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Review Papers: Penicillin: A Potent New Chemotherapeutic Agent

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HISTORY

In 1929 Dr. Alexander Fleming (1) at St. Mary’s Hospital in London observed a clear zone around a mold contaminant on petri dishes inoculated with *Staphylococcus aureus*. This phenomenon stimulated him to isolate and culture the mold on broth to see if the mold would produce a substance inhibitory to the growth of bacteria under these circumstances. His hypothesis proved to be correct; for, whenever he placed a drop or two of the broth on which the mold had grown on plates on which *Staphylococcus aureus* or each of several other bacteria were growing, large clear areas occurred after a short time as the drops diffused through the agar.

Dr. Fleming found by various methods that several different strains of staphylococci were inhibited by the broth with little variation among strains. Other pathogenic organisms which proved to be quite susceptible were *Streptococcus pyogenes*, pneumococci, gonococci, meningococci, *Corynebacterium diphtheriae*, and some strains of *Streptococcus viridans*.

Many organisms were entirely unaffected by the broth. Among these were *Escherichia coli*, *Eberthella typhosa*, *Pseudomonas pyocyaneus*, *Proteus vulgaris*, *Vibrio comma*, *Friedlander’s bacillus*, *Hemophilus influenzae* and Flexner’s dysentery bacillus.

Dr. Fleming found the broth containing the inhibitory substance for certain bacteria to be no more toxic or irritating than the broth itself, when injected into animals. In the summary to this original paper he modestly proposed that the antibacterial substance might find clinical application in infections caused by organisms susceptible to it.

Since the mold which elaborated the antibacterial substance was recognized by Dr. Fleming to be a member of a large genus of molds, *Penicillium*, he named the substance penicillin. Although the word has been pronounced several ways and there has been much discussion as to the correct one, the majority usage among workers in the field appears to be pen-i-til’-lin.

Because penicillin was very unstable and was difficult to produce in large quantities, it excited little interest during the next ten years. Fleming reported its use as a selective bacteriostatic agent for purposes of differentiation of bacteria in two or three papers. In 1932, Clutterbuck et al. (2) devised ways to grow the mold in larger amounts and further described the chemistry of the antibiotic substance.

A sudden new interest in penicillin followed the announcement of Florey, et al. (3, 4) that penicillin is very effective therapeutically and is of extremely low toxicity in contrast to the relatively high toxicity of the antibacterial substances, gramicidin and tyrocidine, isolated by Dubos from soil bacteria. Their papers also included new methods of extracting the antibiotic substance from the mold as well as improved methods of purification.
Since the papers of Florey, et al. appeared in 1940 and 1941, reports of several thousands of cases treated with penicillin have appeared in about three hundred papers.

The startling, almost miraculous, therapeutic results reported by Florey and his co-workers in the successful treatment of hopelessly infected individuals excited such wide interest that in 1941 Florey was brought to the United States by the Rockefeller Foundation to attempt to institute larger scale production than was possible in war-torn England. Here he interested the National Research Council and the Northern Regional Laboratory of the Department of Agriculture at Peoria along with several large pharmaceutical concerns in the possibility of large-scale production. At that time his yields were only about two units per ml. which were too small to be commercially practicable when amounts of the order of one million units were apparently necessary to treat deep-seated infections effectively.

The next important advance, which made large-scale production feasible, came with the discovery by Dr. Andrew Moyer at the Northern Regional Laboratory that addition of corn-steep water (a by-product of the corn refining industry) to the medium on which the mold was grown increased the yields of penicillin to better than forty units per ml.

**PRODUCTION**

At present all penicillin is produced by growing highly selected strains of the mold, *Penicillium notatum* (ordinary untested green bread or cheese mold is entirely unsatisfactory) on a suitable medium from which the penicillin is then extracted.

Probably the foremost problem is the maintenance of sterility. It seems paradoxical that such a potent antibacterial agent as penicillin must be produced by a mold grown under aseptic conditions; but it is well known that organisms which are not susceptible to penicillin, may destroy it very readily. Thus Abraham and Chain (5) extracted a substance from *Escherichia coli* which they named penicillinase since it destroyed penicillin and it had some of the properties of an enzyme.

The optimal culture temperature for penicillin production by the mold appears to be 24° C. The carbon source in the medium is usually lactose; sodium nitrate may be used as the nitrogen source. A host of trace elements has been claimed to be essential for maximum yields by various workers. Among these zinc ions seem to be very important. As stated above, corn-steep water, the composition of which is incompletely known, greatly increases yields.

Three devices are commonly employed to effect aeration (the mold will not grow anaerobically) and the method of culture is named accordingly.

1. **Surface culture method.** Until several months ago most of the penicillin was produced by this method in commercial installations. Aeration is by simple diffusion and a fundamental requirement is that the medium be not more than two cms. deep for best yields. The essential problem is then one of sterilization, inoculation and maintenance under aseptic conditions for several days of the large surfaces of medium required for commercial production of penicillin. To do this, racks filled with shallow trays are sometimes used but more commonly the flat-type half-gallon milk bottle is used. Some establishments are said to be using several hundred thousands of these. After autoclaving the bottles containing about 400 ml. of medium each, they are inoculated under aseptic conditions with suspensions of the mold spores by means of spray guns. The bottles are piled on their sides so that the maximum surface of medium in the bottle is presented to the mold for growth. After a suitable time (usually 5-7 days) the pH of the medium will have changed from 4 or 5 to about 7, when the contents are dumped into screening devices to separate the mold which is then discarded. The medium filtrate containing the penicillin is then ready for extraction.

2. **Submerged Culture Method.** Several plants are now using this method which promises to supplant all other methods for commercial production. One establish-
ment uses several tanks which hold thousands of gallons each. Although the yields per ml. are probably somewhat lower than by the surface culture method, the labor requirements are very much less because the filling, sterilization, inoculation and handling of individual bottles is obviated. In this method aeration is accomplished by vigorous bubbling of sterile air through the inoculated medium. The essential problem seems to be one of sterilization of the huge volumes of air required.

3. Bran Method. In this method aeration is brought about by growing the mold on coarse bran wetted with the medium in large trays or in long rotating cylinders.

According to a report given by Coghill (10) the total production of penicillin in this country is increasing rapidly and it was predicted that sufficient would be available to meet the essential needs of both the military and civilian populations before the end of the year. In March, alone, approximately forty billion units were produced by 21 companies; this is greater than the total amount produced in 1943. When all of the plants are producing to capacity it was predicted that at least 200 billion units per month will be produced by the end of 1944.

The present standard is a pure crystalline solid equivalent to 1650 of the old Oxford or Florey units per milligram. Hence the predicted output of the 21 plants will be only about 9 pounds per day.

EXTRACTION AND PURIFICATION

Several methods have been described for the extraction of penicillin from the culture medium on which the mold has grown. Among the simplest of these is that described by Abraham and Chain (7). The pH of the solution containing the penicillin is adjusted to 1.9-2.0 with phosphoric acid when it is immediately shaken with some solvent immiscible with water such as amyl acetate or chloroform. After separation of the phases the aqueous portion is discarded. The amyl acetate containing the penicillin in acid form is then shaken with small portions of water-containing base such as sodium bicarbonate. This reconverts the penicillin to the sodium salt which is selectively soluble in the aqueous phase.

The resulting solution of the sodium salt of penicillin may be purified further by chromatographic adsorption on columns of alumina or other suitable adsorbents. For clinical use further purification is usually unnecessary (although desirable) beyond removal of amyl acetate dissolved in the solution. Usually the solution is filtered through Seitz bacterial filters and then dried by sublimation of the water from the frozen state under reduced pressure. The product is a yellow powder which is quite stable when perfectly dry and especially if kept in the refrigerator. For therapeutic use it is then readily dissolved in water or normal saline.

CHEMICAL PROPERTIES OF PENICILLIN

Needless to say there probably never has been a compound whose isolation and determination of structure has stimulated a more intensive and concerted effort by individual chemists and groups of chemists in universities and pharmaceutical houses. Announcement of pure penicillin in the form of its crystalline sodium salt was first made in October, 1943, by McPhillamy and Wintersteiner of the Squibb Institute for Medical Research (6). This was, of course, a most important step forward and an essential one preliminary to chemical scrutiny of the compound. For obvious reasons publication of the structure and synthesis of penicillin is not anticipated for the duration even though they may be discovered before the war ends.

Because all reports on the chemistry of penicillin which have been published, and notably the splendid work of Abraham and Chain (7), have been of work on impure preparations, only general properties which are unlikely to be affected by the presence of impurities will be summarized here.
In aqueous solution penicillin is most stable in the pH range 5 to 7. Its antibacterial properties are destroyed at increasing rates as the pH of the solution is varied increasingly widely from the above stability range. It is this sensitivity to high acidities which results in loss of its activity in contact with gastric juice and makes inefficient its administration by mouth. The higher the temperature of the aqueous solution at a given pH the more rapidly the antibacterial properties are lost. Electrometric titration indicates that a lactone ring is opened during inactivation by alkalis.

Penicillin is readily destroyed by hydrogen peroxide and potassium permanganate but is stable to air oxidation.

In the free (acid) form it is readily soluble in fat solvents such as ether, acetone, esters and dioxane. It is less soluble in chloroform and only slightly soluble in benzene and carbon tetrachloride. The free acid dissolved in ether or amyl acetate is stable several days at room temperature; it is inactivated by passing dry hydrogen chloride through the solutions. It is also inactivated by such primary alcohols as methyl, ethyl, benzyl and by ethylene glycol.

The sodium, potassium and ammonium salts are very hygroscopic and hence soon decompose in contact with air. The calcium, strontium, and barium salts are non-hygroscopic relatively stable white powders, especially if kept cold.

Elementary analysis of the barium salt of penicillin obtained by Abraham and Chain with 500 units of activity per milligram corresponded to the formula $\text{C}_{24}\text{H}_{32}\text{O}_{10}\text{N}_{2}\text{Ba}$ with a molecular weight of 645; 508 of this is due to the penicillin itself, free from barium.

Meyer, et al. (8) give the formula $\text{C}_{4}\text{H}_{14}\text{NO}_{2}$ as best fitting their data. Later (9) they succeeded in obtaining the methyl, ethyl, n-butyl and benzohydryl esters by reacting with the corresponding diazo compounds. In vitro the methyl and ethyl esters were quite active, the ethyl more so than the methyl ester. Moreover there was some indication that the stability was increased as evidenced by successful use in treating mice infected with virulent hemolytic streptococci by giving the esters by mouth.

**ASSAY**

The concentration of penicillin is measured by a bioassay method which depends on its antibiotic properties. Two of the several methods used will be described briefly.

**Cup Assay Method.**—A susceptible strain of bacteria (usually *Staphylococcus aureus* 209) is cultured on petri dishes on which are evenly distributed five porcelain or glass cylinders so as to form a seal with the inoculated agar. Into two of the cups thus formed is placed a solution of penicillin of known concentration, usually one unit per ml. Into the other three cups is placed the solution whose content of penicillin is to be determined, after having diluted the solution so that its concentration will also be about one unit per ml. The plates are then incubated at 37° C. overnight. In the morning it will be found that wherever the penicillin has diffused through the agar the growth of the cultured bacteria will have been inhibited. There thus results a clear zone of inhibition around each of the cups. A suitable comparison of the average of the diameters of the zones produced by the solution of unknown concentration with the average for the knowns is the basis of the assay. This method is the most widely used because a sterile preparation is unnecessary and because a large number of assays can be made per person per day.

**Turbidimetric Method.**—Where greater accuracy or greater speed is essential, turbidimetric methods based on the proportionality between penicillin concentration and inhibition of bacterial growth are used. The necessity of using bacterial filters to sterilize the penicillin solutions prior to assay by this method as it is usually employed is its chief drawback.
Recently a bioassay method was described (29) by the use of which penicillin concentrations may be determined in 60–90 minutes with an error of less than 5%. It depends upon the inhibition of the reduction of nitrate to nitrite by *Staphylococcus aureus* in the presence of penicillin. The nitrite is determined colorimetrically by a modification of the method of Shinn (30).

The Oxford and Florey unit was originally defined as that amount of penicillin which, when dissolved in 50 ml. of meat extract broth, just inhibits completely the growth of the test strain of *Staphylococcus aureus* (11). At the present time penicillin is measured in terms of a pure crystalline standard assigned the value of 1650 units per milligram. From this it is seen that 0.6 gram of crystalline penicillin is equivalent in antibiotic activity to one million units.

**ANTIBACTERIAL ACTION OF PENICILLIN**

Many lists have appeared showing the susceptibility or insusceptibility of various bacteria and fungi to the action of penicillin in vitro. The one below is that of Hobby, Meyer and Chaffee (12).

<table>
<thead>
<tr>
<th>SUSCEPTIBLE STRAINS</th>
<th>INSUSCEPTIBLE STRAINS</th>
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</thead>
<tbody>
<tr>
<td><em>Diplococcus pneumoniae</em></td>
<td><em>Hemophilus influenzae</em></td>
</tr>
<tr>
<td><em>Streptococcus hemolyticus</em></td>
<td><em>Escherichia coli</em></td>
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<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td><em>Eberthella typhosa</em></td>
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<tr>
<td><em>Neisseria meningitidis</em></td>
<td><em>Shigella dysenteriae</em></td>
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<tr>
<td><em>Streptococcus viridans</em></td>
<td><em>Proteus vulgaris</em></td>
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<tr>
<td><em>Bacillus subtilis</em></td>
<td><em>Salmonella paratyphi</em></td>
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<tr>
<td><em>Clostridium welchi</em></td>
<td><em>Salmonella enteritidis</em></td>
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<tr>
<td><em>Clostridium septicum</em></td>
<td><em>Pseudomonas aeruginosa</em></td>
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<tr>
<td><em>Clostridium histolyticum</em></td>
<td><em>Pseudomonas fluorescens</em></td>
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<tr>
<td><em>Clostridium sporogenes</em></td>
<td><em>Serratia marcescens</em></td>
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<tr>
<td><em>Clostridium oedemantiens</em></td>
<td><em>Klebsiella pneumonieae</em></td>
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<tr>
<td><em>Clostridium sordelli</em></td>
<td><em>Staphylococcus albus</em>—1 strain</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td><em>Micrococcus albus</em>—1 strain</td>
</tr>
<tr>
<td><em>Cryptococcus hominis</em></td>
<td><em>Monilia albicans</em></td>
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<tr>
<td><em>Bacillus anthracis</em></td>
<td><em>Monilia krusei</em></td>
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<tr>
<td><em>Corynebacterium diphtheriae</em></td>
<td><em>Monilia candida</em></td>
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<tr>
<td><em>Clostridium tetani</em></td>
<td></td>
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<tr>
<td><em>Actinomyces bovis</em></td>
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<tr>
<td><em>Salmonella schottmuelleri</em></td>
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<tr>
<td><em>Salmonella typhimurium</em></td>
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<tr>
<td><em>Brucella abortus</em></td>
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<tr>
<td><em>Brucella melitensis</em></td>
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<tr>
<td><em>Mycoplasma tuberculosis</em></td>
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<tr>
<td><em>Pasteurella pestis</em></td>
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<tr>
<td><em>Anaerobic streptococi</em></td>
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<tr>
<td><em>Vibrio comma</em></td>
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<tr>
<td><em>Lepiospira icterohaemorrhagiae</em></td>
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</tbody>
</table>

In addition to some of the above organisms, Abraham et al. (4) found the following to be susceptible or insusceptible respectively.

Bacillus anthracis
*Corynebacterium diphtheriae*
*Clostridium tetani*
*Actinomyces bovis*

Salmonella schottmuelleri
*Salmonella typhimurium*
*Brucella abortus*
*Brucella melitensis*
*Mycobacterium tuberculosis*
*Pasteurella pestis*
*Anaerobic streptococi*
*Vibrio comma*
*Lepiospira icterohaemorrhagiae*

In preliminary animal experiments followed by administration in four cases of early syphilis in men, Mahoney et al. (13) found that penicillin possessed spirochetical activity. Susceptibility of the spirochetes which cause relapsing fever (*Borrelia novyi*) was observed by Heilman and Herrell in experimentally infected mice (14).

Evidence is at hand to indicate that penicillin is ineffective in virus infections. Myers and Lenahan (15) observed the development of chicken-pox in a patient under penicillin therapy for osteomyelitis. Robinson (16) found that penicillin was ineffective in protecting mice from the influenza virus PR8.

The foregoing list of organisms which are susceptible to penicillin contains the names of the greatest killers of man. The number of men who have died in all of the wars since the beginning of history is small in comparison to the number who have succumbed to the diseases commonly caused by these organisms. Men-
tion of only a few would strike terror into the hearts of the bravest armies; pneumonia, meningitis, bacteremia, osteomyelitis, empyema, tetanus, gas gangrene, brain abscess, mastoid and middle ear infections, gonorrhea, et cetera.

That penicillin is not a panacea is attested to by the above list of insusceptible organisms. Moreover, recent reports show that there are numerous strains of some of the organisms, which appear in the susceptible list, that are not affected by penicillin. Fisher (17) found 12 insusceptible strains of staphylococci of 102 strains in his in vitro studies with crude penicillin. He also encountered several strains of hemolytic streptococci which were not affected by penicillin. Florey (18) reports that Major Scott Thomson found 4 per cent of staphylococci are resistant to penicillin.

The mode of action of penicillin is not clearly understood. Hobby, Meyer and Chaffee (19) found that penicillin usually acts as a bacteriostatic agent but it may not be bactericidal. Actual killing of hemolytic streptococci was not accompanied by lysis. It seemed that penicillin was effective only during active multiplication of the organisms. The rate of killing increased as the penicillin concentration increased within certain limits, when a given number of organisms was present originally; but no penicillin was destroyed or removed from solution in detectable amounts by the organisms. Abraham et al. (4) found no inhibitory effect on the action of penicillin in the presence of pus, blood, serum, tissue autolysates or peptones; this is in contrast to the sulfonamides.

Gardner (20) describes microscopic changes due to a failure of fission in rod-shaped organisms acted upon by penicillin. There occurred a lengthening of the cells due to growth without division and separation of the cells. In the case of Staphylococcus aureus Smith and Hay (21) observed an increase in size of the cells which were cultured in broth containing penicillin. As the cells increased in size, imperfect division occurred as evidenced by the appearance of a clear zone across the middle of the cell.

Welshimer (28) found in studies in the Warburg apparatus that dismutation of pyruvic acid by Staphylococcus aureus was inhibited by penicillin.

Several studies have appeared concerning the development of a tolerance of susceptible organisms when exposed to penicillin concentrations which would just permit growth. McKee and Houck (23) reported growth of a type III pneumococcus culture, highly virulent to mice, in a thirty-fold increase of penicillin after 55 serial passages in a broth containing penicillin in concentrations that would not completely inhibit growth. Type specificity of these resistant cultures was retained although the colonies grew more slowly and the capsule was smaller. There occurred abnormal forms with a tendency to clump and chain formation. Simultaneously there was a marked decrease in virulence for mice; before passage a lethal dose was about ten organisms but after the fifty-fifth passage, approximately 900 million organisms were required to kill the mice. Nor was the virulence restored after nine mouse passages. Similar treatment with three strains of staphylococci, one strain of Streptococcus pyogenes and a type I pneumococcus culture produced the same results. Keefer et al. (24) found that resistance of Staphylococcus aureus and other cocci may develop during treatment of patients with insufficient doses of penicillin. However, the penicillin-resistant organisms remained susceptible to the sulfonamides and vice versa.

Penicillin has the widest margin of safety of any known chemotherapeutic agent. Tissue culture studies by Herrell and Heilman (25) showed that the toxicity for lymphocytes, granulocytes, phagocytes and erythrocytes was extremely low with crude preparations containing more than 97 per cent of impurities. Robinson (16), using an impure preparation, found the toxic dose of penicillin to be about 64 times the effective dose when injected subcutaneously into mice. Clinical evidences of toxicity encountered by Keefer et al. (24) such as urticaria, flushing of the face, and local pain at the site of injection were attributable to
impurities which had not been removed. Numerous recent reports in which purer preparations of later and improved manufacture describe no unfavorable clinical reactions whatsoever. In the light of this recent clinical experience with the drug, it seems safe to say that overdosage with pure penicillin is virtually impossible.

ADMINISTRATION

Penicillin has been administered locally as wet dressings, via catheters into wounds and body cavities, in ointments, and as a powder—either as the calcium salt or by insufflation with one per cent penicillin diluted with sulfonamide powder (26). It has also been injected subcutaneously, intramuscularly, intravenously, intraperitoneally, intrathecally and into various body cavities and spaces. In general the present practice is to use it locally for burns, surface infections, wounds, etc.; and intramuscularly at the rate of 5,000–20,000 units every 2–4 hours for systemic administration. Occasionally it is given by continuous intravenous drip in overwhelming infections or where an established bacteremia exists. A distinct disadvantage is that it cannot be given efficiently by mouth because of the rapid destruction of most of it by the acid in the stomach.

Fig. 1, a and b. Front and side views of patient at onset of penicillin therapy. Extensive facial cellulitis and edema of both eyes may be noted; patient moribund; c, appearance of child ninety-six hours later.

The striking effectiveness of penicillin in overwhelming infections susceptible to it is well demonstrated in the case of a patient treated by Herrell (22). Fig. 1 (a and b) are front and side views of the patient at the start of penicillin treatment. According to her father, this four-year-old girl had bitten the inside of her left cheek prior to the appearance of swelling and redness of the left jaw six days before the pictures were taken. On admission, her temperature was 104°F. and breathing and swallowing were difficult. Examination revealed necrosis in the left alveolar lingual region; this was drained by means of a stab wound into which was introduced a small rubber drain. Blood culture was strongly positive for hemolytic *Staphylococcus aureus*. The leukocyte count was 4500 with 37% neutrophils, 62% lymphocytes and 1% monocytes. During the next five days she was given between 20,000 and 30,000 units of penicillin daily intra-

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Venously. Thirty-six hours after the penicillin was started, the blood became sterile. At the time X-ray revealed pneumonia of the right upper lobe, presumably staphylococcal in origin. Fig. 1 (c) was taken 96 hours after initiation of the treatment, at which time she could again swallow and breathe with little difficulty. Fig. 2 (a) shows the child on the ninth day of treatment when her temperature had become normal. Fig 2 (b and c) show her on dismissal, completely recovered. During the twelve days of treatment the patient had received a total of 196,000 units of penicillin or only 0.12 gram of the drug on the basis of the pure crystalline salt!

**OTHER ANTIBIOTICS**

As Professor Fleming has remarked, it would be a most unusual accident if the first successful antibiotic would prove to be the best one. At present there is a wide-spread search for other antibiotics which will be effective against pathogenic organisms that are insusceptible to penicillin; or that will be more easily produced, more stable, can be taken by mouth, or that can be readily synthesized. Among the many which have been described are: gramicidin, tyrocidine, streptothricin, streptomycin, actinomycin, clavacin, gliotoxin, penatin, etc., etc. However, most of them do not combine the lack of toxicity with the effectiveness possessed by penicillin. Of particular interest is streptothricin (27) since it is reported to be effective against the organisms that are responsible for typhoid fever and bacillary dysentery, an important group of gram-negative pathogens against which penicillin is entirely ineffective.

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