THE SPIROCHETICIDAL ACTION OF PENICILLIN* IN VITRO AND ITS TEMPERATURE COEFFICIENT

By HARRY EAGLE, M.D., AND ARLYNE D. MUSSELMAN

(From the Venereal Disease Research and Postgraduate Training Center of the U. S. Public Health Service, Johns Hopkins Hospital, Baltimore)

(Received for publication, August 18, 1944)

In view of the demonstrated efficacy of penicillin in the treatment of syphilis (1) and relapsing fever (2), it becomes of interest to determine whether this therapeutic effect requires the necessary intervention of body cells, or whether it is due to a direct spirocheticidal action. With other organisms, both an inhibitory, bacteriostatic effect (3) and an actual bactericidal action (4) have been described.

Methods and Materials

The pathogenic *Spirochaeta pallida* has unfortunately not been cultivated on artifical media. We have therefore had recourse to cultured strains which are available which purport to be *S. pallida*, but which are non-pathogenic and differ morphologically from that organism. Unless otherwise indicated, the experiments here described were carried out with the so called Reiter strain of *S. pallida* (5). This grows luxuriantly in Brewer's thioglycollate medium (6), enriched with one-tenth volume of heated rabbit serum; and all the experiments of the present paper were carried out in that medium.

Forty-eight hour cultures, in which the organisms are in an active growth phase, provided the inoculum, added simultaneously with penicillin to fresh medium. The size of that inoculum was determined by direct microscopic count, using a measure drop (0.02 cc.) under a coverslip of known area. Knowing the area of the microscopic field and the average number of organisms per field (20 to 50 fields counted), the number of organisms in the culture could be determined with a reproducibility of 20 per cent. To determine the number of viable organisms surviving after incubation with penicillin, advantage was taken of the fact that these spirochetes grow out as discrete colonies in a solid or semisolid medium (7). The material in the experimental tube was subcultured by making serial tenfold dilutions in serum-thioglycollate medium containing 0.125 instead of 0.05 per cent agar. This was sufficiently fluid to permit shaking and pipetting, and viscous enough to permit colony formation. From 25 to 35 per cent of untreated organisms grew out as discrete colonies; and in each experiment the number of organisms surviving the action of penicillin was referred to the number recovered from subcultures made at the beginning of the experiment, to give the percentage of survivors.

Throughout this paper, the concentration of penicillin is expressed as Oxford units per cubic centimeter.

EXPERIMENTAL

Spirocheticidal Action of Penicillin

Our first experiments (*cf.* Table I) strongly suggested that penicillin only temporarily inhibited the growth of the Reiter strain of *S. pallida*. With a

* The work described in this paper was performed with penicillin supplied by the Office of Scientific Research and Development.

fairly heavy inoculum (*e.g.* 1.3×10^6 per cc.), concentrations of penicillin in excess of 0.05 unit per cc. caused an initial inhibition of growth. Subcultures were however positive, and the organisms eventually grew out in large numbers. Although this was apparently a typical bacteriostatic effect, such experiments did not exclude the possibility that penicillin was spirocheticidal, and that the delayed growth was due to the survival and slow multiplication of a small number of relatively resistant organisms.

TABLE I

The Apparent Spirochetostatic Action of Penicillin on S. pallida (Reiter) In Vitro

Inoculum (initial No. of organisms per cc.)	Examined or subcultured after day No.	Units of penicillin per cc. culture							
		0.8	0.4	0.2	0.1	0.05	0.025	0.0125	0
		No. of spirochetes per cc. $\times 10^6$							
1.3×10^6	3	No visible growth					2.3	38	80
	7	No visible growth				Few organisms	Heavy growth		
	14	No visible growth	Few organisms	Heavy growth					
	16 (subculture)	Heavy growth							

Conclusion: Apparent slow multiplication of organisms after initial inhibition.

To test that possibility, inocula of varying density were added to varying concentrations of penicillin, and subcultures were taken at intervals. As shown in Fig. 1, in those mixtures containing small numbers of organisms, all the spirochetes were in fact killed by as little as 0.062 Oxford unit penicillin per cc., or approximately a 1:25,000,000 concentration of the drug. As the number of organisms was increased, progressively more penicillin was required to effect complete sterilization, and in mixtures containing large numbers of organisms (*e.g.* 10^7 per cc.) a few sometimes survived a concentration of even 5 units per cc., as shown by positive subcultures on the 14th day (*cf.* Table II).

The quantitative significance of the data summarized in Fig. 1 was, however, vitiated by the deterioration of penicillin under the conditions of the experiment. As is illustrated in Table III, at penicillin concentrations of 0.1 to 10 units per cc., from 50 to 90 per cent of the penicillin activity was lost within 7 days. The high concentrations of penicillin necessary to sterilize a heavy inoculum were therefore in part due to the deterioration of the drug under the conditions of the test. This deterioration was, however, not quantitatively

sufficient to explain the wide variation in sterilizing concentration indicated in Fig. 1. At least three possible explanations suggest themselves, and are now under study. With a heavy inoculum, so long a time is required to kill off all

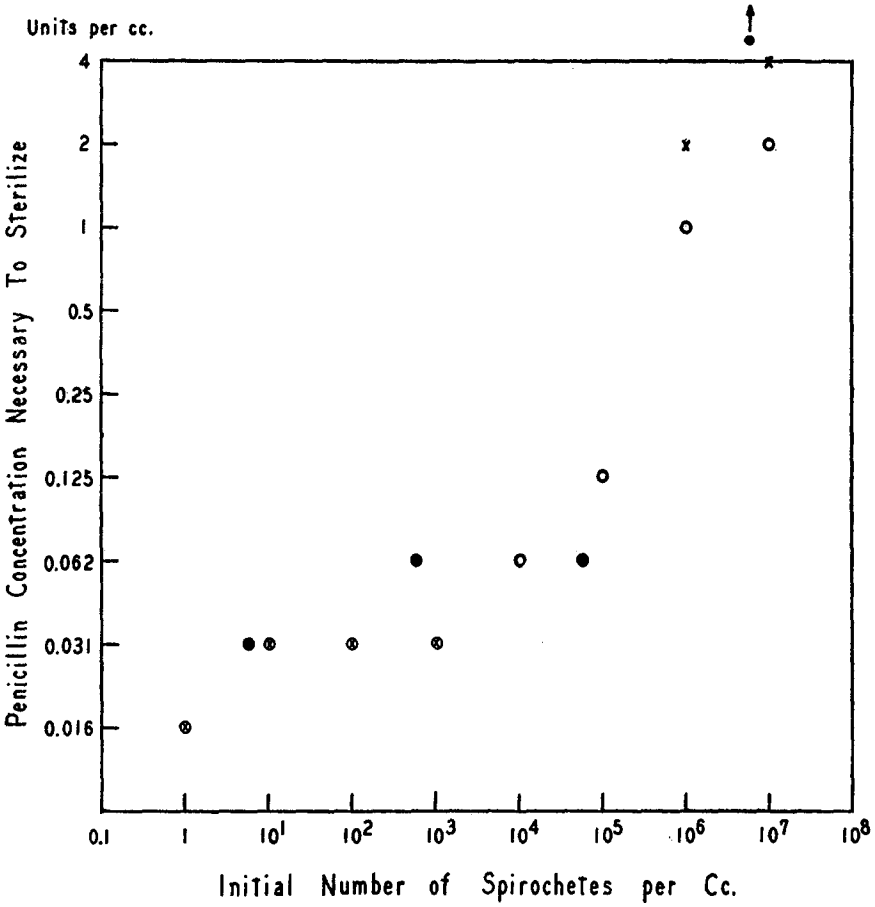

FIG. 1. The concentration of penicillin necessary to sterilize suspensions of *S. pallida* (Reiter) in relation to the initial number of organisms. The indicated number of organisms (48 hour culture) were suspended in serum-thioglycollate-broth medium, and incubated with the indicated concentrations of penicillin for 14 days at 37° C. Tubes showing no growth were then subcultured to assure the absence of surviving viable organisms. O, X, ●, individual experiments done at different times.

the organisms that those surviving after *e.g.* 7 days may have adapted themselves to the drug. Further, they may by then be in a different growth phase, and less vulnerable to the drug (*cf.* 4*c*). Finally, since it is obviously true that the organisms vary in their initial susceptibility, a heavy inoculum may

provide a sufficient number of highly resistant organisms to permit their growth in the presence of significant concentrations of penicillin. This slow multipli-

TABLE II

Spirocheticidal Action of Penicillin on S. pallida (Reiter) In Vitro

Inoculum (initial No. of organisms per cc.)	Units of penicillin per cc. culture							
	5	2	1	0.5	0.25	0.125	0.062	0.031
9×10^6	No visible growth, but subcultures positive				Cultures positive			
9×10^4								
9×10^2	No visible growth: subcultures on 14th day also negative						No visible growth, subcultures positive	Cultures positive
9								
0.9								

Conclusion: Small inocula completely killed by penicillin in 14 days.

TABLE III

The Rate of Deterioration of Penicillin at 37° in Serum-Thioglycollate Broth

Penicillin was added to the sterile broth to a final concentration of 0.8 units per cc. At varying intervals, aliquot portions were tested for spirocheticidal activity with a fixed inoculum of *S. pallida* (Reiter strain).

Time at 37°C.	Cc. of original mixture in total volume of 8 cc.						Approximate loss of activity
	4	2	1	0.5	0.25	0.125	
	Supposed penicillin concentration, units per cc.						
	0.4	0.2	0.1	0.05	0.025	0.0125	
	Result of culture						
							per cent
0 (control)	0	0	0	0	0	+	0
8 hrs.	0	0	0	0	0	+	0
1 day	0	0	0	0	0	+	0
3 days	0	0	0	0	+	+	50
1 wk.	0	0	+	+	+	+	87

cation of a few residual viable spirochetes would become apparent only after the quantitatively preponderant susceptible organisms had been killed.

Rate of Spirocheticidal Action in Relation to Concentration of Organisms and of Penicillin

The course of events when small amounts of penicillin were added to spirochetes *in vitro* is shown in Fig. 2. At low concentrations of penicillin, and with a fixed inoculum, the rate of killing was a function of its concentration. The

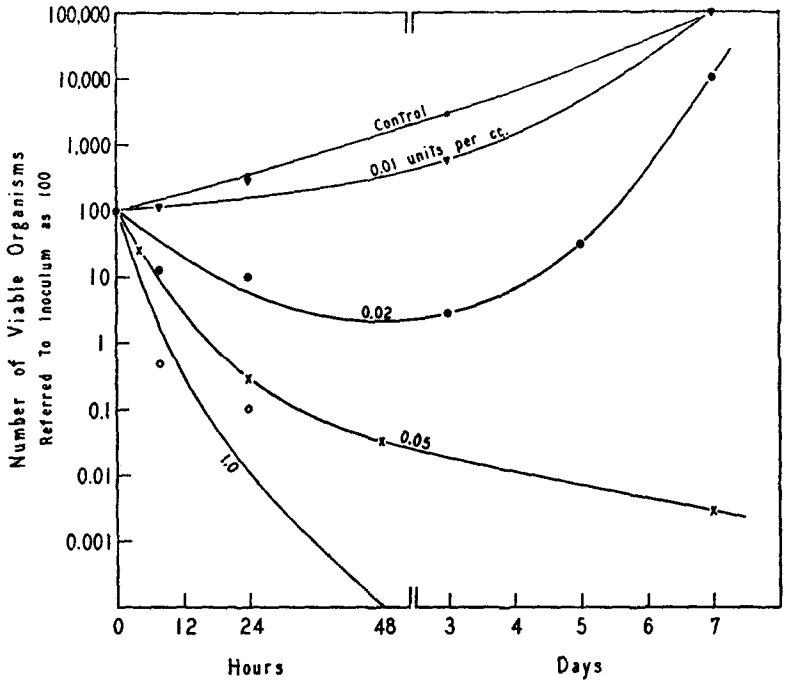

FIG. 2. The rate at which *S. pallida* (Reiter) was rendered non-viable by varying concentrations of penicillin. A 48 hour culture was added to the thioglycollate-serum-broth medium to a concentration of 10^6 organisms per cc., and the indicated concentrations of sodium penicillin then added. Aliquots were drawn off at the intervals indicated in the figure, and the number of surviving viable organisms determined by serial dilutions in the same medium. The ordinates in the figure are the proportion of surviving organisms, referred to the number viable at time 0 as 100.

top curve in Fig. 2 shows the rate of growth in the control tube containing no penicillin. As little as 0.01 unit per cc., or approximately a 1:150,000,000 concentration of penicillin, had a significant initial effect. However, multiplication kept pace with spirocheticidal action, and the number of organisms remained essentially unchanged until the penicillin had deteriorated sufficiently to permit the organisms to grow out. Twice that concentration, or 0.02 unit

per cc., had a more pronounced effect, but again the penicillin deteriorated before the spirocheticidal action was complete, and the culture grew out. An initial concentration of 0.05 unit per cc. killed off 99.99 per cent of the organisms, but a few spirochetes were found to have persisted after 7 days. Concentrations of 1 unit per cc. eventually caused complete sterilization.

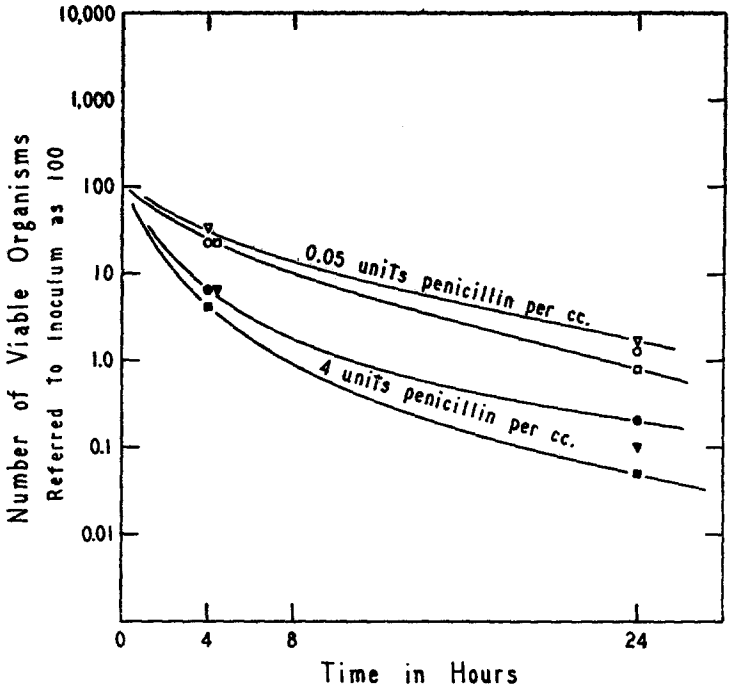

FIG. 3. The initial rate at which *S. pallida* (Reiter) was rendered non-viable by penicillin in relation to the number of organisms and the concentration of penicillin.

Initial No. of organisms	Penicillin concentration, *units per cc.* 4	0.05
10^7	●	○
10^6	▼	▽
4×10^4	■	□

Because of the deterioration of penicillin, the selective survival of resistant organisms, and a possible adaptative change, the time and the amount of penicillin required to sterilize a culture was of less significance than the initial rate at which the organisms were killed. Experiments to determine that rate in relation to the number of organisms and the concentration of penicillin are summarized in Fig. 3.

As is there shown, the initial rate of spirocheticidal action was independent of the initial number of organisms, at least in the range 4×10^4 to 10^7 per cc.

This observation would suggest that, unlike the arsenicals (8), and like the sulfonamides, penicillin is not significantly concentrated by the organisms which it kills. Hobby, Meyer, and Chaffee (4*c*) have shown this to be the case with streptococci, despite the fact that the rate of killing with that organism was a function of the number initially present. In our own experiments we have been unable to demonstrate any measurable binding of penicillin by spirochetes, using suspensions containing 10^9 to 4×10^9 organisms per cc. It must be emphasized that such experiments indicate only that the amount of penicillin bound is too small to be demonstrable by relatively crude methods of assay, and too small to affect the rate at which *e.g.* 10^7 spirochetes per cc. are killed, as compared with 4×10^4 per cc. Minute amounts of the drug may nevertheless combine with the organisms; and the lethal effect of penicillin may be due only to that quantitatively insignificant bound portion.

With respect to the effect of penicillin concentration on the rate of spirocheticidal action, the striking differences shown in Fig. 2 are in part due to the progressive deterioration of penicillin and the selective survival of resistant organisms as previously discussed. It is nevertheless clear from that figure and from the data of Table IV and Fig. 5 that a threshold concentration of penicillin, on the order of 0.01 unit per cc. or a 1:160,000,000 dilution, had to be exceeded before its effect became demonstrable. Beyond that threshold concentration, the *initial* rate at which the organisms were rendered non-viable was markedly increased by increasing the penicillin concentration, up to a concentration level of approximately 0.1 to 0.25 unit per cc. Further increase beyond that level had relatively little effect in accelerating the spirocheticidal action (*cf.* Table IV and Fig. 5). A qualitatively similar but quantitatively different effect on varying the penicillin concentration has been noted by Hobby, Meyer, and Chaffee (4*c*) with *Staphylococcus aureus*.

The Motility of Spirochetes in Relation to Spirocheticidal Action of Penicillin

It should be pointed out that the preceding discussion, and the supporting experimental data, deal with the rate at which the spirochetes in the culture medium were rendered non-viable by penicillin, judged by their ability to grow out in subculture. This did not coincide with the rate at which they were immobilized. Thus, in 8 hours, as little as 0.25 unit of penicillin per cc. had rendered 90 to 99 per cent of the organisms non-viable; but at that time period, even 500 units penicillin per cc. had produced no apparent change in their motility (*cf.* Fig. 4). In 24 hours, however, most of the organisms treated with penicillin, whether at 0.1 or 500 units per cc., were immobilized, and many showed degenerative changes.

When penicillin acts on this strain of spirochetes *in vitro*, there is thus an initial period of approximately 4 to 8 hours during which the drug produces irreversible changes in the organisms as the result of which they eventually

die. The metabolic system affected is, however, not immediately essential to the life of the cell, and there ensues a period of many hours during which motility and presumably other vital functions remain unchanged. This observation may provide an experimental approach to the metabolic system primarily affected by penicillin.

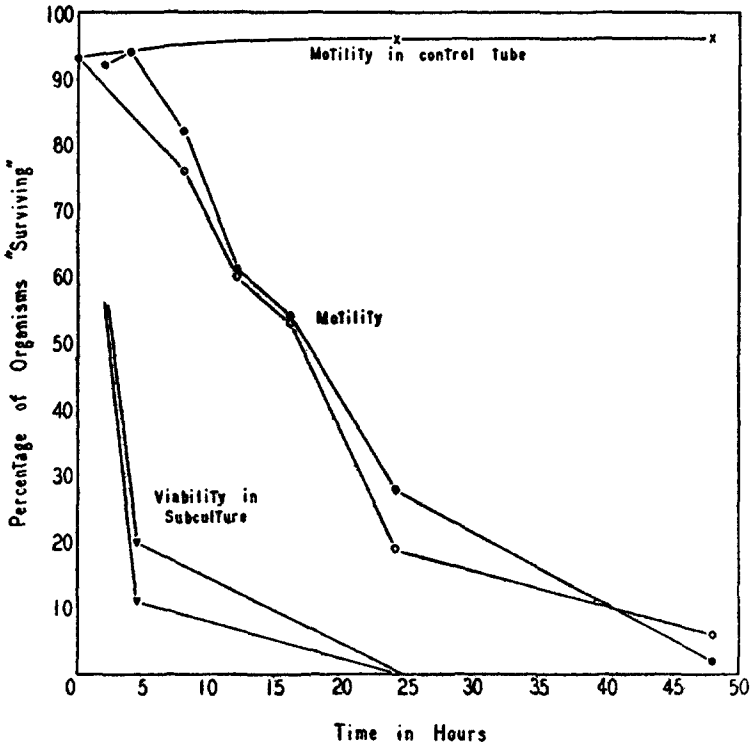

FIG. 4. Showing that *S. pallida* (Reiter) remains actively motile for many hours after the organisms are rendered non-viable by the action of penicillin.

Motility—	●	500 units per cc.
	○	0.25 unit per cc.
Viability—	▼	4 units per cc.
	▽	0.25 unit per cc.

The Effect of Temperature on the Spirocheticidal Action of Penicillin

The spirocheticidal action of sodium penicillin was simultaneously determined at 39–40°, 36–37°, 32–33°, 22–23°, and 8°C. At each of these temperatures a fixed inoculum of spirochetes was added to varying concentrations of sodium penicillin in the serum-thioglycollate medium. After 24 hours, the approximate number of viable organisms surviving in each tube was determined

by making serial tenfold dilutions in the same medium. One of three experiments, all with qualitatively similar results, is summarized in Table IV.

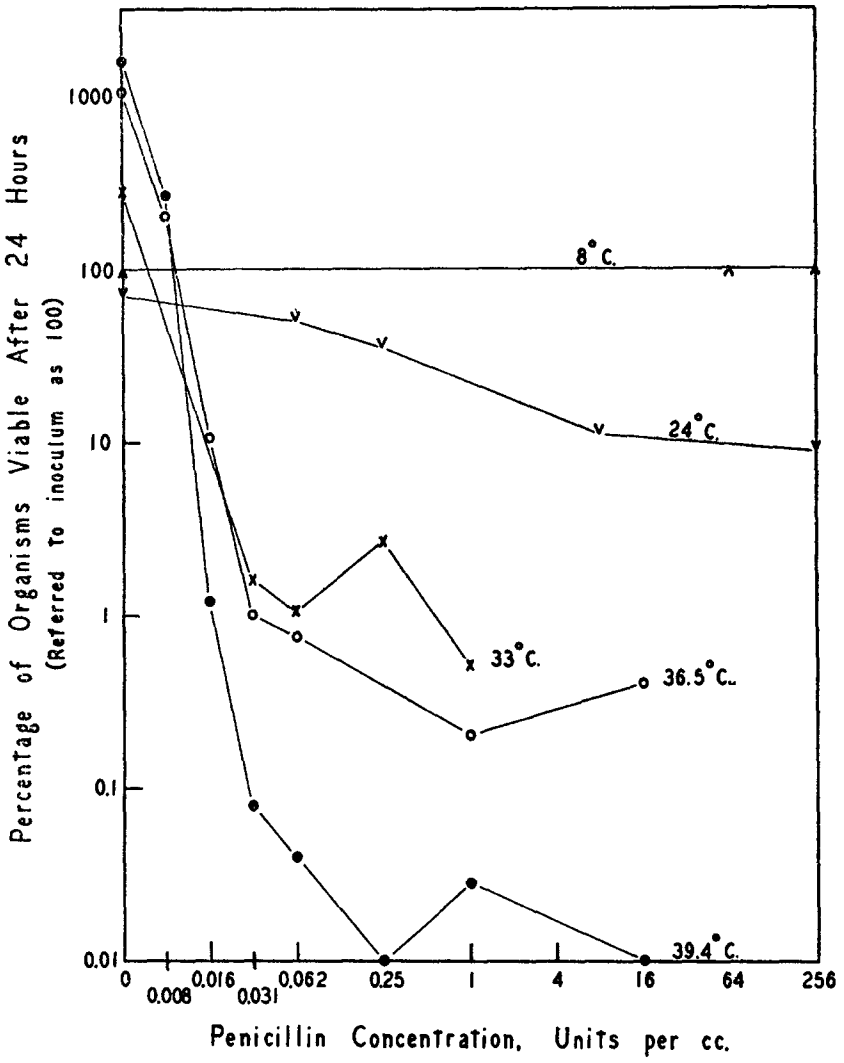

FIG. 5. Illustrating the effect of temperature on the spirocheticidal action of penicillin *in vitro* (*S. pallida*—Reiter strain).

A second similar experiment is graphically summarized in Fig. 5, in which the percentage of organisms surviving at various temperatures is plotted as a function of the concentration of penicillin.

As is there shown, and as has been indicated in a previous section, increasing the concentration of penicillin beyond a threshold level of 0.1 to 0.25 unit per cc. had little effect on the proportion of organisms surviving after 24 hours. At any one temperature, penicillin in excess of a limiting minimum level killed the organisms at a rate more or less independent of its concentration. That rate was, however, affected to a marked degree by temperature. Thus,

TABLE IV

Illustrating the Effect of Temperature on the Spirocheticidal Action of Penicillin

Culture tubes freshly inoculated with 10^6 spirochetes per cc. and containing varying concentrations of penicillin were kept at the indicated temperatures (±0.5°C.) for 24 hours. At that time, serial dilutions were made in the same thioglycollate-serum medium, and the number of colonies counted after 7 to 10 days at 37°.*

Temperature	Penicillin concentration, units per cc.												
	256	64	16	2	1	0.5	0.25	0.125	0.062	0.031	0.016	0.008	0
	Per cent of organisms surviving after 24 hours* (Referred to number of colonies at time 0 as 100)												
°C.													
39.7								0.02		0.04	11	320	700
36.2					0.16		0.36		0.2	0.24	14		1700
31.9				0.6		0.8	0.8	1.2	1.6	2.4			500
22.5	13	10	8										100

* The method of enumerating organisms is illustrated in following example:

Tube subcultured	Cc. of original mixture in 10 cc. of subculture								No. of viable organisms per cc.	Per cent of viable organisms, referred to time 0 as 100
	10	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
	No. of colonies									
Control tube at time 0....	+	+	+	+	26	4			260,000	100
Experimental tube after 24 hrs.....	0	11	1	0	0	0			110	0.04
Control tube after 24 hrs.....	+	+	+	+	+	22	2		2,220,000	850

with a fixed inoculum of 10^6 spirochetes per cc., at 8°C. concentrations of penicillin up to and including 256 units per cc. had no demonstrable effect. In one experiment not shown in Table IV, spirochetes exposed at that temperature to 16 units penicillin per cc. for 7 days were wholly unaffected, the tube containing as many viable organisms as the control tube containing no penicillin. At 22–23°, 32–33°, 36–37°, 39–40°C., the number of viable organisms after exposure to penicillin for 24 hours averaged 10, 1, 0.2, and 0.02 per cent of the original inoculum respectively.

DISCUSSION

Although the experiments of the preceding sections were carried out with a single strain, the so called Reiter strain of *S. pallida*, penicillin was found to be equally active against the Nichols, Noguchi, and Kazan strains, also purporting to be cultures of *S. pallida*, and against a culture of mouth spirochetes isolated by Doctor Ruth Wichelhausen of the Department of Bacteriology, Johns Hopkins Medical School.

The degree to which results obtained with cultured organisms may be carried over to the pathogenic *S. pallida* is an open question. These pathogenic organisms have as yet not been cultivated. Suspensions obtained from rabbit testicular chancres (9) were not immobilized in 2 to 6 hours by concentrations of penicillin up to and including 500 units per cc.[1] However, since the motility of the highly susceptible cultured spirochetes was also unaffected in that time period and in those concentrations, this observation does not exclude a direct spirocheticidal action. The drug may be actively spirocheticidal for the pathogenic organism in minute concentration, and immobilization may be as inadequate a criterion of its action as it is in the case of the cultured organisms here studied. Under such circumstances, the therapeutic effect of penicillin in syphilis could be primarily referable to its direct spirocheticidal action. Studies to determine this point are now in progress.

Within wide limits, the rate at which spirochetes were killed by penicillin was independent of its concentration. If this observation with a non-pathogenic organism *in vitro* applies to the pathogenic organisms *in vivo*, it suggests that if a given schedule of treatment results in the maintenance of an effectively spirocheticidal concentration of penicillin in the tissue fluids, further increase in the size of the individual injections may have but little effect on the rate at which the spirochetes are killed, and thus, on the end results of treatment.

Of practical interest also is the marked effect of temperature on the rate of spirocheticidal action. The difference between the results obtained at 37° and at 40°C. is sufficiently great to suggest that artificially induced fever used in conjunction with penicillin in the treatment of early syphilis may make it possible either to reduce the total dosage, shorten the duration of treatment, or both. The known therapeutic action of fever in general paresis and other forms of central nervous system involvement, coupled with the possibly enhanced activity of the drug at the higher temperature, suggests that fever should be tried in conjunction with penicillin in those conditions.

[1] Dunham, Hamre, McKee, and Rake (10) have recently reported the immobilization of pathogenic *S. pallida* under such conditions by penicillin concentrations in excess of 800 units per cc., but not at lower levels. These are, however, concentrations of a different order of magnitude from those attained *in vivo*.

SUMMARY

1. Penicillin was found to be actively spirocheticidal *in vitro* against the Reiter, Kazan, Nichols, and Noguchi strains of so called *S. pallida*, and a strain of mouth spirochetes. The threshold concentration was 0.01 unit per cc. (1–160,000,000 penicillin). The rate and degree of action increased with the concentration of penicillin up to a level of approximately 0.1 to 0.25 unit per cc., which rendered more than 99 per cent of the organisms non-viable within 12 hours. Higher concentrations did not appreciably accelerate the effect.

2. Within the range 4×10^4–10^7 organisms per cc., the initial rate at which the spirochetes were killed was not affected by their number. Consistent with that observation, no demonstrable penicillin was bound or inactivated by thick suspensions. The amount of penicillin required to sterilize suspensions of varying density nevertheless varied to a large extent with the initial number of organisms. This was only in part due to the progressive deterioration of the penicillin with prolonged incubation; and the persistence of organisms resistant to the drug, and perhaps an adaptative change after prolonged exposure to penicillin, may be contributing factors.

3. The organisms remained actively motile for a period of 8 to 24 hours after they had been rendered non-viable by the action of penicillin. Even 500 units of penicillin per cc., or approximately 10,000 times an effectively spirocheticidal concentration, did not accelerate that delayed immobilization. It follows that, although penicillin rapidly renders the organisms non-viable, the metabolic system affected is not immediately essential to the life of the cell, and the motility and presumably other vital functions remain unaffected for a significant number of hours.

4. The rate at which the organisms were killed by penicillin increased with temperature in the range 8–40°C. With an original inoculum of 10^6 spirochetes per cc., the percentage of organisms surviving after 24 hours at 39–40°, 36–37°, 32–33°, 22–23°, and 8°C. was 0.02, 0.2, 1, 10, and 100 respectively; and those results were independent of the concentration of penicillin in the range 0.25 to 250 units per cc. If these observations with a non-pathogenic organism *in vitro* are applicable to the pathogenic organism *in vivo*, they suggest that the combined use of fever and penicillin in the treatment of syphilis may be more effective than either alone.

BIBLIOGRAPHY

1. Mahoney, J. F., Arnold, R. C., and Harris, A., *Vener. Dis. Inform.*, 1943, **24**, 355.
2. Heilman, F. R., and Herrell, W. E., *Proc. Staff Meetings Mayo Clinic*, 1943, **18**, 457. Augustine, D. L., Weinman, D., and McAllister, J., *Science*, 1944, **99**, 19. Eagle, H. and Magnuson, H. J., *Public Health Rep., U.S.P.H.S.*, 1944, **59**, 18.

3. Chain, E., Florey, H. W., Gardner, A. D., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G., *Lancet*, 1940, **2,** 226. Reid, R. D., *J. Bact.*, 1935, **29,** 215. Fleming, A., Chain, E., and Abraham, E. P., *Biochem. J.*, 1942, **36,** 1.
4. (*a*) Fleming, A., *Brit. J. Exp. Path.*, 1929, **10,** 226. (*b*) Dawson, M. H., Hobby G. L., Meyer, K., and Chaffee, E., *J. Clin. Inv.*, 1941, **20,** 434. (*c*) Hobby, G., Meyer, K., and Chaffee, E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50,** 281. (*d*) Rammelkamp, C. H., and Maxon, T., *Proc. Soc Exp. Biol. and Med.*, 1942, **51,** 386. (*e*) Hobby, G. L., Meyer, K., Chaffee, E. and Dawson, M. H., *J. Bact.*, 1943, **45,** 65.
5. Reiter, H., *Deutsch. med. Woch.*, 1925, **51,** 303; *Klin. Woch.*, 1926, **5,** 444.
6. Brewer, J. H., *J. Am. Med. Assn.*, 1940, **115,** 598.
7. Wichelhausen, O. W., and Wichelhausen, R. H., *J. Dental Research*, 1942, **21,** 543.
8. Eagle, H., and Magnuson, H. J., *J. Pharmacol. and Exp. Therap.*, in press.
9. Eagle, H., *J. Pharmacol. and Exp. Therap.*, 1940, **69,** 342.
10. Dunham, W. B., Hamre, D. M., McKee, C. M., and Rake, G., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55,** 158.