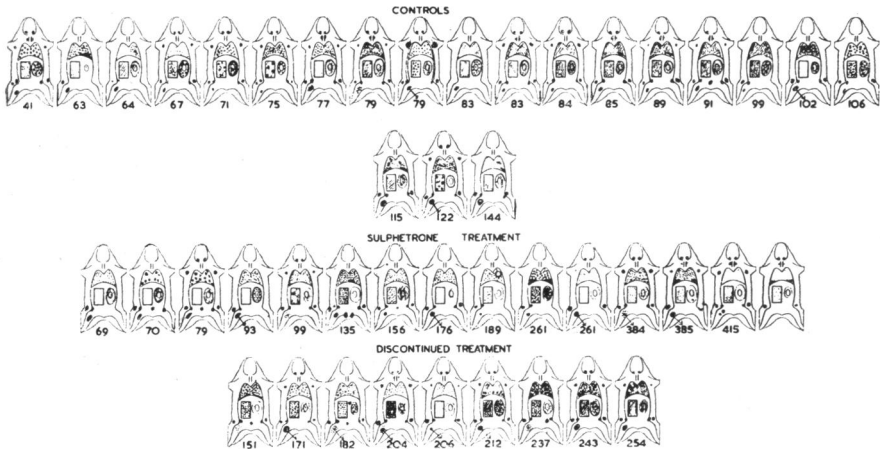

FIG. 5.—*Second experiment.* Guinea-pigs infected with 0.25 mg. of the virulent human strain of tubercle bacilli CN. 271 (H. 37). A pictorial record of the gross amount and distribution of tuberculosis seen at death in untreated and treated animals. The number beneath an animal shows the days of life after infection. Although observed until death from tuberculosis, there is less gross disease in the treated group.

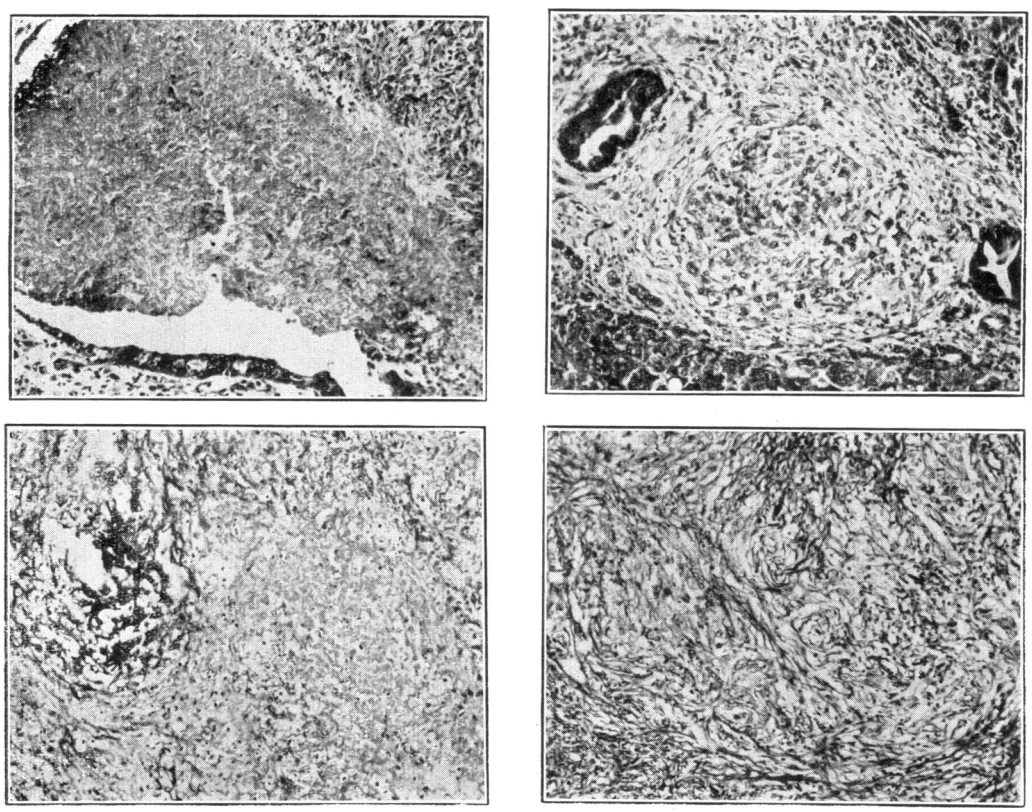

FIG. 6.—*Second experiment, H.* 37, *human strain.* (Top left) Area of progressive destructive tuberculosis in the liver of an untreated guinea-pig which died 143 days after infection with tubercle bacilli (× 66). (Top right) Fibrotic healing tubercles in the liver of a guinea-pig treated for 384 days with sulphetrone (× 66). (Bottom left) Progressive destructive tuberculous area in an inguinal lymph node of an untreated infected guinea-pig which died 143 days after infection (× 123). (Bottom right) Non-progressive, markedly fibrotic involution in an inguinal lymph-node of a guinea-pig treated for 384 days with sulphetrone.

his collaborators to evaluate the status of chemotherapeutic antituberculous drugs. It is their opinion, with which we agree, that the sulphone drugs are not capable of eliminating virulent organisms from the organs of predilection in the experimental animal.

In making this first report of the antituberculous activity of sulphetrone it seemed proper to record tests which presented the drug in its least favourable light

In the experiments reported here, the amounts of the virulent strains inoculated were large, and in addition to an experiment with a human strain, a test with a bovine strain is reported. Also, the animals were observed until death. In another place comparative tests with other drugs will be reported.

Sulphetrone is a derivative of diaminodiphenylsulphone of low, acute and chronic toxicity. As will be reported elsewhere, its lack of toxicity is such that doses equivalent to those given to guinea-pigs and sufficient to give blood concentrations of 5 to 7.5 mg. per 100 c.c. have been administered to man for continuous periods of 12 or more months. In this respect it differs from promin and diasone whose toxicity is such that they may be given for only short and intermittent periods. Since others have considered that the therapeutic action of promin and diasone may be due to some extent to their degradation to diaminodiphenylsulphone, it is interesting that with sulphetrone the evidence, both chemical and pharmacological, is that this breakdown does not occur.

In the first test the experimental conditions, both in respect to the amount of the virulent infecting organism and to its bovine origin, were severe. Nevertheless, the group of infected guinea-pigs receiving 2 per cent of sulphetrone in their diet showed a significant prolongation of survival-time. For example, at a time when half of the control animals were dead, only one-quarter of the treated group were dead ; when all control animals were dead, one-quarter of the treated group were still alive. Expressed in terms of average survival days the figure for the treated group was 77 days, and for the untreated group 45 days.

The inoculum used in the second experiment was the large injection of 0.25 mg. of the human virulent Saranac strain H. 37. In this instance the protection given by drug treatment was large. For example, when the last of the control animals had died, three-quarters of the treated animals were still alive, and as Fig. 4 shows, were in very good physical condition. In contrast, the animals from the control group showed gross tuberculosis, and histological examination revealed the presence of

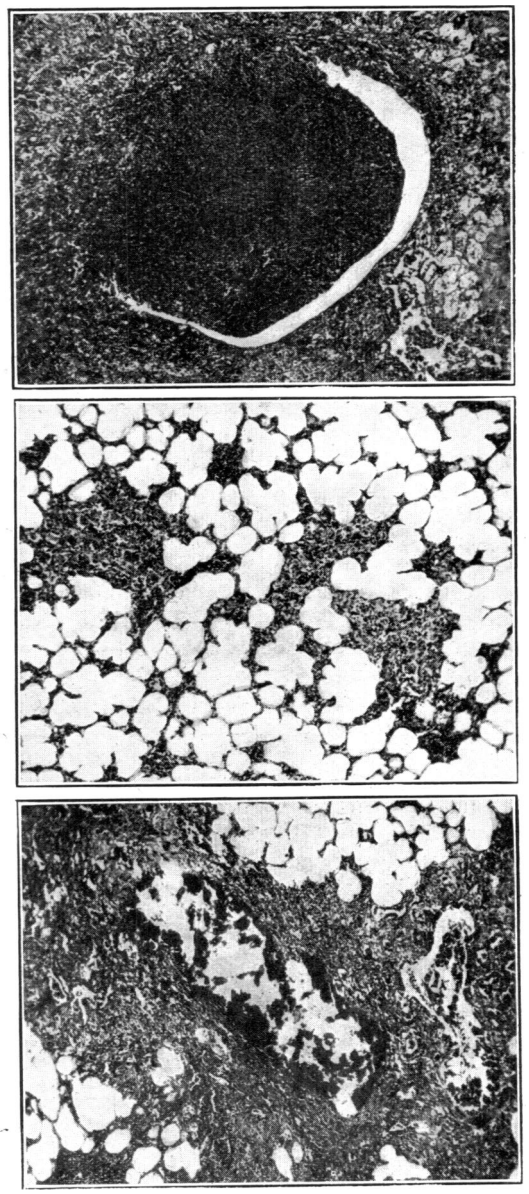

FIG. 7.—*Second experiment, H. 37, human strain.* (Top) Severe necrotizing progressive tuberculosis in the lungs of an untreated guinea-pig which died on the 143rd day after infection with tubercle bacilli (×123). (Centre) Discrete tuberculous nodules consisting of coalescent young miliary tubercles from the lungs of a guinea-pig treated for 154 days with sulphetrone and then treatment discontinued. Died on the 204th day (×123). (Bottom) Calcified fibrotic nodule in the lungs of a guinea-pig treated with sulphetrone until death on the 189th day (×123).

numerous tubercle bacilli. By dividing the survivors into two groups, one in which drug was continued and one in which drug was withdrawn, a very significant observation was made. The order of protection conferred by the drug was continued in one group and withheld in the other. Expressed in figures, it is shown that at the time when all members of the drug-discontinued group were dead, one-half of the drug-continued group were still alive. Throughout the entire drug-treated group macroscopic evidence obtained at necropsy showed very much less tuberculosis than in the untreated group. This was confirmed by histological examination and amplified by the observation that acid-fast organisms were very much less in number than in the untreated group, where they were numerous. The most significant histological evidence was the repeated finding of healed tuberculous lesions in spleen, liver, lungs, and lymph nodes; often they were calcified, particularly in lungs and lymph nodes.

In both experiments there is evidence of a therapeutic effect present only in the groups treated with sulphetrone. With both the bovine and the human strains the results suggest that sulphetrone exerts a retarding effect on the progressive nature of established experimental tuberculosis in the guinea-pig. It should be remembered that the conditions of the tests gave the infection the optimum advantage. In addition, in the experiment using the human strain, there was impressive histological evidence of a reversal from progressively destructive disease to one in which the morbid process was retarded; there was also evidence of resolution and calcification.

The therapeutic effect is shown most markedly by the difference between percentage survivors in the treated and untreated groups, but it is evident that sulphetrone, in common with other similar chemotherapeutic agents, is incapable of eliminating the causative organism.

SUMMARY

1. The chemotherapeutic value of 4,4′-bis(γ-phenyl - *n* - propylamino) - diphenylsulphone tetrasodium sulphonate, given the trade name of "sulphetrone," in treating experimental tuberculosis in guinea-pigs is described.

2. When sulphetrone was added to the diet in 2 per cent, average blood concentrations of 5 mg. per 100 c.c. were found throughout the experiments. The average amount of drug eaten was 0.6 g. daily.

3. In an experiment in which two groups of 20 animals were infected with a heavy inoculum of the virulent bovine strain of tubercle bacilli CN. 858, the survival time of the drug-treated group was prolonged. The average survival time was 45 days in the untreated group and 77 days in the treated group.

4. In a second experiment in which the infection was a heavy inoculum of the human virulent H. 37 strain (CN. 271), the treated group of 24 animals survived considerably longer than the untreated group of 21 animals.

5. There was a resurgence of tuberculosis of a miliary kind in a group of guinea-pigs in which drug treatment was stopped.

6. In the drug-treated animals which died there was impressive histological evidence of healed tubercles.

7. Under the severe conditions of the experiments, sulphetrone was capable of exerting a suppressive effect on the progressive nature of the experimental infections. Sulphetrone is bacteriostatic and, in common with other derivatives of diaminodiphenylsulphone, is not capable of eliminating the infective organism from animal tissue.

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REFERENCES

Brownlee, G., Green, A. F., and Woodbine, M. (1948). *Brit. J. Pharmacol.*, **3**, 15.

Bratton, A. C., and Marshall, E. K., Jnr. (1939). *J. biol. Chem.*, **128**, 537.

Coward, K. (1937). *Biological Standardization of the Vitamins*, 1st ed., p. 7, London; Baillière.

Feldman, W. H., and Hinsaw, H. C. (1943). *Amer. Rev. Tuberc.*, **48**, 256.

Feldman, W. H., and Hinsaw, H. C. (1945). *Amer. Rev. Tuberc.*, **51**, 268.

Feldman, W. H., Hinsaw, H. C., and Mann, F. C. (1944). *Amer. Rev. Tuberc.*, **50** 418.

Feldman, W. H., Hinsaw, H. C., and Moses, H. E. (1940). *Proc. Mayo Clin.*, **15**, 695.

Feldman, W. H., Hinsaw, H. C., and Moses, H. E. (1942). *Amer. Rev. Tuberc.*, **45**, 303.

Steenken, W., Jnr., and Gardner, L. U. (1946). *Amer. Rev. Tuberc.*, **54**, 62.