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THE CHEMOTHERAPEUTIC ACTION OF STREPTOMYCIN, SULPHETRONE, AND PROMIN IN EXPERIMENTAL TUBERCULOSIS*

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The accumulated evidence from both experimental chemotherapeutic and clinical sources makes it possible to say that while streptomycin approaches closer than any other known chemotherapeutic agent to the concept of a completely effective antituberculous drug, it fails to eliminate unconditionally the causative organism. Of significance in this connection is the clinical evidence presented elsewhere (Madigan, Swift, and Brownlee, 1947b) that the antibiotic appears to be most effective in rapid progressive disease, and least effective in chronic disease. The metabolism of the organism seems to be implicated in these effects, since in young dividing cells streptomycin is markedly effective, while it is ineffective against old resting cells. It appears to be significant that when smaller infections of tubercle bacilli are used (0.1 mg. H.37; Feldman, Hinshaw, and Mann, 1945) the results are better than when larger infections are used (1.0 mg. A.27; Smith and McClosky, 1945).

From theoretical considerations of what is known of the mode of action of diaminodiphenylsulphone drugs, it is not surprising that sulphones and streptomycin show a synergism of action. The synergism between streptomycin and promin demonstrated by Smith and McClosky (1945), and between streptomycin and diasone by Callomon, Kolmer, Rule, and Paul (1946), is of a high order and, in view of the relative failure of streptomycin by itself, suggests the possibility of combined clinical therapy. Sulphetrone, the pharmacology and chemotherapy of which has been recently described (Brownlee, Green, and Woodbine, 1948), is a diaminodiphenylsulphone derivative of low chronic toxicity which is effective in experimental tuberculosis and which may be used to maintain effective blood concentrations in man for continuous periods of 12 or more months (Brownlee and Kennedy, 1948).

The present report describes a comparison between the chemotherapeutic antituberculous activity of streptomycin, sulphetrone, promin, and combined streptomycin and sulphetrone.

Methods and Materials

A recent account (Brownlee and Kennedy, 1948) has appeared of the methods adopted in the management of guinea-pig tests. The same report should be consulted for the composition of the dry powdered Tubercle Diet No. 2 which constitutes the basic ration.

Mycobacterium tuberculosis strains

The strain CN.844 used in the present tests is a virulent human strain isolated in 1942 from a case of tuberculous adenitis, and maintained on synthetic fluid medium by implant transfer at 21 days. This virulent strain gives rise to a more chronic type of disease than the virulent Saranac strain CN.271 (H.37) previously used. This change was dictated by a temporary loss of virulence in the H.37 strain, a feature discussed by Steenken and Gardner (1946), who make suggestions for avoiding this complication.

Streptomycin

The sample of streptomycin sulphate used in this test was drawn from W.F. Batch 17 and is of 33 per cent purity. The chemotherapy and pharmacology of this batch together with the method of assay have been described (Madigan *et al.*, 1947a). The potency of streptomycin is stated in terms of weight of pure streptomycin base $C_{21}H_{37}N_7O_{42}$ (mol.wt.579) and is derived by assay with a working standard of streptomycin sulphate ($1\frac{1}{2}$ H_2SO_4; mol.wt.726) of known potency; it follows that streptomycin sulphate contains 79.8 per cent of base.

Sulphetrone

"Sulphetrone" is the registered name of the Wellcome Foundation Ltd. for 4:4′-bis(γ-phenyl-*n*-propylamino)diphenylsulphone tetrasodium sulphonate. It

* The development of sulphetrone is part of a programme of work on antituberculous compounds carried out by the Therapeutic Research Corporation of Great Britain.

is available as a cream-coloured amorphous powder containing 9–10 per cent of water. Assays in this report are in terms of anhydrous material. Promin, 4:4′-diaminodiphenylsulphone-N, N′-didextrose sodium sulphonate, is a cream-coloured product containing, when freshly prepared, 10 per cent of water; assays are also given in terms of anhydrous material.

The estimation of drugs in body fluids

The estimation of sulphetrone in body fluids has been described (Brownlee, Green, and Woodbine, 1948), and is based upon diazotization and coupling to N(1-naphthyl)-ethylenediamine hydrochloride (Bratton and Marshall, 1939). The essential points are an over-all dilution of 1 in 15 and the concentration of acid, both of which are critical. Promin was estimated by the same method, which gave recoveries of some 75 per cent, to which a correction factor was applied. Streptomycin in blood was estimated by the slide technique of Fleming (1943), using a susceptible haemolytic strain of *Escherichia coli* CN.1360. Blood, in quantities up to 0.5 c.c., was obtained from the ear of the guinea-pig by drawing it into a clean heparinized capillary tube.

Experimental

Eighty-three young adult guinea-pigs of mixed sexes, weighing 650–700 g., were inoculated deeply into the right thigh muscles with 0.25 mg. of a fourteen-day-old sub-culture of human tubercle bacillus, CN.844. Seventeen days after infection the animals were tested for their reaction to an intradermal injection of 0.1 c.c. of 1/10,000 Old Tuberculin. All animals gave a positive reaction; eight reactions were classed as mild. Tuberculin tests were repeated at the 15th and 23rd week of infection. On the twenty-first day, liver samples were obtained from two animals from each group by biopsy under pentobarbital sodium anaesthesia. On the following day the animals were divided into five groups, four of 18 animals and a control group of 11 animals.

Streptomycin treatment

One group of 18 guinea-pigs received the equivalent of 10 mg. of pure streptomycin base daily. Four four-hourly intraperitoneal injections each of 2.5 mg. of streptomycin base contained in 0.25 c.c. of pyrogen-free distilled water were given with aseptic precautions at 8 a.m., 12 noon, 4 p.m., and 8 p.m.; no drug was given during the intermediate 12 hours. Treatment was continued for 168 days, during which time each guinea-pig received 1.68 g. of the antibiotic. Concentrations of streptomycin per c.c. of plasma were estimated at intervals throughout the test and the average figures 1 hour after injection were 7.0 μg. $\pm$1.0 (S.E. of mean of 60 observations), and at 3.5 hours 4.0 μg.$\pm$1.0 (72); at 4 hours no streptomycin could be detected in the plasma. Two animals in this group died from intraperitoneal haemorrhage as a result of accidental puncture of the spleen, one at the 7th week after infection, and one at the 8th week.

Combined streptomycin and sulphetrone treatment

In addition to treatment with 10 mg. of streptomycin base daily, a group of 18 guinea-pigs also received 2 per cent of sulphetrone incorporated in the dry diet.

Plasma concentrations of streptomycin were estimated at intervals throughout the test, and the average figures were similar to those of the streptomycin-treated group; these were 7.0 μg.$\pm$1.5 (90) at 1 hour, and 4.0 μg.$\pm$1.0 (90) at 3.5 hours. The average blood sulphetrone concentration per 100 c.c. was 5.1 mg.$\pm$ 3.8 (90).

Sulphetrone treatment

A group of 18 guinea-pigs received 2 per cent of sulphetrone in the dry diet. This corresponded to an average drug intake of 600 mg. daily and resulted in an average blood sulphetrone concentration per 100 c.c. of 5.9 mg.$\pm$3.5 (86).

Promin treatment

A group of 18 animals was treated with promin incorporated to a concentration of 0.5 per cent in the diet. The average drug intake was 150 mg. daily and gave a blood promin concentration per 100 c.c. of 3.5 mg.$\pm$2.2 (60).

Control group

Eleven animals constituted the control group and received no treatment.

Results

Survival times

Inoculation with 0.25 mg. of the CN.844 strain established a slow chronic type of infection well suited to the comparison; for this reason only three animals in the control group of 11 died during the experiment of 168 days, and these from gross generalized tuberculosis. In the same period of time two animals died in the streptomycin treated group at 46 and 56 days owing to intraperitoneal haemorrhage as a result of needle injury to the spleen. Post-mortem examination revealed minimal tuberculosis. An unfortunate accident resulted in the death of five additional guinea-pigs of this group, on the 66th day, thus reducing the effective number of streptomycin guinea-pigs to eleven. Post-mortem examination of these animals showed no detectable lesions. Two animals in the combined streptomycin and sulphetrone group died with intraperitoneal haemorrhage owing to spleen injury; in both animals the tuberculous lesions were minimal. One animal found dead on the 168th day had no tuberculosis other than a small abscess at the site of injection and an enlarged inferior inguinal gland; the cause of death was believed to have been a concurrent salmonella infection. Four animals in the sulphetrone group

died ; in none of them was the tuberculous process so well advanced as to justify the view that death was due to tuberculosis. Within the promin group there were seven deaths: one of these, at 25 days, was due to a streptococcal pneumonia, minimal tuberculous lesions being present ; in the other six animals the tuberculosis was extensive enough to justify the belief that it had been the major cause of death, a contributory feature being the toxic nature of the drug ; guinea-pigs in this group were subdued and in poor condition.

Effects on weight

The influence of the drug treatment on the average weights of the guinea-pigs is shown in Fig. 1, where the effect of the four test substances and combination of substances is compared with the control group. Both groups receiving streptomycin by intraperitoneal injection show a loss of weight which lasts for some six weeks and is then followed by recovery. The fact that the final weight records show the greatest gains within this group suggests that the temporary loss of weight may have been caused by the repeated intraperitoneal injections at the commencement rather than by a specific toxic effect.

The group receiving sulphetrone gained in weight, and the group receiving combined sulphetrone and streptomycin, though they barely maintained their weight, were in good condition and more alert than any other group. The promin group lost as much weight as did the controls, with which they compared unfavourably in general condition. When the experiment ended their condition was exceedingly poor and suggestive of toxaemia.

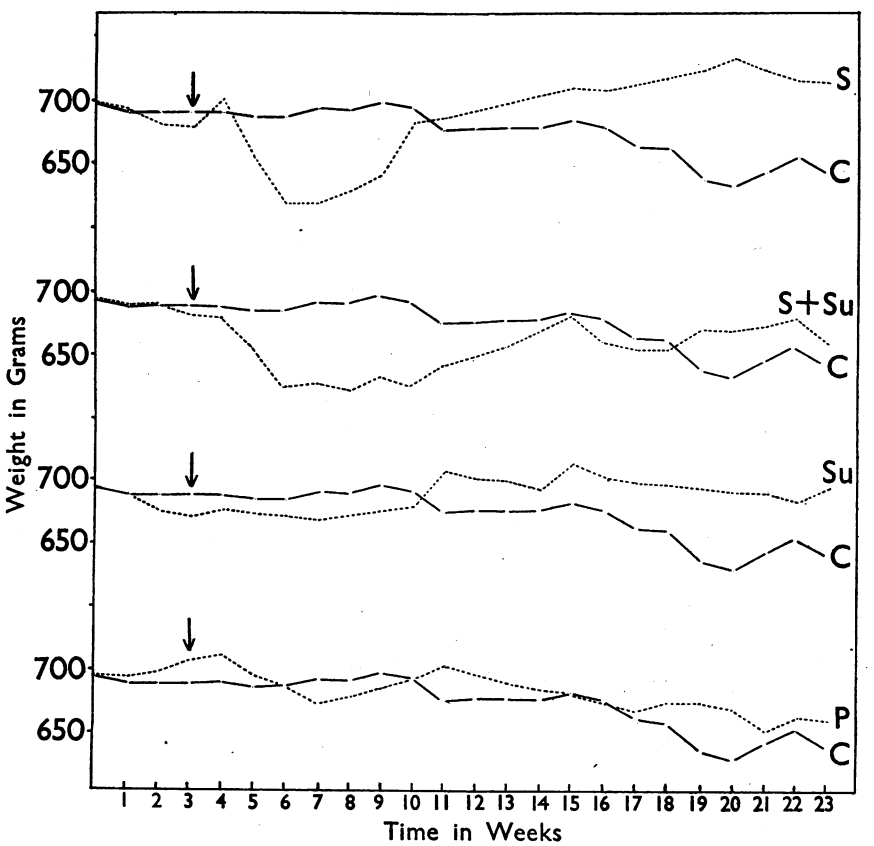

FIG. 1.—The influence of drugs on the average weights of groups of guinea-pigs infected with 0.25 mg. of a virulent strain of human tubercle bacilli. Drug therapy begun at arrow. S, streptomycin. S+Su, streptomycin with sulphetrone. Su, sulphetrone. P, promin. C, control untreated group.

Clinical

Differences between the treated and untreated groups, and within the treated groups themselves, were apparent by 90 days after infection. In the untreated group the superior and inferior inguinal glands were grossly enlarged. In the groups treated with streptomycin alone and with streptomycin and sulphetrone these glands were not enlarged, or only slightly so. This difference was less obvious between the sulphetrone and control groups and not evident at all in the comparison of the promin and control groups. The normal appearance of the groups on streptomycin alone and on combined therapy contrasted strikingly with the toxic appearance of the group treated with promin.

Tuberculin tests

Throughout the course of the experiment three tuberculin tests were made. The first, for the purpose of rejecting non-reactors, was on the 17th day; a second was made on the 105th day, and a third on the 147th day. On each occasion 0.1 c.c. of a 1 in 10,000 dilution of Old Tuberculin was injected intradermally and the subsequent reaction read at 72 hours. At the second test the severity of the reactions within the different groups appeared to be related to the extent of the disease suggested by the records of enlarged glands, losses in weight, and general health of the animals.

Reducing the clinical observations to a simple numerical basis gave the figures shown in Table I for both 105 and 147 days. The numerical notation is as follows: 1, negative; 2, query negative, or a needle injury; 3, weak positive such as mild diffuse staining of skin or a small reddened area of 3 mm. or less in diameter; 4, positive, without necrosis; 5, positive, with necrosis of mild limited appearance; 6, positive, with necrosis of moderate extensive appearance; 7, positive, severe reaction. The Table shows the mean numerical indices corresponding to degrees of severity of actions.

TABLE I

TUBERCULIN REACTIONS IN DRUG-TREATED AND UNTREATED GUINEA-PIGS

Group	Number of guinea-pigs	Mean numerical index of reactions ± S.E.	
		105 days	147 days
Streptomycin	12	3.8 ± 1.2	4.5 ± 1.6
Streptomycin + Sulphetrone ..	16	2.5 ± 0.9	3.7 ± 1.3
Sulphetrone ..	15	4.1 ± 1.4	4.5 ± 1.4
Promin ..	16	4.5 ± 1.3	4.9 ± 1.8
Control ..	11	5.2 ± 1.6	4.5 ± 1.4

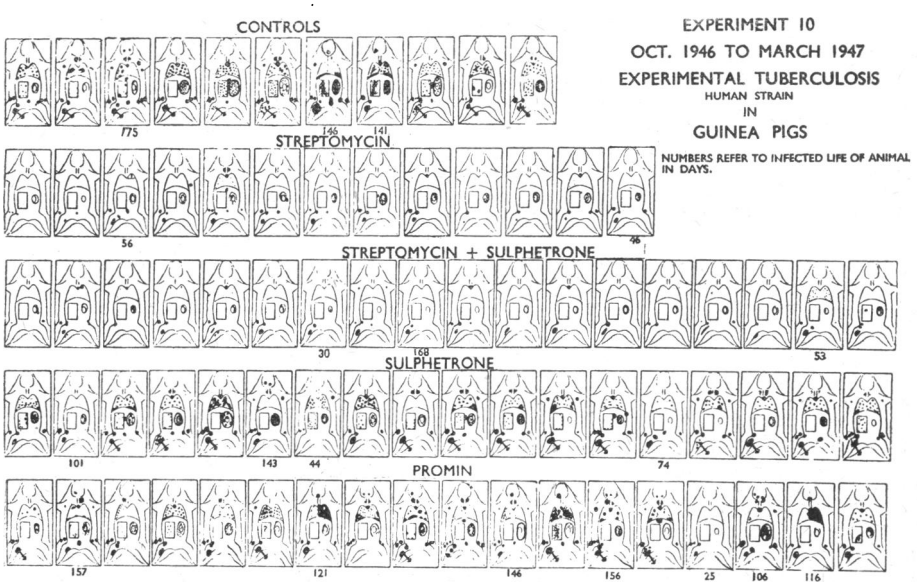

FIG. 2.—A pictorial representation of the degree and distribution of experimental tuberculosis in guinea-pigs *post mortem* 178 days after infection with 0.25 mg. of a virulent strain of tubercle bacilli. The drug-treated groups received 10 mg. streptomycin base daily, 10 mg. streptomycin base daily plus 2 per cent sulphetrone in the diet, 2 per cent sulphetrone in the diet, and 0.5 per cent promin in the diet. The control group was untreated.

Although the disease appears to have been progressive during the course of treatment, the comparative effectiveness of each drug in limiting the spread of the disease appears to have remained constant. Statistically, the differences are probably real.

The enlargement in the accessory glands also shows that the disease was progressive in all groups during the interval between the second and third tests. The decrease in the tuberculin reaction in the control group is probably explained by the common observation, both in animals and man, that in the course of progressive disease skin reactions often become less sensitive.

Necropsy studies

The experiment was terminated on the 178th day and the animals brought to post-mortem examination. We have become used to observing and recording differences between treated and untreated animals, but have not met previously such striking differences as were observed between the group which received combined treatment and the controls, and to a less extent between the streptomycin and control groups. In these treated groups both spleen and liver were normal in size and colour and tuberculosis was minimal in extent.

There was gross, extensive, and progressive generalized tuberculosis in all animals of the untreated group. The promin treated group showed, in general, markedly less tuberculosis, although in many animals spleens were grossly tuberculous. Within this group five animals had disease comparable to that seen in the control group, with extensive caseation and cavitation of the lungs. The sulphetrone group showed much less tuberculosis than the control group and less tuberculosis than the promin group. This was particularly evident in the spleens and liver.

The extent and distribution of the disease in the drug treated and the untreated groups is shown pictorially in Fig. 2 and numerically in Table II in which, following the excellent notation of Feldman (1943), scores are given for the extent and character of the tuberculosis observed macroscopically in spleen, liver, lungs, and glands.

TABLE II

AVERAGE SEVERITY AND DISTRIBUTION OF TUBERCULOSIS EXPRESSED NUMERICALLY

Group	Macroscopic examination of the tissues involved					
	Number of animals	Spleen (maximum 35)	Lungs (maximum 30)	Liver (maximum 25)	Site and lymph nodes (maximum 10)	Average index (maximum 100)
Controls	11	30.5	25.5	19	10	85.0
Streptomycin ..	13	13.9	4.6	0	9	27.5
Streptomycin and sulphetrone ..	18	9.4	2.5	0.5	8.3	20.7
Sulphetrone ..	18	20.0	18.6	9.4	9.4	57.4
Promin ..	18	24.2	22.2	11.7	10	68.1

Bacteriology

Post mortem the spleens were removed aseptically and divided for histological examination and examination for tubercle bacilli. For the latter purpose they were ground in a previously sterilized mortar in 6*N* HCl, spun, neutralized with a calculated amount of NaOH and seeded on to egg-medium. Acid-fast organisms of typical morphological appearance were recovered from all the control animals; within the promin group there were 16 out of 18 recoveries, with sulphetrone 12 out of 18, with streptomycin 4 out of 13, and from the combined therapy group 2 very scanty growths from 18 cultures. It was noticeable that the cultures obtained from the promin treated pigs grew luxuriantly, in contrast to all other treated groups.

Sensitivity of strain and cultures to drugs

The CN.844 strain when grown in Long's Synthetic Medium is inhibited by 0.5 μg./c.c. of streptomycin, by 65 μg./c.c. of sulphetrone, by 125 μg. of promin, and by a mixture of 30 μg./c.c. sulphetrone and 0.25 μg./c.c. streptomycin, in which mixture an additive, not a synergistic, effect is seen. Significant, in view of the therapeutic effects, was the finding that all strains recovered *post mortem* were unchanged in sensitivity to streptomycin and sulphone drugs.

Histology

Examination of histological material from the untreated control group revealed a uniform picture of severe, progressive infection. Confluent tuberculous interstitial pneumonia, with frequent caseation and many acid-fast bacilli, was seen in the lungs; the livers exhibited multiple tubercles spreading outwards from the portal tracts with many acid-fast bacilli in infarcted areas. Spleens had massive infarcts, haemorrhages, and coalescent small tubercles, mostly with caseous centres, and many acid-fast bacilli were present. The

lymph nodes were occupied by confluent caseating tubercles and generally acid-fast organisms were seen.

The treated groups differed, more or less, in the amount and distribution of the tuberculosis and in the numbers of acid-fast bacilli seen.

The promin group differed least from the controls. Involvement of the lungs was the rule, but there was evidence of localization and repair. Livers showed little frank tuberculosis, but there were extensive foci of recent necrosis; acid-fast organisms were rarely seen. Spleens were extensively involved, with numerous tubercles, extensive infarction and congestion; acid-fast organisms were rare.

The restraining powers of the sulphone drugs were carried a demonstrable step forward in the sulphetrone group. Lungs were less extensively involved, the lesions being limited at the most to patchy tuberculous interstitial pneumonia coalescing in areas into large tubercles, with rare necrosis; at the least, there was congestion and oedema; acid-fast bacilli were not seen. Livers showed small areas of miliary tubercles and an occasional fibrosing tubercle; acid-fast bacilli were not seen. Spleens showed numerous small tubercles in a congested oedematous pulp but without acid-fast bacilli being identified.

The streptomycin group differed markedly from the controls. Only 5 out of 13 lungs showed tuberculous lesions: in two they were restricted to single fibrotic calcified tubercles. In only one animal was there a tuberculous lesion in the liver. With the exception of one animal in which the disease appeared to be non-progressive, tubercles were found in all spleens. Mostly the tubercles were fibrotic (5 cases), or calcified (7). Tubercles were seen in all the contiguous lymph nodes; in three animals they were limited to small fibrotic areas

The group treated with streptomycin and sulphetrone differed remarkably from the controls and presented an impressive degree of protection even when compared with the streptomycin group. Only 6 out of 18 lungs showed tuberculous lesions; in five of these the lesion was calcified and fibrotic. All lungs showed evidence of many healed lesions. In only one liver was a tubercle found and this was a small hardened epithelioid node. The spleens were involved in 14 out of 18 animals, but all the lesions were non-progressive, consisting of areas of diffuse fibrosis or of repressive lesions with calcification. Lymph nodes adjacent to the inoculation site were minimally involved in all animals; all the lesions were non-progressive and consisted almost entirely of fibrous tissue with some calcification.

Discussion

Although incapable of eliminating the causative organism unconditionally, streptomycin is capable of modifying favourably the course of inoculation tuberculosis in the experimental animal (Smith and McClosky, 1945; Feldman, Hinshaw, and Mann, 1945; Youmans and McCarter, 1945). Viewed from the base-line laid down by experimental and clinical sulphone therapy the significant advance in efficiency made by this new antibiotic justified a careful clinical assessment. From the clinical reports now available it is apparent that the antibiotic is able to influence tuberculosis to only a limited extent and under certain conditions. Ironically enough these appear to be the conditions under which the bacillus is multiplying rapidly, for example in miliary tuberculosis. The importance of appreciating the reasons for these failures needs no stressing. Thus, Baggenstoss, Feldman, and Hinshaw (1947) record the death of 5 cases of miliary tuberculosis in spite of healing in the lungs, and they note that streptomycin could not be demonstrated in brain substance in which tuberculous lesions were found. Madigan, Swift, and Brownlee (1947b) had similiar failures with miliary tuberculosis to report, and in addition they could not record healing in phthisis of diverse origin. If the disease in the experimental animal is regarded as different from the diseases due to *M. tuberculosis* in man, this difference reflects the metabolic adaptability of the organism and it is to countering this aptitude that we must address our efforts. The acute experiments, here described, offer no evidence of adaptation of the organism to any of the chemotherapeutic agents; significant in this connection is the record of Madigan *et al.* (1947b), that in chronic disease (phthisis) the organisms acquired resistance to streptomycin, while in acute infection (miliary tuberculosis) in which healing was seen the susceptibility of the organism was unchanged. Experimental evidence is presented by the same authors, that streptomycin is most effective against young dividing bacilli and ineffective against old resting cells. A similar observation is made by Middlebrook and Yegian (1946). A practical approach to the attack on the metabolic adaptability of the tubercle bacillus is that of Smith and McClosky (1945), who showed the synergistic action of streptomycin and promin in experimental tuberculosis; Callomon, Kolmer, Rule, and Paul (1946) also demonstrated a similar effect with diasone and streptomycin.

The present report is concerned with the synergism of streptomycin and sulphetrone, a diaminodiphenylsulphone derivative which combines efficiency of action with freedom from the chronic toxicity associated with the use of promin and diasone. Groups of animals treated with promin, with sulphetrone, and with streptomycin enable a comparison to be made.

On the basis of survival time, change in weight, response to tuberculin tests, macroscopic evidence of gross tuberculosis *post mortem* or microscopic examination there is presented a uniform picture of the marked superiority of the combined therapy. The order of efficiency is streptomycin with sulphetrone, streptomycin, sulphetrone, and promin. It should be noted that oral promin exhibits the drug by its most toxic route and that the resulting blood levels are consequently low; this remains, however, a practical problem. It is interesting that a relation was found between the degree of protection and the severity of intradermal tuberculin reactions, an observation in contrast to that of Smith and McClosky (1945). A careful sifting of all evidence here presented leaves the impression that the disease was progressive in all groups, albeit at a much suppressed rate in those groups where protection was greatest. Nevertheless, with this implication in mind, the experimental effects produced by the combined streptomycin and sulphetrone therapy are believed to justify a careful clinical evaluation in selected cases. The need meanwhile is for improved antituberculous bacterial antibiotics, which will not induce resistant strains.

SUMMARY

1. Four groups of 18 guinea-pigs, and one control group of 11, infected with 0.25 mg. of a human virulent strain CN.844 of *M. tuberculosis* were treated 22 days later with drugs for 168 days. One group received 0.5 per cent of promin in the diet, one 2 per cent sulphetrone in the diet, one 10 mg. of streptomycin parenterally daily, and a fourth 2 per cent sulphetrone and 10 mg. of streptomycin. The control group of 11 animals remained untreated.

2. Twice during treatment all animals were tested for reactions to the intradermal injection of Old Tuberculin. There appeared to be a simple relation between the degree of severity of the intradermal reactions and the course of the disease judged by weight records and enlargement of lymph nodes.

3. Records of losses in weight, enlargement of glands, relation to tuberculin tests, macroscopic evidence of distribution of tuberculosis *post mortem*, and the evidence of histology, all presented a uniform picture of degrees of protection. All drug-treated groups showed evidence of protection. It was least in the promin group, became greater in the sulphetrone group, still greater in the streptomycin group, and was greatest in the group treated with sulphetrone and streptomycin. In the latter group the protection was so marked as to be clearly synergistic.

4. The opinion is expressed that the disease was progressive in all groups.

5. Strains of the tubercle bacillus recovered from the drug-treated groups were unchanged in their susceptibility to the antibacterial action of the drugs.

6. The experimental effects produced by combined streptomycin and sulphetrone therapy are believed to justify a careful clinical evaluation in selected cases.

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