The early work of Wright and Minot[6] showed that a factor associated with the serum globulins was important in bringing about the metamorphosis and disintegration of platelets in the presence of calcium. This would account for the relative stability of washed platelets. It will be of interest to inquire how the globulin factor as detected by platelet metamorphosis is related to the substance or substances which function as co-factor for platelets in the activation of prothrombin.

6 Wright, J. H., and Minot, G. R., *J. Exp. Med.*, 1917, **26**, 395.

*Summary.* 1. Twice-washed platelets were relatively stable in the presence of calcium ions and in contact with glass.

2. In the presence of calcium, washed rabbit platelets plus a small amount of bovine globulin activated bovine prothrombin. Activation was much slower with platelets alone, and no activation was detected with the globulin alone.

3. Water-clear platelet extracts could be substituted for platelet suspensions in this test system. In such case, the presence of the globulin was still necessary for prompt activation of prothrombin.

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## The Rickettsiostatic Action of Crystalline Penicillin Fractions in Embryonate Eggs.*

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In 1944, we found that crude commercial penicillin strikingly inhibited the growth of typhus rickettsiae in the yolk sac,[1] and markedly reduced or even completely prevented the mortality from typhus infection in mice.[2] In fertile eggs, the total dosage used was 975 units per egg, given in 3 doses on the 2nd, 4th and 6th, or on the 3rd, 5th and 7th days after rickettsial injection. Doses of 100 units had little or no effect.[3] In mice, the most impressive results were obtained with daily doses ranging from 640 to 1100 units, starting 6-48 hours after injection with lethal doses of rickettsiae. (The unit age content of these samples of penicillin was determined by assay against *Staphylococcus aureus*.)

The rickettsiostatic and chemotherapeutic action of penicillin in eggs and mice was found in the same year to compare favorably with that of para-aminobenzoic acid.[2,3] Uniformly good rickettsiostasis by penicillin was noted in at least 5 different experiments.

In 1945 and 1946, the samples of penicillin which we obtained were usually completely ineffective against rickettsial infection in doses of 1000 *S. aureus* units per egg. Similar observations, indicating variability in the therapeutic action of commercial penicillin against bacterial and spirochetal infections, in spite of standardization *in vitro* in terms of bacteriostatic units, have been reported by others.[4] We considered the possibility that the satisfactory rickettsiostatic action of the earlier samples might have been caused by some impurity other than penicillin. The realization that commercial penicillin has contained varying amounts of several chemical types of penicillin, including penicillins G, K,

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1 Greiff, D., and Pinkerton, H., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 116.

2 Moragues, V., Pinkerton, H., and Greiff, D., *J. Exp. Med.*, 1944, **79**, 431.

3 Greiff, D., Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1944, **80**, 561.

4 *J.A.M.A.*, 1946, **131**, 271.

F and X[4] suggested another possible explanation of our inconstant results. The experiments to be reported here were planned to test the comparative rickettsiostatic action of these penicillin fractions.

*Material and Methods.* For the samples of penicillin, we are indebted to Dr. Leonard Karel of the National Institute of Health. The information furnished indicated that the purity of these compounds was relative rather than absolute. The penicillin G contained 1707 *S. aureus* units per mg and was estimated to be about 99.5% pure (chromatographic analysis with differential assay of various cuts). The penicillin K was really a mixture, containing 65% K, 18% dihydro-F, 7% F, and 7% G. The penicillin F was estimated by distribution curve to contain 6% of inactive material; otherwise the curve agreed well with the theoretical for penicillin F. The penicillin dihydro-F was said to contain 85% dihydro-F, 6% F, 7% K, and 2% G. The penicillin X by iodometric chemical assay gave figures indicating that it was about 92% pure; it was believed to contain no penicillins other than X; it was found to contain 918 *S. aureus* units per mg.

The samples used were not assayed in our laboratory for bacteriostatic action. From the work of others,[5,6,7] penicillin G, when assayed *in vitro* against *Staphylococcus aureus* or *Bacillus subtilis* is known to contain about 1667 units per mg (our sample 1707). Penicillin K contains (approximately) 2300 units per mg against *S. aureus* and 700 units per mg against *B. subtilis;* penicillin F 1500 units per mg against *S. aureus* and 970 against *B. subtilis;* penicillin X 900 units per mg against *S. aureus* (our sample 918) and 1500 against *B. subtilis.*[7] Because of this variable potency against different organisms, comparison of the samples for rickettsiostatic action was made primarily on a gravimetric basis.

[5] Williamson, J., and Lourie, E. M., *Brit. Med. J.*, 1946, June 1, page 829.

[6] Schmidt, W. H., Ward, G. E., and Coghill, R. D., *J. Bact.*, 1945, **49**, 411.

[7] Robertson, P., and Dufrenoy, J., *Bact. Rev.*, 1948, **12**, 79.

Penicillin G (benzyl-penicillin) is presumably identical with the crystalline product designated penicillin II by British workers. Penicillin F (Penicillin I in British nomenclature) is $\Delta^2$ pentenyl-penicillin, while penicillin dihydro-F is n-amyl-penicillin. Penicillin X (penicillin III in British nomenclature) is a p-hydroxy-benzylpenicillin. Penicillin K is n-heptyl-penicillin. The penicillin G was in the form of the potassium salt. All other samples used were in the form of sodium salts. It seems unnecessary to discuss the chemistry of the penicillins in detail, since no correlation between chemical structure and therapeutic action has been established.

A murine strain of typhus, maintained chiefly in fertile eggs for several years but recently passed through a guinea pig to establish its virulence, was used. The penicillin fractions were injected into the yolk sacs in two doses on the 2nd and 4th days after rickettsial injection. Groups of 24 to 36 eggs were used for each compound. The degree of infection in eggs dying or killed at various intervals was estimated by examining stained film preparations of the yolk sac membranes. Survival periods were also used as an index of chemotherapeutic action. The technics for this type of study have been described in detail in previous publications.[1,3]

The results of two separate experiments are shown in Tables I and II. In the experiment represented by Table I, the gravimetric dosage of penicillin X given was about twice as great as that of the other types, since we wished to make the unitage dosages approximately equal in terms of the activity of the compounds *in vitro* against *S. aureus.* This was done in order to facilitate comparison with samples of crude penicillin previously used. Table II represents an attempt to outline further the ranges of activity of the penicillin fractions. In a third experiment, the results of which are not given in tabular form, 1.2 mg of penicillin di-hydro F was found to have rickettsiostatic activity approximately equal to that shown by 0.6 mg of penicillin G. A more detailed assay of these compounds was not made because of insufficiency of the samples obtained.

TABLE I.
Rickettsiostatic Action of Penicillins G, Di-hydro F, F, X, and K.

| Age of embryo, days | Control | G 0.40 mg 666 units† | Di-F 0.40 mg | F 0.40 mg 666 units† | X 0.79 mg 666 units† | K 0.40 mg |
|---|---|---|---|---|---|---|
| 5 | Rickettsiae injected. | | | | | |
| 7 | 333 units of penicillin injected. | | | | | |
| 9 | 1112 | 112 | | 23 | 00 | 1 |
| 9.5 | 333 units of penicillin injected. | | | | | |
| 10 | 23344 | 11222 | 11222 | 2333 | 11 | 22333 |
| | | 233 | 33 | 445 | | 334 |
| 11 | 333333 | 111233 | 23333 | 33334 | 0* (+)* | 33344 |
| | 44555 | | 444 | 45 | (+)* (+) | |
| 13 | | | | | 0* (+)* 1 | |
| 14 | | | | | 0* (+) | |
| 15 | | | | | 0* 1* | |
| 16 | | | | | 0* | |

Each figure represents an individual egg.
* Embryo alive at time of examination.
0 No rickettsia recognized.
(+) Less than one rickettsia per oil immersion field.
1 1-10 rickettsiae per oil immersion field.
2 10-100 rickettsiae per oil immersion field.
3 100-1000 rickettsiae per oil immersion field.
4 1000-5000 rickettsiae per oil immersion field.
5 5000-10,000 rickettsiae per oil immersion field
† Against *S. aureus* (estimated).

TABLE II.
Rickettsiostatic Action of Penicillins G, F, X, and K.

| Age of embryo, days | Control | G 0.6 mg 1000 units† | G 1.2 mg 2000 units† | F 0.67 mg 1000 units† | X 0.60 mg 506 units† | K 0.60 mg |
|---|---|---|---|---|---|---|
| 5 | Rickettsiae injected. | | | | | |
| 7 | Penicillin injected. | | | | | |
| 8 | | 000 | 0 (+) | 00 (+) 2 | 00 | 12 |
| 9 | 13 | (+) | (+) | 33 | (+) | 12233 |
| 9.5 | Penicillin injected. | | | | | |
| 10 | 35 | (+) 111 | 0 (+) (+) | 33333 | (+) 111 | 3333 |
| | | | (+) + | 44445 | | 45 |
| 11 | 444444 | (+) 12222 | 0 (+) (+) | 34444 | 11122 | 233333 |
| | 555555 | 33334 | (+) (+) | 44555 | | 344444 |
| | 5555 | | | 55 | | 55555 |
| 12 | 444444 | (+) (+) | (+) (+) | 3344 | 0 (+) (+) | (+) (+) |
| | 444555 | 111222 | (+) (+) | 555 | (+) (+) | 11 |
| | 555 | | (+) (+) | | | 222 |
| | | | 113 | | | |
| 13 | | 0 (+) (+) | 0 (+) (+) | | 0 (+) | |
| 14 | | | 0* 0 (+) | | 0* 0* | |
| | | | (+) 1 | | | |
| 15 | | | 0* (+)* | | 0* 0* (+)* | |
| | | | (+) 11 | | (+) 2 | |
| 16 | | | | | (+)* 2* 222 | |
| 17 | | | | | 0* 0* 0* 0* | |

For explanation of symbols see footnote of Table I.

The results of these experiments suggest that penicillin X, on a gravimetric basis or on an estimated unitage basis against *B. subtilis*, may be about twice as active against typhus rickettsiae as penicillin G, while the other penicillins tested are much less potent. On an estimated unitage basis against *S. aureus*, penicillin X appeared to show about 4 times as much rickettsiostatic activity as penicillin G.

It is also worth noting that for the first time since 1944, effective rickettsiostasis was obtained by penicillin. Estimated doses of 1000 (*S. aureus*) units of penicillin G were

definitely less effective than 1000 units of the crude penicillin mixtures used in 1944, but 2000 units of penicillin G and 506 units of penicillin X were about as effective as 1000 units of the crude mixture. Since the crude mixtures which we used in 1944 presumably did not contain significant amounts of penicillin X,[8] and probably did have fairly large proportions of penicillins other than G, the reason for their high effectiveness is not clear. It is conceivable that the penicillin fractions when mixed in certain proportions might have an effect greater than the sum of their individual effects.

It is now well known that the efficiency of the crystalline penicillins, as measured *in vitro*, varies with different bacteria and even with different strains of the same organism. Relationships found *in vitro* often have not held in experimental infection. Important factors *in vivo* seem to be the relative stability of the different molecular types, and their relative affinity for plasma proteins. Penicillin K has been shown to lose much of its potency because of the latter factor.[9] Rotman-Kauka and his co-workers[10] found that blood levels remained higher with penicillin X than with either commercial G or crystalline G, and that urinary excretion was higher for G than for X. In our experiments, levels of penicillin activity in the yolk sac were not determined. Loss by urinary excretion is largely eliminated by the use of the embryonate egg, but chemical disintegration and inactivation by protein absorption are both possible.

Penicillin X has been found to be much more active than the other types when tested *in vivo* against several bacterial infections. Penicillin G, however, has been found to be about 4 times as active as X against experimental syphilis[11] and experimental relapsing fever.[12] It may be noted that the results which we obtained in rickettsial infection are qualitatively and quantitatively similar to those obtained in experimental infection with several bacteria, and differ from those obtained with spirochaetal infections.

The figures given by Hobby and her co-workers[8] for example, against hemolytic streptococcus *in vivo* are X, 500; G, 100; F, less than 100; and K, 60 (on a unitary basis) and X, 270; G, 100; F, 92; and K, 82 (on a gravimetric basis). When tested *in vitro* against the same organism, all four of the samples which they used were about equally potent. It is also of interest that these workers found crude penicillin to be 3-5 times as effective *in vivo* as penicillin G and approximately equivalent to penicillin X. Here again their results parallel those which we have obtained against typhus rickettsiae. On the whole, the results which are reported here suggest that the samples of crude penicillin which we used in 1944 may have owed their effectiveness primarily to penicillin fractions. The possibility that impurities may have played a part directly, or indirectly by enhancing the action of penicillin has, however, not been ruled out. The possible nature and mode of action of such impurities has been discussed by others.[7]

As far as we are aware, adequate trials of penicillin in human rickettsial infection have not been made. Blake and his co-workers[13] have reported unencouraging results in two cases of tsutsugamuchi disease. One case (moderately severe) received about 266,000 units per day from the 4th to the 7th days of illness. This patient recovered, but there was no evidence that the penicillin altered the course of the illness. The second case received 120,000 units per day from the 5th to the 9th day of illness. This patient became critically ill on the 12th day of illness, but recovered eventually. Since the dosage

[8] Hobby, G. L., Burkhart, B., and Hyman, B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 296.

[9] Eagle, H., and Newman, E., *J. Clin. Invest.*, 1947, **26**, 903.

[10] Rotman-Kauka, G., Hirsh, H. I., and Dowling, H. F., *New Eng. J. Med.*, 1947, **236**, 314.

[11] Turner, T. B., Cumberland, M. C., and Huan-Ying Li, *Am. J. Syph., Gonor., and Ven. Dis.*, 1947, **31**, 476.

[12] Cumberland, M. C., and Turner, T. B., *Am. J. Syph., Gonor., and Ven. Dis.*, 1947, **31**, 485.

[13] Blake, F. G., Maxcy, K. F., Sadusk, J. F., Jr., Kohls, G. M., and Bell, E. J., *Am. J. Hyg.*, 1945, **41**, 243.

which we found effective in mice translates to about 3,000,000 units per day for a patient weighing 150 lbs., and since as many as 10,000,000 units per day are at present used in subacute bacterial endocarditis, it is obvious that the trial of penicillin in these 2 cases had little value. Yeomans *et al.*[14] report the administration of penicillin to 4 cases of human typhus (dosages and other data not supplied) and were unable to decide whether or not the treatment was of value.

Several compounds having rickettsiostatic activity in animals have been tried thoroughly in human typhus infection, with definitely disappointing results.[15,16] In view of the relatively high toxicity of these compounds for man, and the very low toxicity of penicillin, it is rather surprising that adequate trials of the latter compound, using samples with experimentally proven potency, have not been made.

*Summary and Conclusions.* Samples of 5 relatively pure crystalline penicillin fractions have been tested for rickettsiostatic activity in typhus-infected embryonate eggs. Penicillin X appeared to be about twice as effective on a gravimetric basis, and about 4 times as effective on a (*S. aureus*) unitage basis as penicillin G. Penicillin F, di-hydro F, and K were much less effective. On a (*S. aureus*) unitage basis, penicillin X showed a potency approximately double that of samples of crude penicillin used in 1944. Penicillin G showed a very much higher potency (on a unitage basis) than most samples of partly purified penicillin which we tested in 1945 and 1946. The differential rickettsiostatic potency of these 5 penicillins is roughly parallel to their differential activity against certain bacteria *in vivo* as determined by other, but differs from that reported in experimental spirochaetal infections, in which penicillin G has been found most effective.

Irregular results obtained by us in the past with crude and partially purified commercial penicillins may perhaps be explained on the basis of varying proportions of penicillin fractions, although the possible existence of impurities having independent rickettsiostatic action or capable of enhancing the action of penicillin has not been ruled out. The factors of stability and protein absorption of the penicillins were not evaluated.

The effectiveness of penicillin in human rickettsial infection apparently remains undetermined. The only clinical trials in which data are available are invalidated by the use of low doses, started late in the course of the disease, by the small number of cases, and by the fact that samples of proven potency against the organism involved were not used.

[14] Yeomans, A., Snyder, J. C., Murray, E. C., Zarafonetis, C. J. D., and Ecke, R. S., *J. Am. Med. Assn.*, 1944, **126**, 349.

[15] Steele, J. M., McLimans, W. F., Grant, C. W., and Tullis, J. L., Report No. 6, January 14, 1946, Research Project X-222, Naval Medical Research Institute.

[16] Andrewes, C. H., King, H., van den Ende, M., and Walker, J., *Lancet*, 1944, **1**, 777.