The administration of 10 mg per kilo of the same compound to 2 normal dogs for 6 days did not affect their blood pressure.

Discussion of Results. Our previous results on hypertensive rats have shown that 1,2-cyclohexandione is inactive, but that 1,4-cyclohexandione possesses moderate antipressor activity. Although 1,4-cyclohexandione is much less active than some of the quinones previously investigated, it is so much less toxic to rats and dogs that it offers greater promise as an antipressor agent than any of the other 34 compounds we have studied thus far.

The mode of action of all these antipressor agents is still obscure. The antipressor activity of the dicarbonyl compounds declined in the order, *p*-quinones, *o*-quinones, dialdehydes, cyclic diketones, straight-chain diketones, and their toxicity decreased in the same order. The cyclic diketones are the first group of compounds in this series which are not too toxic for use.

Summary. Daily intramuscular injections for one week of the diketone, 1,4-cyclohexandione, into hypertensive dogs resulted in a moderate reduction of blood pressure. In the dosage thus far employed there has been no gross evidence of local or general toxic effect.

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An Improved Method for Testing of Bacteriostatic Agents Using Virulent Human Type Tubercle Bacilli.*

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The demonstration by Feldman and his coworkers[1-3] and others[4,5] of the therapeutic effect of various compounds on experimental tuberculosis of guinea pigs increases the necessity for a rapid and accurate *in vitro* method for testing the relative growth inhibitory powers of compounds on *M. tuberculosis var. hominis.*

Several investigators[5-15] have used virulent human tubercle bacilli for this purpose, employing a variety of media both liquid and solid. The use of complex solid or liquid media is unsatisfactory because of the possible presence of antagonists; agar when added to a synthetic medium is in itself slightly inhibitory.[16] The use of liquid synthetic media has previously required relatively large volumes of medium in flasks, to provide sufficient surface growth, inoculated on the surface with flakes of pellicle growth. The results are usu-

* This work was aided by a grant from Parke Davis and Co., Detroit, Mich.

1 Feldman, William H., Mann, Frank C., and Hinshaw, H. Corwin, *Am. Rev. Tuberc.*, 1942, **46**, 187.

2 Feldman, William H., and Hinshaw, H. Corwin, *Am. J. Clin. Path.*, 1943, **13**, 144.

3 Feldman, William H., Hinshaw, H. Corwin, and Moses, Harold E., *Arch. Path.*, 1943, **36**, 1.

4 Callomon, Fritz F. T., *Am. Rev. Tuberc.*, 1943, **47**, 97.

5 Smith, M. I., Emmart, E. W., and Westfall, B. B., *J. Pharm. and Exp. Therap.*, 1942, **74**, 163.

6 Boissevain, C. H., *Nat. Tuberc. Assn. Tr.*, 1940, **36**, 88.

7 Ballon, H. C., and Guernon, A., *J. Thorac. Surg.*, 1938, **8**, 184.

8 Ballon, H. C., and Guernon, A., *Tubercle*, 1940, **21**, 153.

9 Birkhaug, K. E., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 275.

10 Follis, R. H., *Am. Rev. Tuberc.*, 1940, **41**, 117.

11 Heise, F. H., and Steeken, W., *Am. Rev. Tuberc.*, 1941, **44**, 635.

12 Rist, Noel, *C. R. Soc. de biol.*, 1939, **130**, 972.

13 Middlebrook, Gardner, and Lloyd, John B., *Am. Rev. Tuberc.*, 1944, **49**, 535.

14 Middlebrook, Gardner, and Lloyd, John B., *Ibid.*, 1944, **49**, 539.

15 Saz, Arthur K., and Bernheim, Frederick, *J. Pharm. and Exp. Therap.*, 1941, **73**, 78.

16 Drea, W. F., *J. Bact.*, 1940, **39**, 197.

ally read over a period of 4-8 weeks in terms of inhibition of pellicle growth. The above methods are cumbersome and time consuming, do not permit the use of a uniform inoculum and the results are difficult to read.

These technical difficulties have led several investigators[17-19] to substitute rapidly growing avirulent acid-fast bacilli as test organisms. The validity of the use of such organisms for this purpose would depend upon there being a correlation between the inhibitory action of chemotherapeutic compounds upon the virulent and avirulent strains. So far this correlation has been assumed without being demonstrated.

The demonstration by Drea[16,20] and by Youmans[21] that suspensions of virulent human tubercle bacilli will grow rapidly beneath the surface and eventually form pellicle growth when inoculated into a synthetic medium permits the utilization of these virulent bacilli as test organisms in the same manner that avirulent rapidly growing acid-fast organisms have hitherto been employed.

Methods. In view of the fact that the H37Rv strain has been extensively utilized as the test organism for *in vivo* work[1-3] it was chosen as the *in vitro* virulent test organism. For comparison we chose National Type Culture Collection organism 607, an avirulent rapidly growing acid-fast bacillus, employed by both Fitzgerald and Feinstone[19] and Freedlander[17,18] as an *in vitro* test organism. Suspensions were made as previously described[21] from 21-day-old surface growths except that in addition all cultures were washed 3 times with double distilled water before suspensions were made. The medium employed was the synthetic one previously described.[21†] Suspensions were standardized by centrifugation in Hopkins vaccine tubes.

The compounds used were 4,4'diaminodiphenyl sulfone, sulfanilamide, sulfathiazole, and sulfadiazine. These were diluted serially, starting with concentrations of 10 mg %, each dilution being half the preceding one. Separate pipettes were used for each dilution. The final volume of each concentration was 10.0 cc and sterilization was accomplished by autoclaving at 10 lb for 20 minutes. Each tube was inoculated, including suitable controls, with 0.1 cc of suspension containing the desired amount of tubercle bacilli. Readings of growth inhibition of the rapidly growing avirulent 607 strain were made at 7 days, and of the slower growing virulent H37Rv culture at 21 days. The latter cultures were shaken at 7 and 14 days since more regular development of surface growth occurred in the controls following this treatment. Readings were made by comparing the growth in the tubes containing the drug with the growth present in medium containing no drug. All tests were run in duplicate and were usually found to check within limits (plus or minus) of one dilution when using the avirulent 607 strain. With the H37Rv culture greater variations were sometimes encountered. Since it was observed that the growth-inhibiting concentrations were not identical for surface and subsurface growth, separate recordings were made for each.

Results. The lowest concentrations necessary to completely inhibit subsurface growth were usually higher than those necessary to completely inhibit surface growth. (Table I.)

The amount of inoculum used was important. With inocula of 1.0 mg H37Rv and 0.1 mg 607 there was subsurface growth in all of the concentrations of all 4 compounds, whereas surface growth was completely inhibited at some point in the series. One-tenth mg H37Rv and 0.01 mg 607 usually gave endpoints of complete growth inhibition for both surface and subsurface growth. Smaller inocula of H37Rv were unsatisfactory because of the prolonged growth period and irregular

[17] Freedlander, B. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 153.

[18] Freedlander, B. L., *Am. Rev. Tuberc.*, 1944, **49**, 543.

[19] Fitzgerald, Robert S., and Feinstone, W. Harry, PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 27.

[20] Drea, W. F., *J. Bact.*, 1943, **44**, 149.

[21] Youmans, Guy P.

† The troublesome precipitates sometimes encountered on autoclaving this medium can be prevented by substituting K_2SO_4 for the $MgSO_4$ in the formula. Growth of the organisms is not affected by this substitution.

TABLE I.
Lowest Molar Concentrations of Compounds Completely Inhibiting Surface and Subsurface Growth of H37Rv and 607 and the 4,4'diamino diphenyl sulfone Coefficients.

	607		H37Rv	
	Molar concentration	Coefficient	Molar concentration	Coefficient
	Subsurface Growth			
4,4'diamino diphenyl sulfone	2.20×10^{-4}	1.0	2.82×10^{-5}	1.0
Sulfanilamide	$>2.18 \times 10^{-4}$	< 1.02	$>1.16 \times 10^{-3}$	<0.0244
Sulfathiazole	5.32×10^{-6}	41.5	3.06×10^{-5}	0.927
Sulfadiazine	1.34×10^{-5}	17.7	1.04×10^{-4}	0.272
	Surface Growth			
4,4'diamino diphenyl sulfone	2.34×10^{-5}	1.0	6.28×10^{-6}	1.0
Sulfanilamide	1.45×10^{-4}	0.161	5.36×10^{-4}	0.0144
Sulfathiazole	3.06×10^{-6}	7.47	7.64×10^{-6}	0.824
Sulfadiazine	1.24×10^{-5}	1.87	3.74×10^{-5}	0.168

results, whereas smaller inocula of 607 behaved in much the same manner as the 0.01 mg inoculum.

Table I shows the molar concentrations of the 4 compounds which completely inhibited surface and subsurface growth of both H37Rv and 607 using 0.1 and 0.01 mg inocula respectively. These figures are averages of 6 separate duplicate tests run at different times. In addition, the table gives the "4,4'diaminodiphenyl sulfone coefficient," calculated by dividing the inhibiting molar concentration of this drug by the inhibiting molar concentrations of the other compounds. This gives the relative effectiveness of these compounds in inhibiting growth as compared with the effectiveness of diaminodiphenylsulfone.

None of the compounds inhibited equally the growth of the two organisms. Sulfathiazole and sulfadiazine were far more effective in inhibiting the growth of M. 607 than was 4,4'diaminodiphenylsulfone, although they were somewhat less effective, relatively, in inhibiting surface growth of this organism, than subsurface growth. On the other hand both surface and subsurface growth of H37Rv was more effectively inhibited by 4-4'diaminodiphenylsulfone than by any of the others although sulfathiazole was almost equal to it in effectiveness.

Fitzgerald and Feinstone[19] also found that sulfathiazole and sulfadiazine were more effective in inhibiting growth of 607 than 4,4'diaminodiphenylsulfone. However, they found sulfadiazine and sulfathiazole equally effective in inhibiting growth of this organism whereas we have consistently found sulfathiazole to be more effective than sulfadiazine. Smith and Emmart[5] using the moderately virulent H37 strain also reported that sulfathiazole and sulfadiazine were less effective than 4,4'diaminodiphenylsulfone in preventing surface growth.

The significance of the results reported in this paper is more striking when it is considered that of the 4 compounds used 4,4'diaminodiphenylsulfone is the only one which shows marked chemotherapeutic action on experimental tuberculosis of guinea pigs produced by H37Rv.[22] This clearly illustrates the possible fallacy involved in using an avirulent rapidly growing acid-fast organism for *in vitro* testing of potential chemotherapeutic agents for tubercle bacilli of human type.

The technic described has the following advantages over those previously described: (1) it is less cumbersome; (2) permits the use of virulent human type of tubercle bacilli; (3) it results in a more homogeneous inoculum; and (4) permits the use of a uniform inoculum for all tubes; (5) makes results easier to read; (6) reduces the time necessary for completion of the tests.

Results obtained with any one compound not infrequently differ from time to time, probably due in the main to two factors: (1) difficulty in preparing suspensions of the same homogeneity or degree of dispersion, and (2) biological variation in cultures used for inoculation even though cultures of the same age are employed. These factors can be

[22] Feldman, W. C., Hinshaw, H., and Moses, H. E., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 60.

controlled by including in the test, as a control, a standard compound such as 4,4'diaminodiphenylsulfone each time unknown compounds are tested and evaluating the results in terms of this as a standard. Improvements in the methods of preparing suspensions and in determining the amount of inoculum would also be helpful. For example, in a limited number of tests, micro-Kjeldahl nitrogen determinations have proved to be far more accurate for the latter purpose than Hopkins vaccine tubes.

The time of the completion of the tests can be reduced to 7-14 days providing only the readings of subsurface growth are recorded and a slightly larger inoculum employed.

Summary. A new technic for rapidly and efficiently testing the growth-inhibiting action of substances on virulent human type tubercle bacilli is described. The order of effectiveness of four compounds in inhibiting the growth of the avirulent acid-fast rapidly growing organism 607 was found to be: Sulfathiazole, sulfadiazine, 4,4'diaminodiphenylsulfone, and sulfanilamide, while for the virulent human strain of tubercle bacillus H37Rv the order was found to be: 4,4'diaminodiphenylsulfone, sulfathiazole, sulfadiazine, and sulfanilamide.

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Subsurface Growth of Virulent Human Tubercle Bacilli in a Synthetic Medium.*

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While there are scattered publications reporting growth of virulent human tubercle bacilli beneath the surface of liquid media, it is generally believed that these organisms will grow only on the surface, and precautions are always taken to float the organisms when subculturing. Drea[1,2] reported that one strain (H37) would grow rapidly in the depths of Long's synthetic medium when suspensions containing as little as 10^{-6} to 10^{-8} mg were inoculated. These findings with H37 have recently been confirmed by Cohn.[3] The present paper reports subsurface growth in a synthetic medium of 5 other virulent human strains of tubercle bacilli and one avirulent rapidly growing acid-fast organism.

Methods. Five virulent human strains were employed: H37Rv, 3637,† 3632,† G-2,‡ and G-5.‡ All 5 strains were pathogenic for guinea pigs. H37Rv, G-2, and G-5 were also pathogenic for mice on intravenous inoculation, and G-2 has been used to produce pulmonary tuberculosis in dogs.[4] No. 607, an avirulent rapidly growing acid-fast organism, was obtained from the National Type Culture Collection.

A synthetic medium was employed containing: Asparagin, 0.5%; monopotassium phosphate, 0.5%; magnesium citrate, 0.25%; magnesium sulfate, 0.05%; glycerol, 2.0%, made up with water redistilled from glass. The pH was adjusted to 7.0 with 40% sodium hydroxide. This medium was tubed in the desired amount, usually 10.0 cc, in 200 × 25 mm new soft glass or pyrex test tubes and sterilized by autoclaving at 10 lb for 20 minutes.

Suspensions of tubercle bacilli were pre-

* This work was aided by a grant from Parke Davis and Co., Detroit, Mich.

1 Drea, W. F., *J. Bact.*, 1940, **39**, 197.

2 Drea, W. F., *J. Bact.*, 1942, **44**, 149.

3 Cohn, Maurice L., *Am. Rev. Tuberc.*, 1944, **49**, 463.

† Obtained from Dr. William H. Feldman, Mayo Clinic, Rochester, Minn.

‡ Isolated from patients with active pulmonary tuberculosis by Dr. Francis D. Gunn, Department of Pathology, Northwestern University Medical School.

4 Mills, Moore A., Barth, E. E., and Gunn, F. D., *Am. Rev. Tuberc.*, 1940, **48**, 28.