negative organisms may be greatly enhanced upon addition of methionine, threonine and methionine sulfoxide. The action of amino acids appears to be synergistic rather than additive. Methionine is essential for the enhancement. Threonine and methionine sulfoxide facilitate the effect of methionine following a reciprocal quantitative relationship.

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THE BENZYL ESTER OF PENICILLIN

DURING the past several years, various types of esters of penicillin have been made in this laboratory in a search for derivatives possessing greater stability, a depot action or effectiveness by mouth. Crude preparations of the methyl, ethyl, n-butyl and benzhydryl esters have been described by others,[1] but were reported to hold "no great promise." However, we have found that the benzyl ester promises to be extremely valuable because of its ease of preparation, enhanced stability, ready oral absorption and powerful chemotherapeutic action.

Our benzyl penicillin G has been obtained as a colorless, hard glassy solid which shows a sharp increase in fluidity at 50° C. The ester was prepared by treating free penicillin in an inert organic solvent with an excess of phenyl diazomethane, any unreacted penicillin being extracted with sodium bicarbonate solution. Evaporation of the solvent yielded a resinous product which was readily purified.

Benzyl penicillin is stable at temperatures above 100° C., in contrast to penicillin salts. The ester is also much more stable in alcoholic solvents than any known penicillin salt. It has a very low solubility in water, but is soluble in alcohol, ether, chloroform, ethyl acetate and in the polyethylene glycol type of polymers. It is approximately 2.5 per cent. soluble in sesame oil or propylene glycol.

In vitro, pure benzyl penicillin exhibits about one thirtieth as much bacteriostatic activity as pure sodium penicillin against broth cultures of *Staphylococcus aureus.* This was shown by serial dilution tests in which the bacteriostatic concentration was found to be 1 microgram per cc; under similar conditions, 0.03 microgram or 0.05 international unit of pure sodium penicillin sufficed. By the cup-plate method, benzyl penicillin is relatively much less potent and shows approximately one four-hundredth of the activity of sodium penicillin.

In vitro, a substantial proportion of the theoretical activity can be demonstrated after splitting the ester by incubating with an aqueous extract of rat kidney. Presumably this regeneration is caused by enzymatic cleavage since boiled kidney extract fails to liberate any activity. Rat serum is almost as effective as rat kidney extract, guinea pig serum shows some activity, while human, horse, rabbit and dog sera are inactive. The cup-plate, serial dilution and the Warburg respirometer procedures have been used to measure the activity so liberated. The observed activity is approximately one half that to be expected theoretically from the penicillin content of the ester. The highest value observed was 54 per cent. (using the cup-plate method). Hickey[2] recently reports obtaining a maximum of 26 and 16 per cent. regeneration from methyl and ethyl penicillin, respectively, using alkaline hydrolysis.

Benzyl penicillin dissolved in a vegetable oil and injected subcutaneously or given by mouth protects mice against lethal doses of streptococci and pneumococci. Mice were inoculated intraperitoneally with 0.3 cc of a 1:100,000 dilution of an 18-hour broth culture of *Streptococcus hemolyticus* (Strain C 203). This inoculation kills 92 per cent. of the unmedicated control mice within 18 to 46 hours (average of 15 experiments). In the subcutaneous injection experiments 0.1 cc of a solution containing the desired dose of benzyl penicillin was injected one hour after the inoculation. For the oral experiments 0.1 cc was given by stomach tube one hour before and 4 hours after the inoculation. Observations were made at convenient intervals for the succeeding 72 hours; rarely were there any deaths after this time. The relative efficacies of 2 or more preparations were

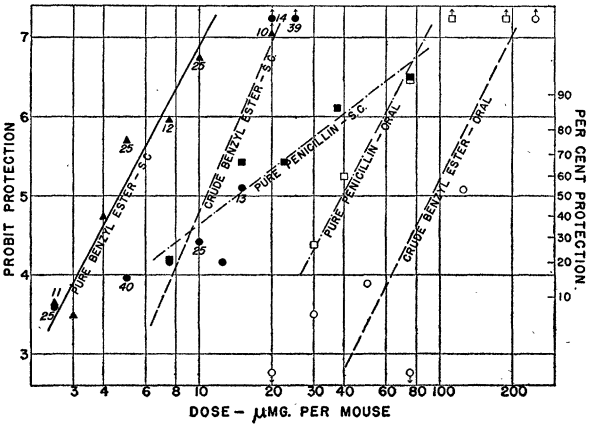

FIG. 1. Graph showing protection afforded by benzyl penicillin and pure penicillin against experimental streptococcal infection in mice.

Legend: ▲—Pure benzyl penicillin given subcutaneously, ●—Crude benzyl penicillin given subcutaneously ■—Pure penicillin given subcutaneously, □—Pure penicillin given orally, ⊙—Crude benzyl penicillin given orally. Each point represents 15 mice unless otherwise indicated. The points to which arrows are attached represent survivals of either 0 per cent. (downward arrow) or 100 per cent. (upward arrow). In these experiments, 5 of the 63 inoculated control mice survived without any penicillin treatment.

[1] K. Mayer, G. L. Hobby and E. Chaffee, SCIENCE, 97: 205–206, 1943.

[2] R. J. Hickey, SCIENCE, 101: 462–463, 1945.

determined by comparing the mortalities observed following graded doses given to comparable groups of mice.

Fig. 1 presents the composite data of *in vivo* experiments in which graded doses were given of both crude and pure benzyl penicillin preparations dissolved in sesame oil and of a crystalline water-soluble salt of penicillin (1,600 μ/mg) suspended in sesame oil. From this log-probit graph[3] the relative potencies of the 3 preparations as well as the efficiency of the two routes of administration may be estimated. In some of these tests the sodium penicillin was dissolved in an aqueous buffer solution, and it was observed that injections of sodium penicillin suspended in oil afford the same protection as the same amounts injected in buffer. When injected subcutaneously, the pure ester appears at least 3 times as potent as the pure salt on a weight basis, although some reservations must be made for a lack of parallelism in the straight lines relating dose to the degree of protection. If allowance is made for the difference in molecular weights the ratio becomes still greater. In other results not shown in the figure, the pure benzyl ester has been found to be 7.5 times as potent as an equimolecular amount of commercial sodium penicillin.

Of great interest also is the effectiveness of benzyl penicillin by mouth. In mice, as may be seen from Fig. 1, about 10 times as much of the crude benzyl penicillin (50 per cent. pure) was required orally as subcutaneously. A less pure preparation (10 per cent.) was better utilized by mouth, since only 4 times as much of it was needed for protection equivalent to that obtained from subcutaneous injections. The mouse appears to absorb water-soluble penicillin quite efficiently, since only three times as much (of either the pure or commercial grade salt) is required by mouth as subcutaneously. It should be noted that the mouse is a poor subject for oral administration experiments because of the relatively rapid passage of the drug through its intestinal tract. This situation favors the quickly absorbed water-soluble penicillin, since there is less exposure to adverse conditions in the stomach, but diminishes the action of the ester, which appears to require digestive cleavage before absorption.

The data presented above indicate that when injected subcutaneously in mice, benzyl penicillin is at least 3 times as potent as ordinary sodium penicillin in aqueous solution or suspended in oil. When taken by mouth, the benzyl penicillin is less active than by injection, but still is sufficiently potent to make it substantially as effective as an equivalent weight of sodium penicillin given by subcutaneous injection. The potential advantages of these favorable properties are obvious. Clinical data are being published elsewhere[4] which demonstrate that these advantages may also be seen in patients.

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[3] L. C. Miller and M. L. Tainter, *Proc. Soc. Exp. Biol. and Med.*, 57: 261–264, 1944.

[4] T. O. Gamble, L. C. Miller and M. L. Tainter, *Am. Jour. Obst. and Gynec.* (in press).

THE FUNCTIONAL PATHOLOGY OF FROSTBITE AND THE PREVENTION OF GANGRENE IN EXPERIMENTAL ANIMALS AND HUMANS[1]

THE functional pathology of frostbite has thus far been obscure. Apart from the excellent studies of Greene,[2] who has approached this problem mainly from the morphologic viewpoint, few basic facts were available. The use of the fluorescein test[3, 4] has thrown more light on the pathologic physiology of the frostbite lesion. In this test small amounts of fluorescein are injected and its migration with the blood stream and into the interstitial spaces is observed under ultra-violet light.

Six rabbits were depilated on the abdomen and exposed to cold by applying the bottom of small glass beakers filled with dry ice to the skin. The periods of exposure varied from 5 to 90 minutes. Under this exposure the skin freezes solid and thaws after intervals varying between 5 and 25 minutes, depending on the length of exposure. For periods of 30 to 120 minutes following such refrigeration no fluorescein appears in the exposed areas indicating a severe spasm of the arterioles. After this time a second stage is initiated during which all blood vessels reopen and fluorescein can be seen throughout the exposed area. The diffusion of fluorescein into the surrounding tissues in the second stage is many times greater than in the non-exposed skin giving the picture of intense hyperfluorescence in the previously frozen areas. This period is also characterized by marked swelling of the exposed areas. Eight to fourteen hours after exposure a repeat fluorescein injection shows that now the exposed spots are not fluorescent, indicating a pre-gangrenous state. This non-fluorescence increases in the next hours until finally the entire spot is non-fluorescent and becomes gangrenous. Biopsies taken at this time show that, in agreement with the findings of Greene[2] and Krey-

[1] Aided by grants from the John and Mary R. Markle Foundation and the Council on Pharmacy and Therapeutics of the American Medical Association.

[2] R. Greene, *Jour. Path. and Bact.*, 55: 259–267, 1943.

[3] K. Lange and L. J. Boyd, *M. Clin N. A.*, 26: 934–952, 1942.

[4] *Idem, Arch. Int. Med.*, 74: 175–184, 1944.