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Erythromycin, Studies of Its Mode of Action

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Edited by

J. E. Potzger

The *Butler University Botanical Studies* journal was published by the Botany Department of Butler University, Indianapolis, Indiana, from 1929 to 1964. The scientific journal featured original papers primarily on plant ecology, taxonomy, and microbiology. The papers contain valuable historical studies, especially floristic surveys that document Indiana's vegetation in past decades. Authors were Butler faculty, current and former master's degree students and undergraduates, and other Indiana botanists. The journal was started by Stanley Cain, noted conservation biologist, and edited through most of its years of production by Ray C. Friesner, Butler's first botanist and founder of the department in 1919. The journal was distributed to learned societies and libraries through exchange.

During the years of the journal's publication, the Butler University Botany Department had an active program of research and student training. 201 bachelor's degrees and 75 master's degrees in Botany were conferred during this period. Thirty-five of these graduates went on to earn doctorates at other institutions.

The Botany Department attracted many notable faculty members and students. Distinguished faculty, in addition to Cain and Friesner , included John E. Potzger, a forest ecologist and palynologist, Willard Nelson Clute, co-founder of the American Fern Society, Marion T. Hall, former director of the Morton Arboretum, C. Mervin Palmer, Rex Webster, and John Pelton. Some of the former undergraduate and master's students who made active contributions to the fields of botany and ecology include Dwight. W. Billings, Fay Kenoyer Daily, William A. Daily, Rexford Daudenmire, Francis Hueber, Frank McCormick, Scott McCoy, Robert Petty, Potzger, Helene Starcs, and Theodore Sperry. Cain, Daubenmire, Potzger, and Billings served as Presidents of the Ecological Society of America.

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ERYTHROMYCIN, STUDIES OF ITS MODE OF ACTION

FRANK STREIGHTOFF

Eli Lilly and Company
Indianapolis, Indiana

Relatively little is known of the mode of action of erythromycin. This antibiotic is widely used in the treatment of infectious diseases and has undoubtedy prolonged the lives of thousands of people. It would seem axiomatic that more knowledge of the mode of action would serve as a foundation for more effective clinical use.

Although Pittenger et al. (1) and Haight and Finland (2) had tested some organic and inorganic compounds for reversal of the inhibitory action of erythromycin, it seemed profitable to use a more informative method. Such tests have usually been carried out in liquid media using a single concentration of the compound in question and one level of the inhibitory substance. It was felt that by the use of agar plates, seeded with a sensitive organism, a solution of the test compound could be introduced on a filter paper disk. Through diffusion an infinite variety of concentrations of the test substance would be present in concentric zones around the filter paper disk. The antibiotic could be added either in the bacteriostatic threshold concentration to the liquid seeded agar medium before pouring the plate, or added on filter paper disks close to the test compound disks on the hardened agar surface. After incubation the zones of growth or inhibition could be interpreted in terms of reversal or potentiation of the inhibition of erythromycin and toxicity of the test compound.

Brown and Emerson (3) found that pantothenic acid, beta-alanine and carnosine reversed the inhibitory action of erythromycin towards the *gravis* strain of *Corynebacterium diphtheriae*. No confirmation has been made to date by other investigators. It would seem desirable not only to attempt to confirm their work but also to look for a similar situation in other organisms. It appeared that organisms in which such an effect would be most apparent would be those which require either pantothenic acid or beta-alanine for growth. In this category *Lactobacillus casei* and *L. arabinosus* require pantothenic acid for growth and numerous strains of yeast require either pantothenic acid or beta-alanine. Since erythromycin is used in the treatment of diseases caused by *Diplococcus*. *Micrococcus* and *Streptococcus,* it seemed pertinent to use those organisms to test for the reversal by pantothenic acid or beta-alanine of the inhibition due to erythromycin. Finally, it seemed desirable to determine whether the resistance of highly resistant strains of *Mi-*

crococcus pyogenes var. *aureus* is due to high production of pantothenic acid.

Many organisms which are resistant to penicillin are resistant by virtue of their production of penicillinase, an enzyme capable of destroying penicillin. This enzyme has been found to be extracellular and intracellular in normally resistant organisms and in organisms in which the resistance has developed (4). As one of the possible mechanisms of resistance to erythromycin, it seemed obvious that an attempt should be made to find erythromycinase. Haight and Finland (2), using many strains of the highly resistant gram-negative organisms, could find no extracellular erythromycinase. Since resistance in staphylococcal infections has become a serious problem, it appeared of value to determine the presence or absence of intracellular or extracellular erythromycinase in the two highly resistant strains of *Micrococcus pyogenes* var. *aureus* available.

One possible mode of action of erythromycin would be its interference with the functioning of one or more enzymes necessary to the growth or reproduction or action of the sensitive organism. Stone et al. (5) recently reported that staphylococci which were highly resistant to erythromycin in vitro were still sensitive when treated in vivo in mice. Since the pathogenicity of the staphylococcus is closely associated with coagulase production, one explanation for this phenomenon would be an inhibition of coagulase by the antibiotic.

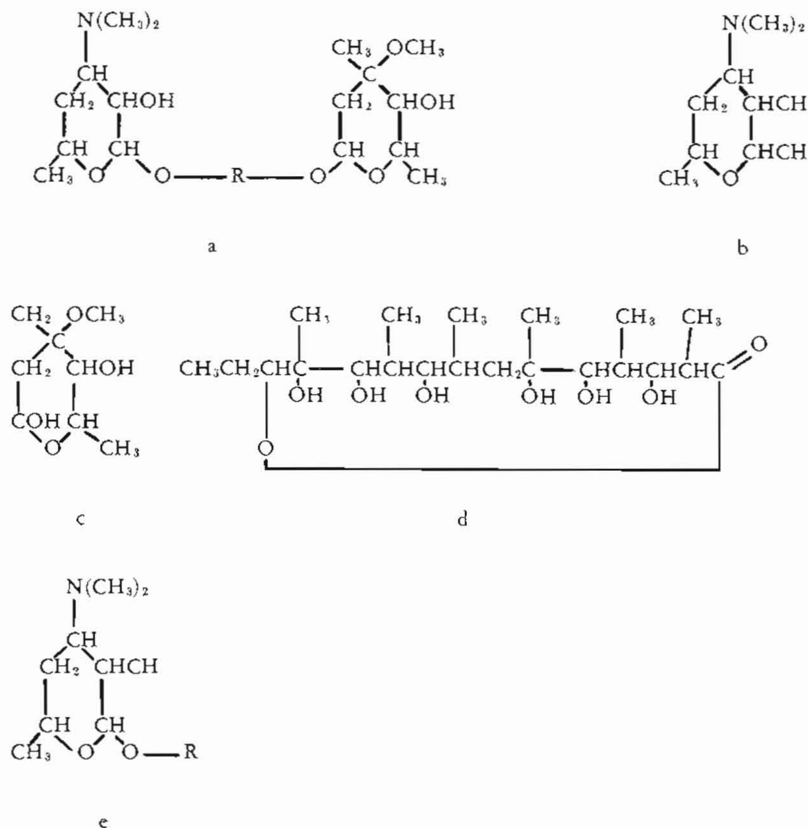
REVIEW OF THE LITERATURE

Erythromycin has the formula $C_{37}H_{67}NO_{13}$ with a structure approximating that shown in Fig. 1 and a molecular weight of 734. It is a base with a pK 8.6. It is slightly soluble in water and more readily soluble in organic solvents. A pH of less than 4 or higher than 10 is highly destructive (6, 7). Degradation studies have led to the identification of large fragments, including erythralosamine, $C_{29}H_{49}NO_8$, and cladenose, $C_8H_{16}O_4$ (Fig.1) (7). One fraction of erythralosamine is desosamine, $C_8H_{17}NO_3$ (Fig. 1) (7). A large fragment, dihydroerythronolide, $C_{21}H_{41}O_8$, has been described (Fig. 1) (8).

Erythromycin appears to be a member of a group of antibiotics which includes: albomycetin ($C_{32}H_{54}NO_9$, mol. wt., 596) (9), amaromycin (10), angolamycin ($C_{49-51}H_{87-91}NO_{18}$, mol. wt., 977-1005) (11), carbomycin ($C_{41}H_{67}NO_{16}$, mol. wt., 830) (12, 13), carbomycin B ($C_{41-42}H_{67-69}NO_{15}$, mol. wt., 814-828) (14), celesticetin ($C_{24}H_{36-40}N_2O_9$, mol. wt., 496-500) (15), erythromycin B ($C_{37}H_{71}NO_{12}$, mol. wt., 721) (16), griseomycin (17), leucomycin ($C_{27}H_{42}NO_{10}$, mol. wt., 540) (18), methamycin ($C_{25}H_{43}NO_7$, mol. wt., 470) (19), narbomycin ($C_{28}H_{47}NO_7$, mol. wt., 510) (20), antibiotic PA-105 (21), pikromycin ($C_{25}H_{43}NO_7$, mol. wt., 477), (22), rhodomycin (23) and spiromycin (24).

In general the members of the erythromycin antibiotic group exhibit

FIGURE 1



a. erythromycin, b. desosamine, c. cladenose,
d. dihydroerythronolide, e. erythralosamine (R is dihydroerythronolide minus 2H; R' is dihydroerythronolide minus $2H_2O$).

similar antibacterial and antiviral spectra. The resemblance of the spectra of carbomycin (12), celesticetin (25), erythromycin B (26), griseomycin (17), antibiotic PA-105 (21) and spiromycin (24) to that of erythromycin has been noted by other investigators. Organisms showing moderate to high sensitivity to erythromycin include the following genera: *Bacillus, Brucella, Clostridium, Corynebacterium, Diplococcus, Hemophilus, Micrococcus, Mycobacterium, Neisseria, Rickettsia, Streptococcus, Vibrio,* lymphogranuloma virus and meningopneumonitis virus. In general the gram-negative organ-

isms, *Escherichia, Proteus, Pseudomonas, Salmonella* and *Shigella* show high resistance. There are variations in the degree of effectiveness of the different members of this antibiotic family. Carbomycin, for example, is usually less effective than erythromycin. There are some marked differences. Amaromycin, for example, inhibits *Brucella abortus* with 1.56 mcg/ml (10) while erythromycin does not inhibit the organism with 1000 mcg/ml (17) even though it is quite active against other members of the genus (6).

Cross-resistance has been demonstrated between erythromycin and erythromycin B (27), carbomycin (28), celesticetin (25), griseomycin (17), antibiotic PA-105 (21), and spiromycin (24). Cross-resistance evidence suggests that leucomycin is closely related to carbomycin and somewhat less closely related to erythromycin (29).

The chemical structure desosamine has been demonstrated in erythromycin (7), erythromycin B (16), pikromycin (23) and narbomycin (20). Carbomycin and carbomycin B contain the very similar desoxydimethyl amino sugar, mycaminose (14). In rhodomycin the order in which the radicals are attached to the desosamine ring has been changed (23). One of the radicals attached to the ring structure in desosamine is a dimethyl amine. It is interesting to note that albomycin (9), angolamycin (11), carbomycin (12), carbomycin B (14), erythromycin (7), erythromycin B (16), leucomycin (18), methamycin (19), narbomycin (20) and pikromycin (22) all have but a single nitrogen atom.

The literature on other members of the erythromycin antibiotic family, reviewed here, indicates no mode of action.

The effect of various inorganic and organic substances on the bacteriostatic action of erythromycin has been investigated. Pittenger et al. (1), working with *Micrococcus pyogenes* var. *aureus* in vitro, found that various concentrations of Cl^-, NO_3^-, SO_4^-, Na^+, K^+, Mg^{++}, Ca^{++}, Mn^{++}, Cu^{++}, Co^{++}, Fe^{++}, Zn^{++}, Na-thioglycollate, cysteine-HCl, acetate, fumarate, alpha-glutarate, malate, oxalactate, pyruvate, succinate, citrate and vitamin mixtures (riboflavin, thiamin, biotin, niacin, p-aminobenzoic acid, calcium panthothenate, pyridoxine, pyridoxal, pyridoxamine and folic acid) had neither appreciable potentiation or reversal of the bacteriostatic ability of erythromycin. Haight and Finland (2) found that various concentrations of NaCl, dextrose, Na-thioglycollate, cysteine-HCl, semicarbozide, urea, glutamic acid, p-aminobenzoic acid and pteroylglutamic acid had no significant effect on the in vitro inhibition of *Bacillus cereus* no. 5 by erythromycin. They also reported that various concentrations of the sodium salts of citric, pyruvic, acetic, lactic, fumaric and succinic acids and the enzyme penicillinase had no effect on the inhibition of *Streptococcus* no. 98. Human sera at various levels had no effect on the antibiotic's inhibition of *Sarcina lutea.* Brown and Emerson (3) reported that 500 mcg/ml beta-alanine or calcium pantothenate, equivalent to 200 mcg/ml beta-alanine, permitted growth of

Corynebacterium diphtheriae in 70 times the original minimal inhibitory concentration of erythromycin. L-carnosine, equivalent to 100-400 mcg/ml of beta-alanine, reversed the inhibition of up to 30 times the minimal inhibitory concentration of the antibiotic.

Haight and Finland (2) found a 530 and 800-fold increase of the in vitro inhibitory activity of erythromycin against *Streptococcus* no. 98 and *Sarcina lutea*. respectively, when the pH was increased from 5.6 to 8.5. Pittenger et al. (1) found a 30-fold increase in the bacteriostatic action against *Micrococcus pyogenes* var. *aureus* with the increase of pH from 6.5 to 8.5. They also noted that an apparent potentiating effect of phosphates on the activity of erythromycin was actually due to the effect of the buffering action on pH. Pittenger et al. (1) suggested that the free base had much more activity than the salt of the antibiotic and that the increase of activity as the pH increased was due to release of the free base.

Haight and Finland (2) found that a change in the pH by replacing the oxygen with carbon dioxide did not affect the bacteriostatic action of erythromycin against *Streptococcus* no. 98, *Streptococcus* C203, *Sarcina lutea, Streptococcus viridans, Diplococcus pneumoniae* type III, *Staphylococcus* no. 195 or an enterococcus.

Resistance has been developed in sensitive strains in the laboratory. Pittenger et al. (1) found that *Micrococcus pyogenes* var. *aureus* increased its resistance to erythromycin about 300-fold in 25 serial transfers in the presence of the antibiotic. The development of resistance to erythromycin was slightly less rapid than the development of resistance to penicillin and much less rapid than the development of resistance to streptomycin under the same conditions. No organism which had become nutritionally dependent on erythromycin was found. Hsie and Kotz (30), however, developed a strain of *M. pyogenes* var. *aureus* which had become nutritionally dependent on carbomycin.

Haight and Finland (2) attempted to determine whether species which are naturally resistant to erythromycin produce erythromycinase. Using the method of Gots (31) numerous strains of *Pseudomonas aeruginosa, Aerobacter aerogenes, Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris, Salmonella* and *Shigella* were checked for the production of an extracellular erythromycinase which would destroy erythromycin which was present in an amount just inhibitory to the test organism, *Sarcina lutea*. No evidence of destruction of the antibiotic was found. By a second method, suspensions of *Proteus vulgaris* and *Escherichia coli* organisms had no destructive effect on a solution of erythromycin over a 72-hour period.

Obviously, there are present in nature mechanisms for the destruction of this antibiotic. The erythromycin content of plasma in vivo drops rapidly without a corresponding amount being excreted in the urine or otherwise being accounted for (2). One scrap of evidence of its metabolism has been

found. Des-N-methyl erythromycin has been isolated from the bile of dogs (32).

Stone et al. (5) found that coagulase-positive strains of *Micrococcus pyogenes* var. *aureus* which had been isolated from human cases showed large variation in in-vitro sensitivity but showed small variation in in-vivo sensitivity to erythromycin. Inhibition in vitro was caused by 0.195 mcg/ml erythromycin for strain no. 3055A, 10,000 mcg/ml, for strain no. 3066A, and 5,000 mcg/ml, for strain no. 3072A. In 20 to 25 g Swiss mice, the effective dose of erythromycin 50 per cent of the time was 18 mcg for strain no. 3055A, 22 mcg for strain no. 3066A and 116 mcg for strain no. 3072A. Of significance, in interpretation of this work, is the widely accepted belief that coagulase production is the best indication of pathogenicity in strains of *M. pyogenes* var. *aureus* (33). Boniece et al. (34) found that erythromycin was effective in reducing the coagulase activity of numerous strains of *M. pyogenes* var. *aureus*.

In one of the few papers on the effect of erythromycin on enzymes, Jacobson and Ayevedo (35) reported that erythromycin did not inhibit fumerase, lipase, cholinesterase and thiaminase from animal sources or the fermentation of alcohol by yeast.

MATERIALS AND METHODS

Materials

ORGANISMS:

- *Bacillus subtilis*, spore suspension, strain X12.1, R. C. Pittenger
- *Corynebacterium diphtheriae*, strain X166, R. C. Pittenger, strain Toronto, McClain
- *Diplococcus pneumoniae*, strain type 1, Park
- *Lactobacillus casei*, no. 7469, American Type Culture Collection
- *Micrococcus pyogenes* var. *aureus*, strain no. 3055, strain no 3066, strain no. 3067, strain no. 3074, all from Boniece
- *Proteus vulgaris*, strain no. 9484, American Type Culture Collection
- *Pseudomonas aeruginosa*, strain X239, R. C. Pittenger
- *Streptococcus pyogenes*, C203, strain no. 8868, American Type Culture Collection
- *Saccharomyces cerevisiae*, "Gebrüder Mayer," strain no. 7752, "Old Process," strain no 7753, "Fleischmann's Baker's," strain no. 7754, American Type Culture Collection

MEDIA:

- Brain heart infusion (Difco) used with *Corynebacterium*
- Hydrolyzed casein medium (36) used with *Micrococcus*
- Mycin assay ager (Difco) used with *Bacillus subtilis*
- Tryptose (Difco), 1 per cent, in nutrient broth (Difco) used with *Corynebacterium*
- Pantothenic acid assay medium (Difco) used with *Lactobacillus*
- Seed agar (Baltimore Biological Laboratories) used with *Micrococcus*, *Proteus* and *Pseudomonas*
- Tryptose medium (37) used with *Diplococcus* and *Streptococcus*
- Yeast synthetic medium (38, 39) used with yeast

INSTRUMENTS:

Laboratory Model G, Beckman pH Meter
Junior Spectrophotometer, Model 6A, Coleman Instruments, Inc.

OTHER MATERIALS:

Alumina A-303, Aluminum Company of America
Discs of very pure, highly absorbent paper for the assay of penicillin and other antibacterial substances, ½-in (12.7 mm) diam, No. 740-E, Carl Schleicher and Schuell
Erythromycin standard solution, 100 mcg/ml. One hundred mg 'Ilotycin' (erythromycin, Lilly), lot no. 652282, was dissolved in 100 ml 1 per cent sodium and potassium phosphate solution adjusted to a pH of 7.0 (Hartman-Leddon Co.) and filtered through a Seitz filter. Solution was made up fresh at least once a month and stored in a refrigerator until used.
"Tes-Tape," a paper impregnated with glucose oxidase and horse-radish peroxidase, Eli Lilly Company

Screening of Compounds to Determine Reversal or Potentiation of Action of Erythromycin

Method of Screening. Work was begun using seeded agar plates to which erythromycin was added on a centrally located paper disk. Solutions of compounds to be tested were added to paper disks which had been placed on the agar. Part of the disk would cover agar in which the antibiotic would diffuse from the erythromycin disk and would be expected to inhibit growth. Part of the disk would cover agar where no inhibition would be expected to occur.

It was decided that seeded plates, in which a bacteriostatic threshold level of erythromycin was present, would constitute a sensitive means of demonstrating any reversal action that a compound might have. In this event the disk to which an active compound had been added would be surrounded by a circular zone or a halo in which growth of the test organism took place. The diameter of the zone would be related to diffusability of the compound as well as to its inherent activity.

Micrococcus pyogenes var. *aureus* no. 3055, a strain sensitive to several antibiotics, and spores of *Bacillus subtilis* were used for these tests. Synthetic media were tried first and found unsatisfactory. Seed agar (BBL) was used with *M. pyogenes* and mycin agar (Difco) was used with *B. subtilis*. Ten ml of heated liquid agar was poured into a Petri dish. After the agar base had hardened, 5 ml additional liquid agar containing 0.5 ml of a 1/100 dilution of either organism and, when indicated, containing 0.5 ml of the appropriate concentration of erythromycin, were layered in on top. When *M. pyogenes* was used, 50 mcg of the antibiotic was either incorporated in the seeded agar or added to the paper disk. When *B. subtilis* was used, 10 mcg of the antibiotic was used. The antibiotic was added to the paper disk in 0.05 ml quantities; solutions of test compounds were added in 0.07 ml quantities. To prevent absorption of moisture from the medium by the paper disks, solutions were added to the disks immediately after they had been placed on

the agar. Plates were incubated at 37°C. for 24 hours and then examined for growth.

Compounds Screened. A large number of compounds were screened for effect on the inhibitory action of erythromycin against *Micrococcus pyogenes* var. *aureus* and the spores of *Bacillus subtilis,* using both techniques. Compounds and their per cent concentration in solution were as follows:

amino acids: DL-alpha-alanine, 1 and 10; beta-alanine, 0.1 and 10; L-arginine, 1; DL-aspartic acid, 1 and 2; hydrolyzed casein (vitamin-free). 10; DL-citrulline, 1; creatine, 1; L-cysteine-HCl, 1 and 10; ethionine, 1; L-glutamic acid, 1 and 2; L-histidine-HCl, 1 and 5; DL-isoleucine, 2; L-leucine-HCl, 1; DL-lysine-HCl, 1; DL-methionine, 1; norleucine, 1; DL-phenylalanine, 1; L-proline, 1; DL-serine, 1; DL-threonine, 1; DL-tryptophan, 1; DL-valine, 2

purines: adenine sulfate, 1; guanine-HCl, 0.7; uracil, 1; xanthine, 1 (All purines were brought into solution with NaOH.)

organic acids: citric acid, 1 and 10; oxalic acid, 1 and 10

vitamins: acetyl choline-HCl, 10; ascorbic acid, 1; beta-alanine, 0.1 and 10; biotin, 0.000,04, Ca-pantothenate, 0.1 and 1; inositol, 0.1; niacin, 0.06; p-aminobenzoic acid, 1; pimelic acid, 0.025; pyridoxine, 0.12, thiamine-HCl, 0.1; yeast extract, 10

carbohydrates: dextrose, 10; lactose, 10; levulose, 10; D-maltose, 1; mannose, 1; mannitol, 10; raffinose, 10, rhamnose, 2, ribose, 1; sucrose, 1 and 10; xylose, 10

miscellaneous organic compounds: adeoylic acid, 1; L-asparagine, 1 and 10; beef extract, 10; betaine, 1; creatinine, 1; desosamine, 10; D-glucosamine, 1; glutathionine, 1; glycerol, 10; mercaptoethyl amine, 10; mucio, 5; Na-thioglycollate, 1; tetra sodium salt of ethylene diamine tetra acetic acid, 9.4

inorganic compounds: $FeSO_4.7H_2O$, 0.1 and 10; KCl, 0.1; $ZnSO_4.7H_2O$, 0.1; $TlSO_4$, 0.1; H_3BO_3, 0.1; NaH_2PO_4 and Na_2HPO_4 (pH 7.0), 10

Hydrogen Ion concentration. The pH was determined for all samples screened which had not been previously discarded.

Since some of the compounds which reversed erythromycin were found to have pH's of 4.0 or less, the pH's of the solutions of seven of these compounds, adenylic acid, ascorbic acid, L-aspartic acid, L-cysteine-HCl, L-glutamic acid, glutathione and L-histidine-HCl, were brought up to the range of 6.0 to 7.0. Solutions of these neutralized compounds were added to paper disks on seeded plates containing a bacteriostatic amount of the antibiotic and checked for reversal of the action of the antibiotic.

L-Cysteine-Hydrochloride. This compound was tested for reaction with erythromycin under mildly acid conditions. A 0.2 per cent L-cysteine-HCl solution was made in erythromycin standard solution and the pH adjusted to 5.2. As a control, erythromycin standard solution was adjusted to a pH of 5.2. The cysteine-erythromycin and control solutions were heated in a 56°C. water bath for 1 hour. Fifteen ml of each solution were filtered through a Seitz filter and compared, using paper disk assay techniques with both test organisms. The diameters of the zones of inhibition were measured and compared as an indication of comparative potency. The remainder of the cysteine-erythromycin and control solutions were stored in a refrigerator at 6°C. for

about 72 hours. The solutions were then filtered and comparative potencies again determined.

Organic and Inorganic acids. In view of the data which showed that compounds which reverse the action of erythromycin were acidic, additional acid substances were tested in the same manner as the original screening. Substances tested were alanine 2 per cent and HCl 1 per cent, glycine 2 per cent and HCl 1 per cent, HCl 1 per cent, lactic acid 1 per cent, malic acid 1 per cent, H_2SO_4 1 per cent and tartaric acid 1 per cent.

Revival. Finally, chemicals with the ability to reverse the action of erythromycin, including acidic substances, were checked for revival of organisms which had been inhibited by erythromycin. Agar plates seeded with either test organism and containing bacteriostatic concentrations of erythromycin were incubated at 37°C. for 24 hours. Solutions of test compounds were added to paper disks on the surface of the agar. The plates were incubated an additional 48 hours and then examined for revival of organisms.

Investigation of the Reversal by Calcium Pantothenate and Beta-Alanine of the Inhibitory Effects of Erythromycin on Several Species of Bacteria

Lactobacillus casei. L. casei was used to determine if Ca-pantothenate and beta-alanine have the ability to reverse the inhibition caused by erythromycin. The procedure followed was that of the Difco Manual (40). Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.02, 0.2, 2 and 20 mcg/ml of the final volume. Four tubes containing each level of Ca-pantothenate received 0, 0.04, 0.2 and 1 mcg erythromycin per ml of the final volume, respectively (Tahle 1). Distilled water was added to bring the volume to 5 ml. Five ml pantothenic acid assay medium was added. The culture tubes were sterilized by autoclaving and then inoculated with a suspension of washed *L. casei.* The cultures were incubated at 37°C. for 72 hours. The pH was determined for each culture. An interpretation was made on the basis of pH as an indication of growth and acid production. This test was repeated. Subsequently, a test was set up in which all tubes received 0.2 mcg/ml Ca-pantothenate. Amounts of beta-alanine added to tubes were 0, 0.2, 2, 20 and 200 mgc/ml of the final volume. Four tubes containing each level of beta-alanine received 0, 0.2, 2, and 20 mcg erythromycin per ml of the final volume, respectively (Table 2).

Saccharomyces cerevisiae. Three strains of *S. cerevisiae,* "Old Process," "Fleischmann's Baker's" and "Gebrüder Mayer," were checked for their requirements of Ca-pantothenate. Using the synthetic medium of Williams et al. (38, 39) "Gebrüder Mayer" gave the greatest response to the addition of Ca-pantothenate to the medium. A test was set up with all tubes in duplicate. Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 mcg/ml of the final volume. Eight

tubes containing each level of Ca-pantothenate received 0, 1, 10 and 100 mcg erythromycin per ml of the final volume for each pair, respectively (Table 3). After inoculation, tests were incubated at room temperature for 5 days. Readings of optical density were made at a wave length of 550 lambdas with a spectrophotometer. Subsequently, another test was run in which amounts of beta-alanine added to the tubes were equivalent to 0, 0.2, 0.4, 0.6, 0.8 and 1 mcg/ml of the final volume. Eight tubes containing each level of beta-alanine received 0, 1, 10 and 100 mcg erythromycin per ml of the final volume for each pair, respectively (Table 4).

Micrococcus pyogenes var. *aureus*. *M. pyogenes* var. *aureus*, strains no. 3055 and no. 3067, were investigated using hydrolyzed casein medium (36). Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.1, 1 and 10 mcg/ml of the final volume; amounts of beta-alanine, were equivalent to 0, 10, 100 and 1000 mcg/ml. For each strain a set of four tubes at each level of Ca-pantothenate or beta-alanine received 0, 0.1, 0.2, 0.5, 1 and 2 mcg erythromycin per ml of the final volume, respectively (Table 5). After inoculation, cultures were incubated at 37°C. for 5 days. Growth was determined by a visual estimate of the turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

Streptococcus pyogenes and Diplococcus pneumoniae. *S. pyogenes* C-203 and *D. pneumoniae* Park type 1 were investigated using tryptose medium (37). Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.1, 1 and 10 mcg/ml of the final volume; amounts of beta-alanine, were equivalent to 0, 10, 100 and 1000 mcg/ml. For each organism a set of four tubes containing each level of Ca-pantothenate or beta-alanine received 0, 0.005, 0.01, 0.02 and 0.04 mcg erythromycin per ml of the final volume, respectively (Table 6). After inoculation, cultures were incubated at 37°C. for 4 days. Growth was determined by a visual estimate of the turbidity.

Corynebacterium diphtheriae. *C. diphtheriae* strains X-166 and Toronto were investigated using a medium of 1 per cent Tryptose (Difco) added to nutrient broth. Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.1, 1 and 10 mcg/ml of the final volume; amounts of beta-alanine, were equivalent to 0, 10, 100 and 1000 mcg/ml. For each strain four tubes containing each level of Ca-pantothenate or beta-alaniue received 0, 0.004, 0.0008, 0.016 and 0.032 mcg erythromycin per ml of the final volume, respectively (Table 7). It was necessary to use a large inoculum to get satisfactory growth. One drop of undiluted 5-day culture was used. After inoculation, the cultures were incubated at 37°C. for 9 days. After vigorous shaking, the growth was determined by a visual estimate of turbidity.

Pantothenic Acid Production by Resistant Strains of Micrococcus pyogenes var. aureus. Two resistant strains of *M. pyogenes* var. *aureus*, no. 3066 and no. 3074, and sensitive strain no. 3055 were grown in hydrolyzed casein

medium without the presence of pantothentic acid and beta-alanine. After inoculation, the cultures were incubated at 37°C. for 24 hours. A 10 ml aliquot of each culture was autoclaved at 15 lb. steam pressure for 15 minutes. To each culture was added 10 ml 4 per cent acetic acid, 2 ml N/1 NaOH and 1 g mylase. The tubes were placed in a 50°C. water bath for 24 hours. The pH was brought to 7.0. These samples were assayed for pantothenic acid using *Lactobacillus casei* in the manner earlier described.

Investigation of the Presence of Intracellular or Extracellular Erythromycinase

Gots' Method, Extracellular Erythromycinase. Using the method of Gots (4), *Proteus vulgaris, Pseudomonas aeruginosa* and resistant strains of *Micrococcus pyogenes* var. *aureus* were checked for the production of extracellular erythromycinase. Seed agar (BBL) plates were prepared which were seeded with sensitive strains *M. pyogenes* var. *aureus*, no. 3055 and no. 3067, and which contained 1.4 mcg erythromycin per ml. The plates were streaked with the above mentioned resistant organisms. The plates were incubated at 37°C. for 24 hours and examined for the growth of the seeded organisms in the agar.

Breakdown of Erythromycin in Solution by Extracellular Erythromycinase. Resistant strains of *Micrococcus pyogenes* var. *aureus*, no. 3066 and no. 3074, were used in a second check for the production of an extracellular erythromycinase. It had been previously determined that these strains could grow in the presence of 200 mcg erythromycin per ml. Cellophane tubes containing 5 ml hydrolyzed casein medium (36) were immersed in 25 ml of the same medium in 40 mm diam culture tubes. The dialyzing tubes were inoculated with resistant strains no. 3066 and no. 3074, respectively, and 100 mcg erythromycin per ml was added to the medium surrounding the dialyzing tubes. A third tube was inoculated with sensitive strain no. 3055, no antibiotic being added to the medium. A fourth tube was prepared in which erythromycin was added but no inoculation was made. After 72 hours incubation at 37°C., dilutions were made of the media outside of the dialyzing tubes. The dilutions were made in hydrolyzed casein medium in 2-fold steps from 1:2 to 1:64. The diluted medium was then inoculated with sensitive strain no. 3055 and incubated at 37°C. for 2 days. The presence or absence of growth in the dilutions was noted.

Solvent Treatment of Cells to Release Intracellular Erythromycin. Abraham and Chain (41) originally found penicillinase as an intracellular enzyme by grinding up cells of *Escherichia coli*. Kirby et al. (42) found that the treatment of *Micrococcus* cells by fat-removing solvents, acetone and ether, was a satisfactory means of making intracellular penicillinase available. Gilson and Parker (43) found that by using the solvents at temperatures of −20°C.

they had less inactivation of the enzyme. Adapting the method of Gilson and Parker to the facilities available, resistant strains, no. 3066 and no. 3074, of *Micrococcus pyogenes* var. *aureus* were checked for the production of erythromycinase.

The entire surface of plates of seed agar (BBL) was inoculated with a suspension of the resistant *M. pyogenes* strains. Two plates were inoculated with each strain. After 24 hour incubation at 37°C., 3 ml sterile saline solution were added to each plate. The organisms were suspended in saline solution. About 5 ml of suspension of each strain were harvested and added to a centrifuge bottle. Each centrifuge bottle was placed in a beaker of alcohol which had been cooled at −20°C. Twenty-eight ml of acetone, cooled to −20°C., were gradually added with shaking. The suspension in the centrifuge bottle was maintained within the range of −10°C. to −20°C. After 75 minutes with frequent shaking, the acetone was removed with suction. An additional 25 ml of −20°C. acteone was added to the residue and the suspension maintained at −10°C. to −20°C. with frequent shaking for 75 minutes. The acetone was again removed with vacuum. This time, 25 ml of −20°C. ether were added to the residue and the suspension maintained at −10°C. to −20°C. with frequent shaking for 75 minutes. The ether was removed with suction. The residue in the centrifuge bottle was dried by evacuation to a pressure of below 100 microns Hg for 1 hour.

Erythromycin was added to hydrolyzed casein medium at dilutions from 1 mcg to 200 mcg per ml of final volume. One series of tubes served as controls, a second series received a suspension of no. 3066 treated cells; a third series received a suspension of no. 3074 treated cells. All tubes were inoculated with a suspension of the sensitive no. 3055 strain, excepting sterility controls. Since sterility controls on inoculation indicated that viable *Micrococcus cells* were present in the residues, another plan was necessary to demonstrate the presence or absence of erythromycin.

An erythromycin standard solution was brought in contact with the defatted cells, sterilized by filtration, and checked for destruction of the antibiotic. A tube containing 3 ml of erythromycin standard solution, 1000 mcg of the antibiotic per ml, received 1.7 mg of defatted no. 3066 cells; a second tube received 4.1 mg of defatted no. 3074 cells; a third tube received no cells and acted as a control. All tubes were left at room temperature for 48 hours. Twelve ml distilled water were added to each tube and the contents then filtered through a Seitz filter. Dilutions of the filterates added to hydrolyzed casein medium were equivalent to 0, 0.10, 0.39, 1.56, 6.25, 25 and 100 mcg of erythromycin originally present per ml of final volume. All tubes were inoculated with the sensitive strain no. 3055 of *M. pyogenes* var. *aureus*. After incubation at 37°C. for 4 days, the tubes were examined for growth.

Physical Destruction of Cell Wall to Release Intracellular Erythromycinase. According to Hugo (44) and McIlwain (45), a very satisfactory

method of preparing cell-free enzymes is by the physical rupturing of the cell wall with fine abrasives. Resistant strains no. 3066 and no. 3074 of *Micrococcus pyogenes* var. *aureus* and the resistant organisms *Proteus vulgaris* and *Pseudomonas aeruginosa* were treated in this manner to determine the presence of intracellular erythromycinase.

The entire surfaces of two plates of seed agar (BBL) were inoculated with each strain of organism used in this experiment. After 24 hour incubation at 37°C., 4 ml sterile saline solution were added to each plate and the organisms therein suspended. About 8 ml of each strain were harvested aud placed in a centrifuge tube. The cells were centrifuged down in an angle head centrifuge and had a final volume of about ¼ ml. The residue was transferred to a cooled sterile mortar. Six-tenths g of Alumina A-303 were added. The cells were ground with a pestle for 1 minute following the procedure described by McIlwain (45). The ground cells were resuspended in 2 ml sterile saline solution and 1 ml erythromycin standard solution. The suspensions were refrigerated at 6°C. for 24 hours and then left at room temperature for 24 hours. Next they were diluted with 27 ml distilled water each and were sterilized by filtration through Seitz filters. The filtrates were added to hydrolyzed casein medium in amounts equivalent to 0, 0.1, 0.4, 1.2, 3.7 and 11 mcg of erythromycin originally present per ml of final volume. All tubes were inoculated with the sensitive strain no. 3055 of *M. pyogenes* var. *aureus*. After incubation at 37°C. for 3 days, growth was determined by visual inspection.

The Effect of Erythromycin on the Action of Coagulase and Other Enzymes

Coagulase tests were carried out in a manner similar to that described in the Difco Manual (46). Plasma was prepared from rabbit blood to which 1 per cent Na-oxalate was added. Dilutions of erythromycin were made in distilled water with erythromycin standard solution. Derivatives of erythromycin were dissolved or suspended in distilled water. *Micrococcus pyogenes* var. *aureus* was grown in Brain Heart Infusion (Difco) at 37°C. for 24 hours. Suspensions of the organisms were staudardized at 42.5 per cent light transmission at a wave length of 550 lambdas in a spectrophotometer. Five-tenths ml plasma were measured into an 11 mm by 65 mm culture tube; 0.1 ml erythromycin solution, derivative solution or water was added; 0.1 ml standardized suspension of *M. pyogenes* var. *aureus* cells was added last. Each tube was mixed by shaking and placed in a 37°C. water bath. Tubes were examined for coagulation every 5 minutes for the first 30 minutes and every 10 minutes up to 3 hours. The amount of coagulation was estimated by the apparent viscosity of the plasma. A numerical value of 4 was assigned when the tube could be inverted without displacement of the plasma from the bottom of the tube; a value of 0 was assigned when there was no apparent change in viscosity.

A preliminary study was made on the effect of the addition of erythromycin or its derivatives at the time of growth on the formation of coagulase by *M. pyogenes* var. *aureus*. Tubes containing 5 ml casamino acid medium to which had been added 12.5, 5 and 1.25 mcg erythromycin, 5 mg desosamine, 5 mg erythralosamine, 5 mg dihydroerythronolide, $C_{21}H_{41}O_8$, and a control were inoculated with strain no. 3055. A control medium and a medium with 100 mcg erythromycin added were inoculated with strain no. 3066. The coagulase action of these cultures was noted for the first 60 minutes. The effect of the addition of 100 mcg erythromycin at the time of the coagulase test on the coagulase activity of strain no. 3055 was also determined.

A second series of tests was carried out using cultures of sensitive strains no. 3055 and no. 3067 and resistant strains no. 3066 and no. 3074 grown in brain heart infusion alone and resistant strains no. 3066 and no. 3074 grown in the presence of 100 mcg erythromycin per ml. The coagulase activity was determined for each of these six cultures alone with the addition of 100 mcg erythromycin at the time of test.

A third series of tests was carried out using cultures of strains no. 3055, no. 3066, no. 3067 and no. 3074 grown in brain heart infusion. At the time of test, 0, 0.1, 1, 10 and 100 mcg erythromycin were added to plasma tubes with each strain of *M. pyogenes* var. *aureus* to determine the effect on coagulase activity of erythromycin.

A fourth series of tests was carried out to determine the effect of several degradation products of erythromycin on the coagulase activity of *M. pyogenes* var. *aureus*. At the time of test 1000 mcg desosamine, 1000 mcg cladenose, 100 mcg dihydroerythronolide, 100 mcg erythralosamine, and 1 mcg erythromycin were added in 0.1 ml distilled water to 0.5 oxalated plasma. One-tenth ml of suspended cells of *M. pyogenes* was added to each tube as well as to a control. Strains no. 3055, no. 3066, no. 3067 and no. 3074 were used. The time of coagulation of each tube was noted.

The new clinical aid, "Tes-Tape," depends on the activity of glucose oxidase and horse-radish peroxidase to determine the presence of glucose in the urine. A 40 mm strip of "Tes-Tape" was wet with 0.05 ml erythromycin standard solution and allowed to air dry. The antibiotic treated strip and a control strip were both wet with a 1 per cent glucose solution. Both strips were dried in the air for 1 minute. The depth of the color of each strip was compared with the color chart on the tape dispenser.

RESULTS

Screening of Compounds to Determine Reversal of Potentiation of Action of Erythromycin

Compounds Screened. Several of the compounds screened showed some form of activity. Some of these substances reversed the inhibition of the test

organisms by erythromycin; other potentiated the action of the antibiotic; still others acted independently of the erythromycin in a toxic effect on the test organisms or reacted with the media to form opaque areas.

The following compounds reversed the inhibition of test organisms by erythromycin. The diameters of the growth zones on plates seeded with *Micrococcus pyogenes* var. *aureus* and spores of *Bacillus subtilis* are here listed:

adenylic acid, 1 per cent: *B. s.*, 14 to 19 mm
ascorbic acid, 1 per cent: *M. p.*, 14 mm; *B. s.*, 14 to 18 mm
DL-aspartic acid, 2 per cent: *M. p.*, 15 to 24 mm; *B. s.*, 19 to 26 mm
citric acid, 1 per cent: *M. p.*, 22 mm, *B. s.*, 18 to 27 mm
L-cysteine-HCl, 10 per cent: *M. p.*, 23 to 33 mm; *B. s.*, 27 to 34 mm
L-glutamic acid, 1 per cent: *B. s.*, 15 to 21 mm glutathione, 1 per cent: *M. p.*, 19mm
L-histidine-HCl, 5 per cent: *M. p.*, 20 mm; *B. s.*, 22 mm
oxalic acid, 1 per cent: *M. p.*, 23 mm; *B. s.*, 27 to 33 mm

The inhibition of *Micrococcus pyogenes* var. *aureus* and spores of *Bacillus subtilis by* erythromycin was potentiated by adenine sulfate, 1 per cent, guanine-HCl, 0.7 per cent, uracil, 1 per cent, and xanthine, 1 per cent.

A number of the compounds which reversed the inhibition of erythromycin had a halo-like growth zone. In fact, the only one of these reversing compounds for which this halo-like effect was not seen was glutathione. Data giving the diameter of the inner zone of inhibition is given in the paragraph 2, page 79. In seeded plates to which erythromycin had not been added, these compounds produced zones of inhibition of about the same diameter as the inner diameter of the halo in plates to which erythromycin had been added.

Oxalic acid reacted with both media to form opaque zones around the paper disks.

Toxic effects of *Micrococcus pyogenes* var. *aureus* and spores of *Bacillus subtilis* produced inhibition zones with diameters as follows:

$FeSO_4.7H_2O$, 10 per cent: *M. p.*, 25 mm; *B. s.*, 27 mm
D-glucosamine, 1 per cent: *M. p.*, 25 mm; *B. s.*, 13mm
mercaptoethyl amine, 10 per cent: *B. s.*, 50 mm (no sharp threshold)
thioglycerol, 10 per cent: *M. p.*, 21 mm; *B. s.*, 16 mm
tetra sodium salt of ethylene diamine tetra acetic acid, 9.4 per cent: *M. p.*, 27 mm; *B. s.*, 34 mm

$FeSO_4.7H_2O$, 10 per cent, and D-glucosamine, 1 per cent, enhanced the growth of spores of *Bacillus subtilis* with zones 39 mm and 22 mm in diameter, respectively.

Hydrogen Ion Concentration. The hydrogen ion concentration was determined on solutions of all compounds screened except Ca-panthothenate, pyridoxine, niacin, biotin, desosamine, $ZnSO_4.7H_2O$, $TiSO_4$ and H_3BO_3. The hydrogen ion concentrations of most solutions were found to lie within the pH range of 5.0 to 7.9. Solutions which had a hydrogen ion concentration above or below this range had pH's as follows:

adenine sulfate, 1 per cent	10.0
adenylic acid, 1 per cent	2.7
ascorbic acid, 1 per cent	2.5
DL-aspartic acid, 2 per cent	2.9
betaine, 1 per cent	4.1
citric acid, 1 per cent	2.2
L-cysteine-HCl, 1 per cent	1.7
L-glutamic acid, 1 per cent	2.9
glutathione, 1 per cent	3.0
guanine HCl, 0.7 per cent	10.4
L-histidine-HCl, 1 per cent	4.0
mercaptoethyl amine, 10 per cent	4.6
oxalic acid, 1 per cent	3.7
p-aminobenzoic acid, 1 per cent	3.7
thiamine-HCl, 0.1 per cent	3.7
uracil, 1 per cent	9.9
xanthine, 1 per cent	10.4

The neutralized solutions of adenylic acid (4 per cent), ascorbic acid (10 per cent), L-aspartic acid (5 per cent), L-cysteine-HCl (10 per cent), L-glutamic acid (4 per cent), glutathione (2 per cent) and L-histidine-HCl (5 per cent) gave no evidence of reversing the bacteriostasis of the test organisms, *Micrococcus pyogenes* var. *aureus* and *Bacillus subtilis*, caused by threshold amounts of erythromycin.

L-Cysteine-HCl. Erythromycin which had been treated with L-cysteine-HCl at a pH of 5.2 at 56°C. for 1 hour gave zones of inhibition with both *Micrococcus pyogenes* var. *aureus* and the spores of *Bacillus subtilis* identical with those obtained from an erythromycin control which had been treated with no L-cysteine-HCl. After a subsequent 3-day storage in the refrigerator, potencies were still identical.

Organic and Inorganic Acids. It was found that the inhibition of *Micrococcus pyogenes* var. *aureus* and the spores *Bacillus subtilis* was reversed by a number of organic and mineral acids. Alanine (2 per cent) and HCl (1 per cent), glycine (2 per cent) and HCl (1 per cent), HCl (1 per cent), lactic acid (1 per cent), malic acid (1 per cent), H_2SO_4 (1 per cent) and tartaric acid (1 per cent) were effective. In the case of *B. subtilis* these acids in the aforementioned concentrations caused halo-like zones of growth in plates containing threshold amounts of the antibiotic. In the case of *M. pyogenes* the zone of growth extended to the paper disk on which the acid had been added.

Revival. It was found that revival of *Micrococcus pyogenes* var. *aureus* and spores of *Bacillus subtilis* by a threshold amount of erythromycin could be effected by the compounds which had shown reversal action when added at the same time as the antibiotic. DL-asparic acid, L-cysteine-HCl and L-histidine-HCl brought about the revival of both organisms. Growth could be seen at 24 hours but was much more evident at 48 hours. Only a small percentage of the inhibited organisms were revived. In the case of *M. pyogenes* var.

aureus most of these appeared to be growing on the surface of the agar. After becoming aware of the importance of the pH of the compounds screened, it was found that alanine (2 per cent) and HCl (1 per cent), ascorbic acid (4 per cent), L-glutamic acid (2 per cent), HCl (1 per cent) and H_2SO_4 (1 per cent) brought about the revival of *B. subtilis*. The revival of *M. pyogenes* var. *aureus* was brought about by alanine (2 per cent) and HCl (1 per cent), ascorbic acid (4 per cent), L-glutamic acid (2 per cent), malic acid (1 per cent) and tartaric acid (1 per cent).

Investigation of the Reversal by Calcium Pantothenate and Beta-Alanine of the Inhibitory Effects of Erythromycin on Several Species of Bacteria

Lactobacillus casei. In the studies with *L. casei* it was found that 0.2 mcg/ml erythromycin caused an increase in the pH of the blank tubes to which no Ca-pantothenate had been added after the tubes had been inoculated and incubated 3 days at 37°C. One mcg/ml of the antibiotic resulted in a still higher pH (less acid production). The minimal inhibitory level of erythromycin, i.e., 0.2 mcg/ml erythromycin, 0.02, 0.2 and 2 mcg/ml Ca-pantothenate caused a reversal of the inhibitory effect and brought about growth. However, when 1 mcg/ml of the antibiotic was present even 20 mcg/ml of the vitamin caused no appreciable reversal of inhibition (Table 1). When 0.2, 2 or 20 mcg/ml of erythromycin was present, up to 200 mcg/ml beta-alanine had no growth effect in the presence of 0.2 mcg/ml Ca-pantothenate (Table 2).

Saccharomyces cerevisiae. With the "Gebrüder Mayer" strain of *S. cerevisiae* 10 mcg/ml of erythromycin caused no inhibition. When 100 mcg/ml of the antibiotic was present, it took 0.02, 0.04 and 0.05 mcg/ml Ca-pantothenate to bring the growth of the yeast to the level, as measured by the spectrophotometer, which 0.005, 0.02 and 0.03 mcg/ml Ca-pantothenate attained in the absence of erythromycin (Table 3). Similarly, it required 0.4 and 0.8 mcg/ml beta-alanine in the presence of 100 mcg/ml erythromycin to bring about growth equivalent to that of 0.2 an 0.4 mcg/ml beta-alanine in the absence of erythromycin (Table 4).

It was necessary to use the level of 100 mcg/ml erythromycin to cause a reduction of growth of the yeast. It was impractical to use higher concentrations of the antibiotic due to its limited solubility.

Micrococcus pyogenes var. *aureus.* Strain no. 3055 of *M. pyogenes* var. *aureus* was inhibited by 0.5 mcg/ml erythromycin in the absence of either Ca-pantothenate or beta-alanine; strain no. 3067 was inhibited by 1.0 mcg/ml. With 0.1, 1 and 10 mcg/ml Ca-pantothenate added to the medium, 1.0, 0.5 and 0.5 mcg/ml of the antibiotic were required for inhibition of strain no. 3055 and 1.0, 1.0 and 1.0 mcg/ml of the antibiotic for strain no.

3067. With 10, 100 and 1000 mcg/ml beta-alanine added, 0.5, 0.5 and 0.5 mcg/ml erythromycin were required for inhibition of strain no. 3055 and 1.0, 1.0 and 2.0 mcg/ml, for strain no. 3067. Neither Ca-pantothenate or beta-alanine reversed the inhibition of these strains by erythromycin appreciably (Table 5).

Diplococcus pneumoniae. The growth of *D. pneumoniae* was partially inhibited by 0.005 mcg/ml erythromycin and completely inhibited by 0.01 mcg/ml in the absence of Ca-pantothenate or beta-alanine. The presence of 0.1, 1 and 10 mcg/ml Ca-pantothenate, the organism was inhibited by 0.01 mcg/ml of the antibiotic at all levels. In the presence of 10, 100 and 1000 mcg/ml beta-alanine, the organism was inhibited by 0.01 mcg/ml erythromycin at all levels. Neither growth factor reversed the inhibition of the organism by the antibiotic (Table 6).

Streptococcus pyogenes. The growth of *S. pyogenes* was partially inhibited by 0.005 mcg/ml and completely inhibited by 0.01 or 0.02 erythromycin in the absence of Ca-pantothenate or beta-alanine. In the presence of 0.1, 1 and 10 mcg/ml Ca-pantothenate, the organism was inhibited at all levels by 0.02 mcg/ml of the antibiotic. In the presence of 10, 100 and 1000 mcg/ml beta-alanine, the organism was inhibited by 0.01, 0.02 and 0.02 mcg/ml of the antibiotic, respectively. Neither Ca-pantothenate nor beta-alanine caused any appreciable reversal of the inhibition of *S. pyogenes* by erythromycin (Table 6).

Corynebacterium diphtheriae. The inhibition of *C. diphtheriae* by erythromycin showed no sharp cut-off point. A 0.004 mcg/ml concentration of the antibiotic permitted maximum growth of the organism; 0.008 mcg/ml, permitted heavy growth; 0.016 mcg/ml, permitted moderate growth; and 0.032 mcg/ml, permitted slight growth. In the presence of 0.1 and 1 mcg/ml Ca-pantothenate or 10, 100 and 1000 mcg/ml beta-alanine, the different concentrations of erythromycin produced the same pattern of inhibition as in the control which received neither nutrient. When 10 mcg/ml Ca-pantothenate was used, the partial inhibition caused by 0.008 and 0.016 mcg/ml erythromycin appeared to be reversed and that caused by 0.032 mcg/ml of the antibiotic was not affected (Table 7).

Gots' Method. Using the two sensitive strains of *Micrococcus pyogenes* var. *aureus*, no. 3055 and no. 3067, in seeded plates, there was no evidence that the resistant strains of *M. pyogenes* var. *aureus*, no. 3066 and no. 3074, or the resistant organisms *Proteus vulgaris* and *Pseudomonas aeruginosa* produced a metabolite which was capable of diffusing through the agar and reversing the inhibition of erythromycin.

Production of Pantothenic Acid by Resistant Strains of Micrococcus pyogenes var. aureus. Mylase-treated, autoclaved suspensions of resistant and sensitive strains of *M. pyogenes* were assayed for pantothentic acid content. The suspension of sensitive strain no. 3055 contained 0.51 mcg/ml panto-

thenic acid; resistant strain no. 3066 contained 0.44 mcg/ml; resistant strain no. 3074 contained 0.43 mcg/ml.

Investigation of the Presence of Intracellular and Extracellular Erythromycinase

Gots' Method. Extracellular Erythromycinase. Using the method of Gots (4), agar seeded with sensitive strains no. 3055 and no. 3067 of *Micrococcus pyogenes* var. *aureus* gave no indication of a diffusible extracellular erythromycinase in either resistant strains no. 3066 and no. 3074 of *M. pyogenes* var. *aureus* or in the resistant organisms *Proteus vulgaris* and *Pseudomonas aeruginosa.* Using bacteriostatic amounts of the antibiotic in the agar, there was no growth of the sensitive seeded organisms in the proximity of the resistant organisms.

Breakdown of Erythromycin in Solution by Extracellular Erythromycinase. After incubation the erythromycin containing media surrounding the dialyzing tubes which were inoculated with resistant strains no. 3066 and no. 3074 of *Micrococcus pyogenes* var. *aureus* and the antibiotic control still inhibited the growth of the sensitive strain no. 3055 at dilutions up through 1:64. The second control, which contained no erythromycin and had been inoculated with sensitive strain no. 3055, produced no substance capable of reducing growth (Table 8).

Solvent Treatment of Cells to Release Intracellular Erythromycinase. Erythromycin solutions which had been treated with the solvent-extracted, resistant strains of *Micrococcus pyogenes* var. *aureus* had identical inhibitory powers. In each case after treatment by defatted cells of strains no. 3066 and no. 3074, dilutions equivalent to an original concentration of 0.39 mcg/ml erythromycin inhibited the growth of sensitive strain no. 3055 of *M. pyogenes* var. *aureus;* dilutions equivalent to an original concentration of 0.10 mcg/ml did not inhibit the growth (Table 9).

Physical Destruction of Cell Wall to Release Intracellular Erythromycinase. Dilutions of erythromycin which had been treated with alumina ground cells of resistant strains no. 3066 and no. 3074 of *Micrococcus pyogenes* var. *aureus* and the resistant organisms *Proteus vulgaris* and *Pseudomonas aeruginosa* and had been subsequently sterilized retained much erythromycin activity. In all four cases dilutions equivalent to 1.2 mcg/ml original erythromycin content inhibited growth of the seusitive strain no. 3055 of *M. pyogenes* var. *aureus;* dilutions equivalent to 0.4 mcg/ml original erythromycin content did not inhibit growth (Table 10).

The Effect of Erythromycin on the Action of Coagulase and other Enzymes

In the preliminary test the presence of coagulase was varied when erythromycin and several erythromycin degradation compounds had been added to

the growing organism *Micrococcus pyogenes* var. *aureus*. Using strain no. 3055, the control with no compound added, cultures grown with 1 mg/ml desosamine, 1 mg/ml erythralosamine and 1 mg/ml dihydroerythronolide all gave a coagulation value of 4 after 60 minutes. The addition of 100 mcg erythromycin at the time of the test reduced the coagulation value of no. 3055 to 3 at 100 minutes. Using strain no. 3066, the control gave a coagulation value of 3 at 60 minutes and the addition of 100 mcg erythromycin at the time of test reduced the coagulation value to 1 at 60 minutes.

In the second series of tests, using suspensions of *M. pyogenes* var. *aureus* which had been standardized by a spectrophotometer, the addition of 100 mcg erythromycin to the mixture of plasma and suspension of organisms at the time of test increased the time required for coagulation. The six suspensions of the organism coagulated the plasma in periods of time varying from 40 to 80 minutes. When 100 mcg erythromycin was added at the time of the test, the time required for coagulation was 80 minutes to greater than 3 hours. The increase in time to bring about coagulation due to the addition of erythromycin at the time of the test varied from 40 minutes to greater than 140 minutes (Table 11).

A third test, with all four strains of *M. pyogenes* var. *aureus*, revealed that amounts of erythromycin as small as 0.1 mcg increased the time of coagulation. With all four strains the addition of 1 mcg erythromycin resulted in still further increases of time required for coagulation. Amounts of erythromycin in excess of 1 mcg, namely 10 and 100 mcg, did not further increase time required for coagulation under test conditions but took longer for coagulation than those tubes which received 0 or 0.1 mcg of the antibiotic (Table 12).

It was found that the addition of 100 mcg/ml erythromycin increased the growth for resistant strains no. 3066 and no. 3074 of *M. pyogenes* var. *aureus* as measured in percentage light transmission from 23 to 20 and 34 to 28, respectively.

In a final test, the effect of several degradation products of erythromycin on the time required for coagulation was found to vary among the same four strains of *M. pyogenes* var. *aureus*. The average time required for formation of a coagulum, firm enough to allow inverting the tube without loss of the plasma, was 82 minutes. The addition of 1000 mcg desosamine required on the average 76 minutes; 1000 mcg cladenose, 80 minutes; 100 mcg erythralosamine, 97 minutes; 100 mcg dihydroerythronolide, 112 minutes; and 1 mcg erythromycin, over 160 minutes (Table 13).

"Tes-Tape," the glucose oxidase and horse-radish peroxidase impregnated paper, was not affected in its reaction to glucose by erythromycin. Tape previously saturated with erythromycin standard solution reacted to give the same color upon being wet by glucose solution as a control.

DISCUSSION

Screening of Compounds to Determine Reversal of Potentiation of the Action of Erythromycin

Nearly one hundred compounds were screend for effect on the inhibition of growth of *Micrococcus pyogenes* var. *aureus* and the spores of *Bacillus subtilis* by erythromycin. Solutions of four of these compounds potentiated the action of the antibiotic and solutions of nine of the compounds reversed the action. The four compounds which had a potentiating effect were purines and had been dissolved, using NaOH, with resulting pH's of 9.9 to 10.4. Solutions of the nine compounds which had shown reversal of the action of erythromycin were all acidic with pH's ranging from 1.7 to 4.0. Since the work of Pittenger et al. (1) and Haight and Finland (2) indicated that an increase in pH resulted in increased activity of erythromycin, it appeared that both the potentiating and reversing phenomena resulted from the hydrogen ion concentration of the solutions screened.

The neutralization of the solutions of screened compounds which showed activity afforded an opportunity to determine whether the structure alone could account for activity. With the four purines, adenine, guanine, uracil and xanthine, neutralization would result in their precipitation and, therefore, no useful data could be obtained. Solutions of seven of the nine active acidic compounds were brought to pH's between 6.2 and 7.0. None of these solutions retained the ability after neutralization to reverse the action of erythromycin. As a complementary measure, various other organic and inorganic acids were found to reverse the action of.the antibiotic.

Revival, apparently, presents a similar situation. Only solutions of acid compounds brought about revival and they were for the most part the same as those which caused reversal. It would appear that the greater proportion of the *Micrococcus pyogenes* var. *aureus* and *Bacillus subtilis* cells had been killed by the "bacteriostatic" concentration of erythromycin. The remaining resistant bacteria resumed growth after the shift to the acid side, by the addition of the solution to the paper disk, reduced the effectiveness of the antibiotic to below the bacteriostatic level.

The halo phenomenon was exhibited in both reversal and revival. In plates seeded with the spores of *Bacillus subtilis* the diameters of the inner zone of inhibition were larger than those of plates seeded with *Micrococcus pyogenes* var. *aureus*. The inner zone of inhibition adjacent to the paper disk appears to be a toxic effect of a low pH. Since *Bacillus subtilis* requires more alkaline surroundings it follows that the zone would necessarily be larger. With an increase in distance from the paper disk one would expect the pH to rise to a level where the bacteria were not inhibited and at which the concentration of antibiotic present would be reversed and, finally, at a still

greater distance the strength of the acid would have been so reduced that the organisms would again be inhibited by the antibiotic.

It had been suspected that the sulfhydryl radical of cysteine or the closely related mercaptoethylamine might react with a ketone group in erythromycin but there appears to be no reaction.

Desosamine, the nitrogen containing sugar which is found in numerous antibiotics of the erythromycin group, exhibited no activity of any kind. The activity of erythromycin is due to other characteristics of the molecule.

Investigation of the Reversal by Calcium Pantothenate and Beta-Alanine of the Inhibitory Effects of Erythromycin on Several Species of Bacteria

Very little has been found to support the thesis that Ca-pantothenate and beta-alanine act as antagonists of erythromycin. Brown and Emerson (4) used Ca-pantothenate and beta-alanine to reverse the inhibition of *Corynebacterium diphtheriae* by erythromycin. Employing the same medium but using different strains of the bacterium, Ca-pantothenate and beta-alanine did not reverse the action of erythromycin. In this study ratios of Ca-pantothenate and beta-alanine to the antibiotic equalled or surpassed those used by Brown and Emerson. The data reported here do not confirm their findings, although other strains were used.

Working with other organisms and various technics, indications of the reversal of the action of erythromycin by Ca-pantothenate or beta-alanine were found in two instances. The "Gebrüder Mayer" strain of *Saccharomyces cerevisiae* showed reversal of inhibition due to the antibiotic in the presence of both Ca-pantothenate and beta-alanine. The reversal of erythromycin was seen at only one inhibitory concentration of the antibiotic since higher levels were not practical due to its limited solubility. Therefore the nature of the reversal cannot be determined. Two alternative explanations of the results can be made. Since the yeast requires either Ca-pantothenate or beta-alanine, the antibiotic cannot be inhibiting the growth and division by blocking the production of Ca-pantothenate or beta-alanine. It is possible, however, that the erythromycin acts by competing with Ca-pantothenate or beta-alanine for a position in an essential enzyme system. As an alternative, erythromycin could be interfering with an essential enzyme system which is distinct from the enzyme system in which Ca-pantothenate and beta-alanine play an important role. In this situation the addition of a sub-bacteriostatic amount of erythromycin and the presence of a low amount of Ca-pantothenate would interfere with the normal operation of the respective enzyme systems. While the reduced action of either enzyme system alone would not prevent growth and reproduction, the reduced action of both enzyme systems would, synergistically, inhibit growth or reproduction. If the reversal is competitive, it may be estimated that either 0.02 mcg Ca-pantothenate or

1 mcg beta-alanine reverse the action of 100 mcg erythromycin. The activity coefficients of Ca-pantothenate and beta-alanine would be 5,000 and 100, respectively. *Lactobacillus casei* showed no reversal of the action of the antibiotic with beta-alanine; but in the presence of the minimal inhibiting concentration of erythromycin, the bacterium showed an apparently non-competitive reversal of the inhibition by Ca-pantothenate.

In the other organisms studied, Ca-pantothenate and beta-alanine did not reverse the inhibitory action of erythromycin. The inhibition by erythromycin of *Diplococcus pneumoniae, Micrococcus pyogenes* var. *aureus,* and *Streptococcus pyogenes* was not reversed by Ca-pantothenate at concentrations as high as 10 mcg/ml or beta-alanine at concentrations as high as 1000 mcg/ml. The average level of pantothenic acid in human blood was found by Pearson (47) to be 0.19 mcg/ml. It appears unlikely that vitamin therapy would increase the pantothenic acid above blood levels at which no reversal of the action of erythromycin would occur.

If the action of erythromycin were reversed by Ca-pantothenate, resistance of organisms might be due to synthesis of large amounts of that vitamin. Amounts of pantothenic acid produced by two highly resistant strains of *Micrococcus pyogenes* var. *aureus* were almost equal to that produced by a sensitive strain. The enzyme mylase made pantothenic acid in a bound form available for assay. These data are convincing in showing the lack of any competitive relationship between erythromycin and pantothenic acid. Since beta-alanine is a precursor of pantothenic acid (48), the above data constitute indirect evidence that synthesis of beta-alanine is not the means by which these strains are resistant to the antibiotic.

The evidence that the resistant strains of *Micrococcus pyogenes* var. *aureus* and the two organisms, *Proteus vulgaris* and *Pseudomonas aeruginosa,* do not produce extracellular erythromycinase, is equally valid as evidence that they do not produce extracellular metabolites competitive with erythromycin.

With the possible exception of data found using the "Gebrüder Mayer" strain of *Saccharomyces cerevisiae,* all results indicated that neither Ca-pantothenate or beta-alanine competitively reversed inhibition caused by erythromycin.

Investigation of the Presence of Intracellular and Extracellular Erythromycinase

One mechanism which might account for the resistance of certain strains and organisms to erythromycin would be the presence of erythromycinase. The enzyme might be present either in an extracellular form or in an intracellular form.

Neither of the two resistant *Micrococcus pyogenes* var. *aureus* strains nor *Proteus vulgaris* nor *Pseudomonas aeruginosa* reversed the inhibition of the

two sensitive seed organisms by erythromycin. In addition, resistant strains of *M. pyogenes* var. *aureus* were grown in dialyzing tubes surrounded by media containing erythromycin. It should be expected that the erythromycin would diffuse through the cellophane tube to the organisms and be broken down if erythromycinase were present. Here again there was no indication of destruction.

It should be noted that if apparent destruction of the antibiotic had occurred it would be necessary to determine whether erythromycinase had destroyed the erythromycin or a competitive metabolite had been produced.

Since no extracellular erythromycinase was found, it can be concluded that the resistant strains of *Micrococcus pyogenes* var. *aureus* are truly resistant. It had been found previously that some "resistant" organisms produce an antibiotic destroying enzyme which changes the environment by reducing the antibiotic concentration below the threshold level so that the truly sensitive organism can grow. Such is not the case here.

The possibility of an intracellular erythromycinase still remained. The treatment of cells by fat solvents has been found to render the walls of the cells permeable enabling molecules as large as penicillin, for example, to come in contact with enzymes in cells (4). There was no evidence, however, of erythromycinase in the solvent-treated strains studied. The use of high frequency sound waves to break down the cell walls was rejected because the cells of *Micrococcus pyogenes* var. *aureus* offer great resistance to this treatment. Confirmation by grinding with alundum was chosen since it is effective and yet causes a minimum of denaturation of the protein of the cell. Apparently there was some absorption of the antibiotic on the alundum and the contents of the cells; but the amount of erythromycin left free in the solution after treatment was still many times that necessary to inhibit the growth of a sensitive strain. Thus, the evidence is strong that intracellular erythromycinase is not present in the two resistant strains of *M. pyogenes* var. *aureus* and in *Proteus vulgaris* and *Pseudomonas aeruginosa*.

No evidence of either extracellular or intracellular erythromycinase was found in the organisms studied.

The Effect of Erythromycin on the Action of Coagulase and Other Enzymes

Originally it had been suspected that the effectiveness of erythromycin in vivo in mice against strains of *Micrococcus pyogenes* var. *aureus* was due to an effect on coagulase by erythromycin. This matter was pursued when Boniece et al. (34) revealed that erythromycin in small amounts reduced the activity of coagulase. From the results obtained using four strains of *Micrococcus pyogenes* var. *aureus* there is no doubt as to the reduction of the speed of coagulase activity when small amounts of erythromycin were added at the time of test. Furthermore, the degradation compounds, cladenose, desosa-

mine, dihydroerythronolide, and erythralosamine, had little if any activity compared with erythromycin. It appears that the property of reducing the activity of coagulase is evanescent as the antibiotic is cleaved into its component parts. The data concerning dihydroerythronolide and erythralosamine was not statistically significant and it would be worth while to make further tests of the effect of these compounds on coagulase. The inhibitory action of erythromycin cannot be associated with its degradation compounds, desosamine, erythralosamine, and dihydroerythronolide.

One conclusion is obvious, erythromycin interferes with sensitive strains of *Micrococcus pyogenes* var. *aureus* in at least two ways: it inhibits portions of the metabolic system which function in the growth and reproduction of the organism; and it interferes with the action of coagulase. It should be noted, however, that coagulase was still formed by resistant strains growing in the presence of the antibiotic. Erythromycin interferes with the action of coagulase not with its production.

It would appear that with the finding of an antibiotic which protects mice in vivo against in vitro resistant *Micrococcus pyogenes* var. *aureus*, and the fact that the antibiotic reduces coagulase activity, one may have found a phenomenon of considerable utility. Let it be assumed that erythromycin counteracts *M. pyogenes* var. *aureus* in vivo by reducing the speed of coagulase activity. Since the antibiotic does not inhibit the reproduction of the organism, but inhibits the action of one of its enzymes acting externally, it would appear that there would be little tendency for the development of an in-vivo-fast strain which would produce a coagulase more resistant to the action of erythromycin. Since this is a most versatile organism, it would appear more likely that the protection afforded to the animal by the use of the antibiotic would favor the development of strains which might be resistant to other natural host defenses and might kill the host by a method in no way connected with the coagulase action.

The effect on the speed of coagulase activity may be an in vitro index for screening drugs and antibiotics for in vivo effectiveness against *Micrococcus pyogenes* var. *aureus*. The finding of active substances by this in vitro method which would be also active in vivo, would, aside from the possibility of clinical usefulness, throw additional light on the manner in which *M. pyogenes* var. *aureus* behaves in vivo.

CONCLUSION

Erythromycin is antibiotic by its interference with the normal functioning of one or more enzyme systems in susceptible organisms.

A screening of nearly one hundred compounds, using *Micrococcus pyogenes* var. *aureus* and the spores of *Bacillus subtilis*, revealed no substances capable of reversing the inhibition caused by erythromycin. Compounds in

acid solution acted to reverse the activity of erythromycin and compounds in alkaline solution acted to potentiate its activity; but the basic cause was the acidity or alkalinity of the solution.

For clinical purposes, the revival of organisms inhibited by erythromycin would appear to be favored by planting into media with as low a pH as would be compatible with the organism.

No evidence was found that Ca-pantothenate or beta-alanine reversed competitively the inhibition by erythromycin of *Corynebacterium diphtheriae, Diplococcus pneumoniae, Lactobacillus casei, Micrococcus pyogenes* var. *aureus* and *Streptococcus pyogenes*.

Both Ca-pantothenate and beta-alanine brought about reversal of the inhibition of *Saccharomyces cerevisiae* by erythromycin. Owing to solubility limitations it was not possible to use larger amounts of the antibiotic and determine the presence of a competitive reversal of the type exhibited by p-aminobenzoic acid and the sulfa drugs over large variations of concentration.

No evidence was found of the synthesis of a metabolite competitive with erythromycin by resistant organisms. Resistant strains of *Micrococcus pyogenes* var. *aureus* synthesized the same amount of pantothenic acid as a sensitive strain. Gots' test demonstrated that resistant strains of *M. pyogenes* var. *aureus, Proteus vulgaris,* and *Pseudomonas aeruginosa* synthesized no extracellular metabolite capable of reversing the action of erythromycin.

Neither extracellular nor intracellular erythromycinase was found in *Micrococcus pyogenes* var. *aureus, Proteus vulgaris,* or *Pseudomonas aeruginosa.* Neither solvent treatment nor mechanical rupturing of cells released such an enzyme.

Desosamine, a structure common to numerous members of the erythromycin antibiotic group, has no effect on the action of erythromycin and has no bacteriostatic effect on *Micrococcus pyogenes* var. *aureus* or on the spores of *Bacillus subtilis*.

The finding of Boniece et al. (34) that erythromycin reduced the activity of the coagulase of *Micrococcus pyogenes* var. *aureus* was confirmed. Desosamine and cladenose, degradation products of erythromycin, did not reduce the activity of coagulase. It is suggested that additional work should be done to determine whether the reduction of activity of coagulase by dihydroerythronolide and erythralosamine is significant. None of the four degradation products of erythromycin tested had an effect on coagulase which approached that of the antibiotic.

Erythromycin had no effect on the enzymes glucose oxidase and horseradish peroxidase.

It is suggested that in view of erythromycin's reduction of the activity of coagulase, there may be little tendency for "in vivo resistant" strains of *Micrococcus pyogenes* var. *aureus* to develop. The coagulase activity test is

recommended as an in vitro method for the testing of drugs for their in vivo effectiveness against *M. pyogenes* var. *aureus*.

The inactivity of various metabolites on the action of erythromycin, the inability of Ca-pantothenate and beta-alanine to reverse the inhibition of numerous organisms by the antibiotic, and the apparent absence of erythromycinase, lead to the conjecture that the most fruitful areas of study of the mode of action would be: 1. the investigation of the effect of erythromycin on the various enzyme systems in the bacterial cell; 2. the comparison of the enzyme systems of sensitive and resistant strains of the same organism.

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TABLE 1

Effect of Calcium Pantothenate and Erythromycin on the Hydrogen Ion Concentration of *Lactobacillus casei*

	erythromycin mcg/ml			
Calcium Panto-thenate mcg/ml	0.0 pH	0.04 pH	0.2 pH	1.0 pH
0.0	5.5	5.5	5.8	6.5
0.02	4 4	4.5	5.1	6.4
0 2	4.1	4.1	4.6	6.4
2.0	4.1	4.2	4.8	6.4
20.0	4.1	4 1	4.8	6.4

TABLE 2

Effect of Beta-Alanine and Erythromycin of the Hydrogen Ion Concentration of *Lactobacillus casei*

Beta-Alanine mcg/ml	erythromycin mcg/ml 0.0	0.2	2.0	20.0
	pH	pH	pH	pH
0.0	4.1	4.7	6.7	6.7
0.2	4.2	4.6	6.7	6.7
2.0	4.1	4.7	6.7	6.7
20.0	4.2	4.7	6.7	6.7
200.0	4.1	4.6	6.7	6.7

TABLE 3

Effect of Calcium Pantothenate and Erythromycin on the Growth of "Gebrüder Mayer" Strain of *Saccharomyces cerevisiae*

Calcium Panto-thenate mcg/ml	erythromycin mcg/ml 0	1	10	100
	(readings in optical density)			
0.000	.014	.016	.015	.010
0.005	.081	073	.072	.044
0.01	.092	.095	.080	.055
0.02	.121	.116	.122	.078
0.03	.127	.122	.126	.102
0.04	.134	.128	.134	.123
0.05	.140	136	.137	.129

TABLE 4

Effect of Beta-Alanine and Erythromycin on the Growth of "Gebrüder Mayer" Strain of *Saccharomyces cerevisiae*

Beta-Alanine mcg/ml	erythromycin mcg/ml 0	1	10	100
	(readings in optical density)			
0.0	024	.009	.004	.003
0.2	.116	.135	—	054
0.4	.216	.211	.195	.126
0.8	.242	.266	.264	.224
1.6	.276	.290	.274	.276
3.2	.300	.294	.296	.282

TABLE 5

Effect of Beta-Alanine and Calcium Pantothenate on the Growth* of *Micrococcus pyogenes* var. *aureus*, Strains no. 3055 and 3067.

	erythromycin mcg/ml Strain no. 3055					Strain no. 3067				
Calcium Pantothenate mcg/ml	0.0	0.1	0.2	0.5	1.0	0.0	0.1	0.2	0.5	1.0
0.0	4	4	4	0	0	4	4	4	0	0
0.1	4	4	4	4	0	4	4	4	0	0
1.0	4	4	4	0	0	4	4	4	0	0
10.0	4	4	4	0	0	4	4	4	0	0
Beta Alanine mcg/ml										
0	4	4	4	0	0	4	4	4	0	0
10	4	4	4	0	0	4	4	4	0	0
100	4	4	4	0	0	4	4	4	0	0
1000	4	4	4	0	0	4	4	4	0	0

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 6

Effect of Beta-Alanine and Calcium Pantothenate on the Growth* of *Diplococcus pneumoniae* Park I and *Streptococcus pyogenes* C203

	D. pneumoniae					*S. pyogenes*				
	erythromycin mcg/ml									
	.000	.005	.010	.020	.040	.000	.005	.010	.020	.040
Ca-Panto-thenate mcg/ml										
0.0	4	2	0	0	0	4	2	2	0	0
0.1	4	2	0	0	0	4	2	2	0	0
1.0	4	2	0	0	0	4	2	2	0	0
10.0	4	2	0	0	0	4	2	2	0	0
Beta-Alanine mcg/ml										
0	4	2	0	0	0	4	2	0	0	0
10	4	2	0	0	0	4	2	0	0	0
100	4	2	0	0	0	4	2	2	0	0
1000	4	2	0	0	0	4	2	2	0	0

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 7

Effect of Beta-Alanine and Calcium Pantothenate on the Growth* of *Corynebacterium diphtheriae*

	Strain X-166					Strain Toronto				
	erythromycin mcg/ml									
	.000	.004	.008	.016	.032	.000	.004	.008	.016	.032
Ca-Panto-thenate mcg/ml										
0.0	4	4	3	2	1	4	4	3	2	1
0.1	4	4	3	2	1	4	4	3	2	1
1.0	4	4	3	2	1	4	4	3	2	1
10.0	4	4	4	4	1	4	4	4	4	1
Beta-Alanine mcg/ml										
0	4	4	3	2	1	4	4	3	2	1
10	4	4	3	2	1	4	4	3	2	1
100	4	4	3	2	1	4	4	3	2	1
1000	4	4	3	2	1	4	4	3	2	1

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 8

Growth* of *Micrococcus pyogenes* var. *aureus* in the Presence of Dilutions of Erythromycin Treated by Contact with Dialyzing Tubes Seeded with Resistant Strains of *M. pyogenes* var. *aureus*

	Not Inoculated	Strain no. 3066	Strain no.3074	Strain no. 3055
	erythromycin mcg/ml (original concentration)			
Dilution of medium from outside of dialyzing tubes	100	100	100	0
1 : 2	0	0	0	4
1 : 4	0	0	0	4
1 : 8	0	0	0	4
1 : 16	0	0	0	4
1 : 32	0	0	0	4
1 : 64	0	0	0	4

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 9

Growth* of *Micrococcus pyogenes* var. *aureus* in the Presence of Dilutions of Erythromycin Treated by Solvent-Extracted Resistant Strains of *M. pyogenes* var. *aureus*

Equivalent of Erythromycin Originally Present after Dilution mcg/ml	Control	Strain no. 3066	Strain no. 3074
100.00	0	0	0
25.00	0	0	0
6.25	0	0	0
1.56	0	0	0
0.39	0	0	0
0.10	4	4	4
0.00	4	4	4

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 10

Growth* of *Micrococcus pyogenes* var. *aureus* in the Presence of Dilutions of Erythromycin Treated by the Ruptured cells of Four Bacterial Strains

Equivalent of Erythromycin Originally Present after Dilution mcg/ml	*Micrococcus pyogenes* var. *aureus* Strain no. 3066	*Micrococcus pyogenes* var. *aureus* Strain no. 3074	*Proteus vulgaris*	*Pseudomonas aeruginosa*
11.0	0	0	0	0
3.7	0	0	0	0
1.2	0	0	0	0
0.4	4	4	4	4
0.13	4	4	4	4
0.0	4	4	4	4

* Growth was determined by a visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 11

Effect of Erythromycin on the Coagulation of Plasma by *Micrococcus pyogenes* var. *aureus*

Strain no.	3055	3066	3066	3067	3074	3074
Erythromycin during growth mcg/ml	0	0	100	0	0	100
Erythromycin added at time of test mcg/ml	minutes required for coagulation					
0	40	40	40	50	80	50
100	80	over 180	130	140	180	180
Increase in time due to erythromycin	40	over 140	90	90	100	130

TABLE 12

Effect of Different Concentrations of Erythromycin on the Coagulation of Plasma by *Micrococcus pyogenes* var. *aureus*

Strain no.	3055	3066	3067	3074
Erythromycin added at time of test mcg/ml	minutes required for coagulation			
0	30	40	40	50
0.1	40	60	80	70
1	70	130	150	over 180
10	70	130	100	130
100	70	90	90	100

TABLE 13

Effect of Degradation Products of Erythromycin on the Coagulation of Plasma by *Micrococcus pyogenes* var. *aureus*

Strain no.	3055	3066	3067	3074
Compound added at time of test.	minutes required for coagulation			
None, control...... .	30	70	130	100
Desosamine, 1000 mcg.	40	60	100	100
Cladenose, 1000 mcg	50	70	100	100
Erythralosamine, 100 mcg.........	60	70	130	130
Dihydroerythronolide 100 mcg.......	60	130	130	130
Erythromycin, 1 mcg....	100	over 180	over 180	over 180

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