Reversal of Cycloserine Inhibition by *D*-alanine.* (25064)

GERALD D. SHOCKMAN (Introduced by Grace Medes)
Institute for Cancer Research, Philadelphia, Pa.

Bondi *et al.*(1) showed that the inhibitory action of cycloserine (*D*-4-amino-3-isoxazolidone)(2,3) on several strains of *Staphylococcus aureus* is competitively antagonized by *D*- or *L*-alanine. According to findings of Ciak and Hahn(4) natural *D*-cycloserine can cause protoplast formation in *Escherichia coli* and accumulation of N-acylamino sugar in *Staph. aureus*, while the synthetic *L*-isomer does not produce these effects. These authors have related the similarity of action of *D*-cycloserine and penicillin on bacterial cell wall formation to their similarity in chemical structure. Park (5) has suggested that cycloserine might "prevent normal incorporation of *D*-alanine into the wall." During studies with *Streptococcus faecalis*, we found that the inhibitory action of natural cycloserine on cells that are not actively growing but are making cell wall substance(6) is specifically reversed by the *D*-form but not by the *L*-form of alanine.

Methods. Growth of *S. faecalis* (ATCC 9790) in complete synthetic medium(7) was stopped during logarithmic growth phase by rapid chilling and the cells harvested and thoroughly washed aseptically at 0 to 2°C. Aliquots of cell crop were then placed in "Wall Medium" which contained/ml : *L*-glutamic acid, 0.3 mg; *L*-aspartic acid, 0.1 mg; *L*-lysine, 0.1 mg; *DL*-alanine 0.2 mg; *L*-cystine, 0.2 mg; guanine, 0.03 mg; uracil, 0.03 mg; ammonium sulfate, 0.6 mg; sodium acetate, 1.2 mg; glucose, 10 mg; sodium phosphate buffer, pH 6.5, 0.3 m mole; magnesium sulfate 0.1 mg; ferrous sulfate 5.5 μg; manganous sulfate 6.8 μg. The cells were incubated at 38°C and their optical density read at frequent intervals(8). In later experiments the organism was grown in Vit. B_6 deficient medium (less than 10^{-6} μg/ml measured as pyridoxamine with *S. faecalis*) with one-third of *DL*-alanine concentration (0.2 mg/ml) previously employed. While this amount is sufficient to give logarithmic growth to high turbidities, it significantly reduces amount of intracellular alanine which, in absence of added alanine, is available for cell wall synthesis. Under these conditions a study was made of the effect of penicillin and cycloserine† on cell wall synthesis.

Results. When *S. faecalis* cells are taken from the log phase and incubated in the relatively simple "Wall Medium" a turbidity increase that is accompanied by synthesis of additional cell wall substance takes place(6). When synthesis of additional wall substance is prevented, such as by lack of a nutrient essential to the cell wall, cells taken from log phase are prone to autolysis either in growth medium or in "Wall Medium"(7,8). Fig. 1 shows turbidity increase in "Wall Medium" and the inhibitory effect of several levels of penicillin (Fig. 1a) on this process. In complete growth medium growth of the same quantity of cells will not be inhibited by 33 μg/ml of penicillin. Contrary to the penicillin effect, as much as 200 μg/ml of cycloserine causes little inhibition in complete "Wall Medium" (Fig. 1b, curve 2). When alanine is omitted from this medium the overall response is only slightly depressed (curve 3) but it is now sensitive to cycloserine (curve 4), and in fact, 25 μg/ml of the antibiotic will lead to rapid lysis. Presence of *L*-alanine (curve 4) has no effect while addition of *D*-alanine partially reverses the cycloserine inhibition (curve 5). Further tests indicated that *D*-alanine was effective in far lower concentration than was *L*-isomer (*i.e.*, 1.6 μg/ml of the *D*-form was approximately equivalent to 12.5 μg/ml of the *L*-form in reversing the action of 25 μg/ml of cycloserine). Since Vit. B_6 is involved in synthesis and racemization of alanine, B_6 deficient cells were employed in

* These investigations were supported by research grants, Nat. Inst. of Allergy and Infect. Dis., U. S. P. H. S., Office of Naval Research and Am. Cancer Soc.

† Cycloserine was supplied by Eli Lilly and Co. (trade name Seromycin).

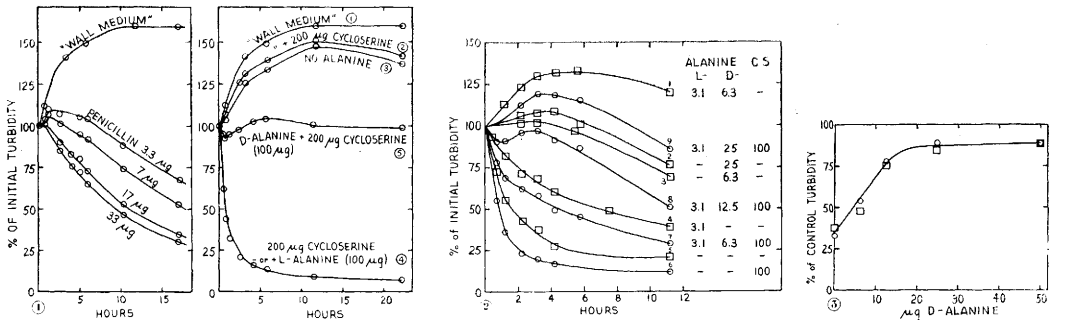

FIG. 1. Effect of penicillin (Fig. 1a) and cycloserine (Fig. 1b) on incubation of log phase *S. faecalis* cells in "Wall Medium." Amounts indicated are μg/ml. Results expressed as % of turbidity of incubated tubes at time 0. *DL*-alanine was omitted from "Wall Medium" for curves 3 to 5 of Fig. 1b, with supplements as indicated.

FIG. 2. Effect of cycloserine on incubation of Vit. B_6 deficient *S. faecalis* cells in "Wall Medium" without *DL*-alanine. Individual alanine isomers were added as indicated. Amounts are μg/ml; CS indicates cycloserine. Curves 1 to 5, with square symbols represent experimental tubes without cycloserine, curves 6 to 9 (circles) with cycloserine (100 μg). Curve 6 also represents 3.1 μg *L*-alanine, 0 μg *D*-alanine, 100 μg CS. Results expressed as in Fig. 1.

FIG. 3. Effect of *L*-alanine concentration on reversal of cycloserine effect by *D*-alanine during incubation in "Wall Medium." Results expressed as % of turbidity attained after 3 hr incubation (Fig. 2) of identical control tubes without cycloserine. Cycloserine concentration, 100 μg/ml. Square symbols represent, per ml, the presence of 3.1 μg *L-alanine*, the circles 50 μg *L*-alanine.

further experiments. B_6 deficient cells require both alanine isomers for wall synthesis (Fig. 2, curves 1 to 5). Curves 6 to 9, Fig. 2, show the effect of increasing concentration of *D*-alanine, in presence of a constant amount of *L*-alanine (3.1 μg/ml), on inhibition produced by 100 μg/ml of cycloserine. Fig. 3 demonstrates that reversal of cycloserine action by *D*-alanine is independent of concentration of *L*-alanine over a wide range (3.1 to 50 μg/ml), and that inhibition is reversed by *D*-alanine at one-fourth the amount of antibiotic by about 90%. *D*-alanine was proportionally effective over the range of cycloserine concentrations tested (25 to 500 μg/ml), indicating a competitive relationship(9).

Discussion. The reversal of cycloserine inhibition seems to be specific for *D*-alanine. It seems probable that the small effect of the *L*-isomer, particularly at high concentrations, is due to a low level of contamination of the product used with *D*-alanine or Vit. B_6 active substances.‡ None of the amino acids of the "Wall Medium" antagonized the action of penicillin, nor was the action of cycloserine antagonized by any of the amino acids except *D*-alanine.

Cycloserine seems to be one of the few examples of an antibiotic that is a logical antagonist. It is a derivative of *D*-serine and a structural analog of a number of neutral *D*-amino acids of which only *D*-alanine has thus far been found to play an active part in bacterial cell synthesis. It would not be surprising if a number of neutral *D*-amino acids were found to act in a manner similar to cycloserine. The action of neutral *D*-amino acids in producing protoplast-like structure of *Alkaligenes faecalis*(11) may be such an example.

The competition of cycloserine and *D*-alanine is not at a permeability site as indicated by the reversing effect of intracellular *D*-alanine. As postulated by other workers(4,5), it may take place at site of incorporation of *D*-alanine into a wall precursor(12). Despite their structural similarity(4,13), and despite the fact that both interfere with the same biosynthetic process, penicillin and cycloserine differ in their mode of action. Their synergistic effect(2) may be due to sequential blocking of cell wall synthesis.

Summary. *D*-alanine, but not *L*-alanine,

‡ Vit. B_6 active substances were found in 5 high quality commercial *L*-alanine products and in 1 *L*-alanine sample obtained from Dr. J. Greenstein in 1951, and these were removed from the *D*- and *L*-alanine used in present experiments by treatment with activated charcoal(10).

competitively reverses the inhibitory action of cycloserine on *S. faecalis* cells that are synthesizing cell wall substance. Both cycloserine and penicillin can inhibit cells that are not growing but that are making cell wall substance. However, they differ in mode of action.

I am indebted to P. Conover for skillful technical assistance and to Dr. G. Toennies for helpful advice.

1. Bondi, A., Kornblum, J., Forte, C., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 270.

2. Harris, D. A., Ruger, M., Reagan, M. A., Wolf, F. S., Peck, R. L., Wallick, H., Woodruff, H. B., *Antibiot. & Chemother.,* 1955, v5, 183.

3. Kuehl, F. A., Wolf, F. S., Trenner, N. R., Peck, R. L., Howe, E. E., Hunnewell, B. D., Downing, G., Newstead, E., Buhs, R. P., Putter, I., Ormond, R., Lyons, J. E., Chaiet, L., Folkers, K., *J. Am. Chem. Soc.,* 1955, v77, 2344.

4. Ciak, J., Hahn, F. E., *Antibiot. & Chemother.,* 1959, v9, 47.

5. Park, J. T., *Symp. Soc. Gen. Microbiol.,* 1958, v8, 49.

6. Shockman, G. D., *Bact. Proc.,* 1959, 128.

7. Shockman, G. D., Kolb, J. J., Toennies, G., *J. Biol. Chem.,* 1958, v230, 961.

8. ———, *Bact. Proc.,* 1957, 131.

9. Woolley, D. W., *A Study of Antimetabolites,* John Wiley & Sons, N. Y., 1952.

10. Snell, E. E., *J. Biol. Chem.,* 1945, v158, 497.

11. Lark, C., Lark, K. G., *Bact. Proc.,* 1958, 108.

12. Park, J. T., Strominger, J. L., *Science,* 1957, v125, 99.

13. McQuillen, K., *Proc. 4th Int. Congr. Biochem., Vienna,* 1958, v13, 406.

Received June 8, 1959. P.S.E.B.M., 1959, v101.

Factors Responsible for Decline of Inflammation in Arthus Hypersensitivity Vasculitis.*† (25065)

CHARLES G. COCHRANE,‡ WILLIAM O. WEIGLE§ AND FRANK J. DIXON
Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pa.

In recent studies of Arthus-type vascular inflammatory reactions(1,2), a rough correlation was found to exist between disappearance of antigen from the vessel and a decrease of inflammation in the reaction. This was noted both macroscopically, and in the vessels, microscopically. These results suggested that removal or perhaps some form of isolation of the antigen-antibody complex eliminated the phlogogenic stimulus and allowed healing of the damaged vessels to take place. However, such factors as a possible exhaustion of cellular or humoral components essential to development of the reaction, and/or production of inhibitors locally might account for the decrease in inflammatory reaction also. In order to find if these latter factors played an essential role in the decline of inflammation, experiments were performed in which antigen was reintroduced into healing Arthus sites at various times. This was performed under the supposition that any inflammation secondary to the presence of the reinjected antigen and its corresponding antibody would be prevented or at least decreased in intensity, if an exhaustion of essential materials had occurred, or if inhibitors played an important role.

Materials and methods. Antigen: Bovine serum albumin (BSA), Armour Laboratories, Lot #T-68204 was used to elicit the Arthus reactions. Aside from BSA, other antigens used for control purposes or to obtain antibodies for the fluorescent antibody studies included rabbit serum albumin (RSA) and rabbit serum globulin (RSG), (Fraction II), Pentex, Inc., Lot #6901 and human gamma globulin (HGG), (Fraction II), Squibb Laboratories, Lot #1615. *Antisera*: Antibodies to the antigens mentioned above were produced, characterized and absorbed to remove con-

* Publication No. 209, Dept. of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

† This work supported by U. S. Atomic Energy Comm. and U.S.P.H.S.

‡ Sarah Mellon Scaife Fellow in Pathology.

§ Public Health Research Fellow of Nat. Inst. of Neurol. Dis. and Blindness.