Classic publications in Antimicrobial Agents and Resistance

The discovery of antibiotics, their clinical use and detection of antibiotic inactivating enzymes by bacteria was heralded by some publications as providing a new paradigm for the medical management of infectious diseases. Though these papers were published more than seven decades ago, they continue to be relevant in contemporary times. Three such landmark papers are brought together in this special issue of the Regional Health Forum, and are reprinted with permission from the respective journals.

1929

On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in isolation of *B.influenzae*.

A.Fleming

British Journal of Experimental Pathology 1929;10:226-236

The discovery in 1929 of *in vitro* action of a product of *Penicillium* on several bacteria of medical importance is believed to be the first published report of the antibacterial action of a fungal product. This was published in 1929 by A.Fleming (who subsequently received the Nobel Prize for this discovery) in the *British Journal of Experimental Pathology* (now the *International Journal of Experimental Pathology)*.

1940

Penicillin as a chemotherapeutic agent.

E Chain, HW Florey, AD Gardner, NG Heatley, MA Jennings, J Orr-Ewing and AG Sanders

Lancet 1940; ii: 226-228

The discovery of antibacterial action of a fungal product by Fleming (1929) was expanded into a product fit for use in human beings by Chain, Florey et al., who demonstrated its clinical efficacy for the first time in 1940. This heralded an era in which new and potent weapons against infectious diseases were developed, and substantially reduced mortality and morbidity from these diseases.

1940

An enzyme from bacteria able to destroy penicillin

EP Abraham and E Chain

Nature 1940;146:837

The world has always celebrated the discovery and clinical use of penicillin– however, the fact that in the same year a study was published showing that an enzyme could be produced by bacteria that *inactivated* penicillin has largely been ignored. This publication should have forewarned us of the ingenuity of microorganisms against antibiotics, which is now manifesting itself as the major problem of antimicrobial resistance in several microorganisms.

ON THE ANTIBACTERIAL ACTION OF CULTURES OF A PENICILLIUM, WITH SPECIAL REFERENCE TO THEIR USE IN THE ISOLATION OF *B. INFLUENZÆ*.

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Received for publication May 10th, 1929.

WHILE working with staphylococcus variants a number of culture-plates were set aside on the laboratory bench and examined from time to time. In the examinations these plates were necessarily exposed to the air and they became contaminated with various micro-organisms. It was noticed that around a large colony of a contaminating mould the staphylococcus colonies became transparent and were obviously undergoing lysis (see Fig. 1).

Subcultures of this mould were made and experiments conducted with a view to ascertaining something of the properties of the bacteriolytic substance which had evidently been formed in the mould culture and which had diffused into the surrounding medium. It was found that broth in which the mould had been grown at room temperature for one or two weeks had acquired marked inhibitory, bactericidal and bacteriolytic properties to many of the more common pathogenic bacteria.

CHARACTERS OF THE MOULD.

The colony appears as a white fluffy mass which rapidly increases in size and after a few days sporulates, the centre becoming dark green and later in old cultures darkens to almost black. In four or five days a bright yellow colour is produced which diffuses into the medium. In certain conditions a reddish colour can be observed in the growth.

In broth the mould grows on the surface as a white fluffy growth changing in a few days to a dark green felted mass. The broth becomes bright yellow and this yellow pigment is not extracted by $CHCl_3$. The reaction of the broth becomes markedly alkaline, the pH varying from 8·5 to 9. Acid is produced in three or four days in glucose and saccharose broth. There is no acid production in 7 days in lactose, mannite or dulcite broth.

Growth is slow at 37°C. and is most rapid about 20°C. No growth is observed under anaerobic conditions.

In its morphology this organism is a penicillium and in all its characters it most closely resembles *P. rubrum*. Biourge (1923) states that he has never found *P. rubrum* in nature and that it is an "animal de laboratoire." This penicillium is not uncommon in the air of the laboratory.

IS THE ANTIBACTERIAL BODY ELABORATED IN CULTURE BY ALL MOULDS?

A number of other moulds were grown in broth at room temperature and the culture fluids were tested for antibacterial substances at various intervals up to one month. The species examined were: *Eidamia viridiscens*, *Botrytis cineria*, *Aspergillus fumigatus*, *Sporotrichum*, *Cladosporium*, *Penicillium*, 8 strains. Of these it was found that only one strain of penicillium produced any inhibitory substance, and that one had exactly the same cultural characters as the original one from the contaminated plate.

It is clear, therefore, that the production of this antibacterial substance is not common to all moulds or to all types of penicillium.

In the rest of this article, allusion will constantly be made to experiments with filtrates of a broth culture of this mould, so for convenience and to avoid the repetition of the rather cumbersome phrase " Mould broth filtrate," the name " penicillin " will be used. This will denote the filtrate of a broth culture of the particular penicillium with which we are concerned.

METHODS OF EXAMINING CULTURES FOR ANTIBACTERIAL SUBSTANCE.

The simplest method of examining for inhibitory power is to cut a furrow in an agar plate (or a plate of other suitable culture material), and fill this in with a mixture of equal parts of agar and the broth in which the mould has grown. When this has solidified, cultures of various microbes can be streaked at right angles from the furrow to the edge of the plate. The inhibitory substance diffuses very rapidly in the agar, so that in the few hours before the microbes show visible growth it has spread out for a centimetre or more in sufficient concentration to inhibit growth of a sensitive microbe. On further incubation it will be seen that the proximal portion of the culture for perhaps one centimetre becomes transparent, and on examination of this portion of the culture it is found that practically all the microbes are dissolved, indicating that the anti-bacterial substance has continued to diffuse into the agar in sufficient concentration to induce dissolution of the bacteria. This simple method therefore suffices to demonstrate the bacterio-inhibitory and bacteriolytic properties of the mould culture, and also by the extent of the area of inhibition gives some measure of the sensitiveness of the particular microbe tested. Fig. 2 shows the degree of inhibition obtained with various microbes tested in this way.

The inhibitory power can be accurately titrated by making serial dilutions of penicillin in fresh nutrient broth, and then implanting all the tubes with the same volume of a bacterial suspension and incubating them. The inhibition can then readily be seen by noting the opacity of the broth.

For the estimation of the antibacterial power of a mould culture it is unnecessary to filter as the mould grows only slowly at 37°C., and in 24 hours, when the results are read, no growth of mould is perceptible. Staphylococcus is a very suitable microbe on which to test the broth as it is hardy, lives well in culture, grows rapidly, and is very sensitive to penicillin.

The bactericidal power can be tested in the same way except that at intervals measured quantities are explanted so that the number of surviving microbes can be estimated.

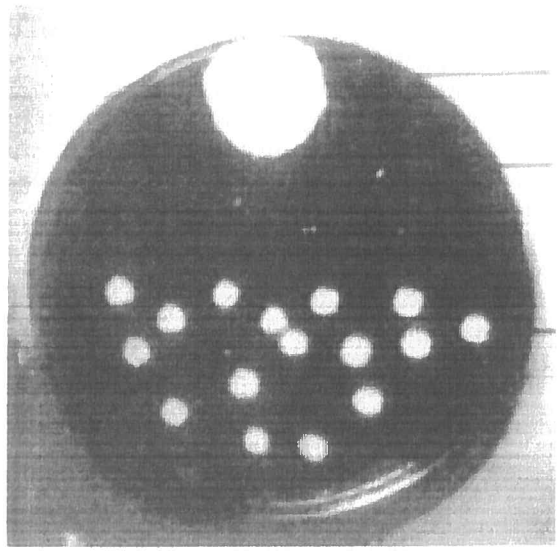

FIG. 1.—Photograph of a culture-plate showing the dissolution of staphylococcal colonies in the neighbourhood of a penicillium colony.

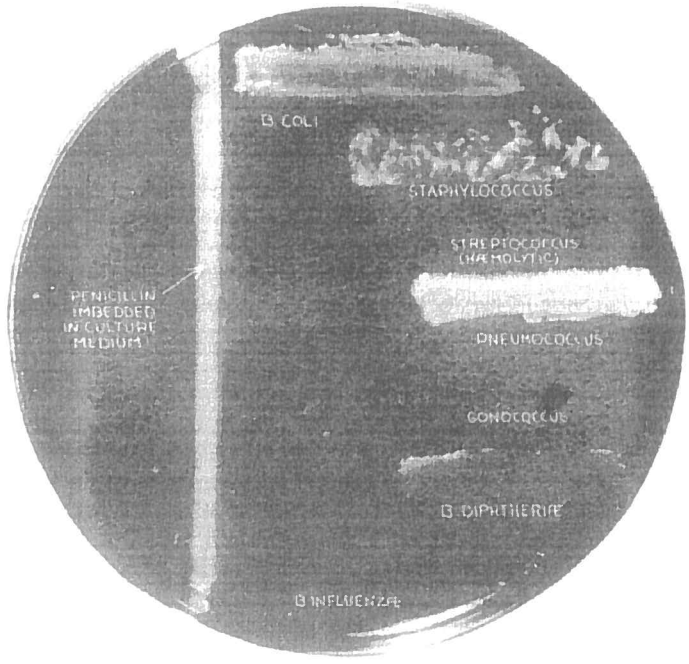

FIG. 2.

Fleming.

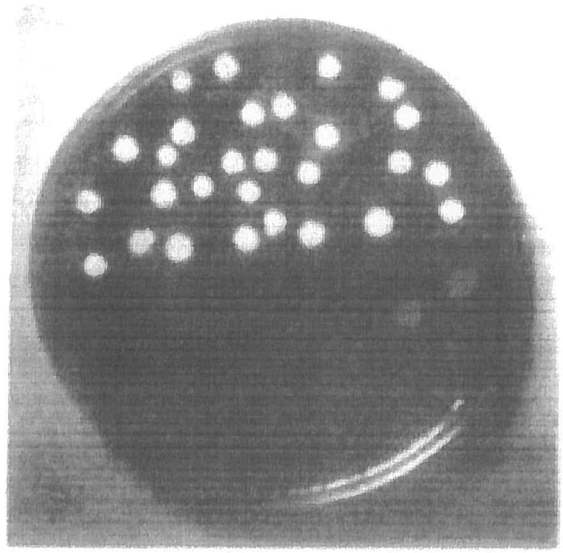

FIG. 3.—Photograph of a culture-plate (Fildes medium) which had been evenly planted with a mixture of staphylococci and *B. influenzæ*. Six drops of penicillin were then spread over the lower half of the plate. Note complete inhibition of staphylococci in the penicillin treated area with resultant pure culture of *B. influenzæ*.

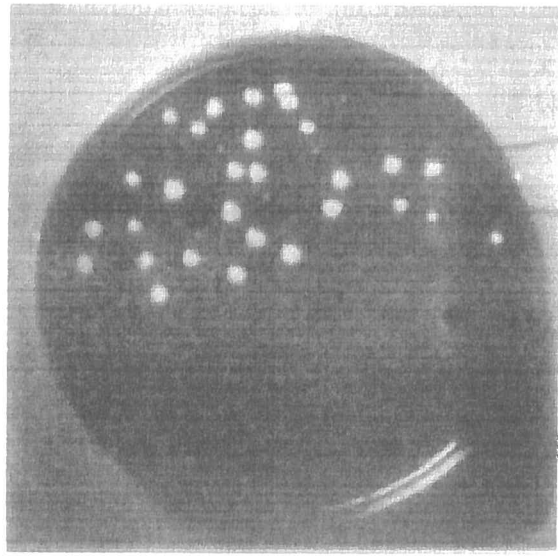

FIG. 4.—Photograph of a culture-plate (Fildes medium) which had been evenly planted with nasal mucus from an individual suffering from a "cold." Six drops of penicillin were spread over the lower half of the plate before incubation. Note profuse growth of staphylococci and diphtheroid bacilli in untreated half, whereas in treated half only some three colonies of *B. influenzæ* are seen.

PROPERTIES OF THE ANTIBACTERIAL SUBSTANCE.

Effect of heat.—Heating for 1 hour at 56° or 80° C. has no effect on the antibacterial power of penicillin. Boiling for a few minutes hardly affects it (see Table II). Boiling for 1 hour reduces it to less than one quarter its previous strength if the fluid is alkaline, but if it is neutral or very slightly acid then the reduction is much less. Autoclaving for 20 minutes at 115° C. practically destroys it.

Effect of filtration.—Passage through a Seitz filter does not diminish the antibacterial power. This is the best method of obtaining sterile active mould broth.

Solubility.—It is freely soluble in water and weak saline solutions. My colleague, Mr. Ridley, has found that if penicillin is evaporated at a low temperature to a sticky mass the active principle can be completely extracted by absolute alcohol. It is insoluble in ether or chloroform.

Rate of development of inhibitory substance in culture.—A 500 c.c. Erlenmeyer flask containing 200 c.c. of broth was planted with mould spores and incubated at room temperature (10° to 20° C.). The inhibitory power of the broth to staphylococcus was tested at intervals.

After 5 days complete inhibition in 1 in 20 dilution.

,, 6 ,, ,, ,, ,, 1 in 40 ,,
,, 7 ,, ,, ,, ,, 1 in 200 ,,
,, 8 ,, ,, ,, ,, 1 in 500 ,,

Grown at 20° C. the development of the active principle is more rapid and a good sample will completely inhibit staphylococci in a 1 in 500 or 1 in 800 dilution in 6 or 7 days. As the culture ages the antibacterial power falls and may in 14 days at 20° C. have almost disappeared.

The antibacterial power of penicillin falls when it is kept at room temperature. The rate of this fall can be seen from Table I.

TABLE I.—*Effect of Keeping at Room Temperature on the Anti-Staphylococcal Power of Penicillin.*

	Growth of staphylococcus in dilutions of penicillin as under.										
	1/20.	1/40.	1/60.	1/80.	1/100.	1/200.	1/300.	1/400.	1/600.	1/800.	1/1000.	Control.
At time of filtration	−	−	−	−	−	−	−	−	−	±	+ +	+ +
After 4 days	−	−	−	−	−	−	−	−	−	±	+ +	+ +
,, 7 ,,	−	−	−	−	−	−	−	±	+	+	+ +	+ +
,, 9 ,,	−	−	−	−	−	−	−	±	+	+	+ +	+ +
,, 13 ,,	−	−	−	−	−	+	+	+	+	+	+ +	+ +
,, 15 ,,	−	:	+	+	+	+	+	+	+	+	+ +	+ +

If the reaction of penicillin is altered from its original pH of 9 to a pH of 6·8 it is much more stable.

The small drops of bright yellow fluid which collect on the surface of the mould may have a high antibacterial titre. One specimen of such fluid completely inhibited the growth of staphylococci in a dilution of 1 in 20,000 while the broth in which the mould was growing, tested at the same time, inhibited staphylococcal growth in 1 in 800.

If the mould is grown on solid medium and the felted mass picked off and

TABLE II.— *Inhibitory Power of Penicillin (Heated and Unheated) on Various Microbes (Agar Plate Method).*

Type of microbe.	Extent of inhibition in mm. from penicillin embedded in agar, serum agar, or blood agar plates.	
	Unheated.	Boiled for 1 minute.
Experiment 1 :		
Staphylococcus pyogenes	23	21
Streptococcus ,,	17	17
,, *viridans* (mouth)	17	15
Diphtheroid bacillus	27	22
Sarcina	10	10
Micrococcus lysodeikticus	6	7
,, from air (1)	20	16
,, ,, (2)	4	9
B. anthracis	0	0
B. typhosus	0	0
Enterococcus	0	0
Experiment 2 :		
Staphylococcus pyogenes	24	...
Streptococcus ,,	30	...
,, *viridans* (mouth)	25	...
Pneumococcus	30	...
Diphtheroid bacillus	35	...
B. pyocyaneus	0	...
B. pneumoniæ (Friedlander)	0	...
B. coli	0	...
B. paratyphosus A	0	...
Experiment 3 :		
Staphylococcus pyogenes	16	...
Gonococcus	16	...
Meningococcus	17	...
Experiment 4 :		
Staphylococcus pyogenes	17	...
,, *epidermidis*	18	...
Streptococcus pyogenes	15	...
,, *viridans* (fæces)	5	...
B. diphtheriæ (2 strains)	14	...
Diphtheroid bacillus	10	...
Gram-negative coccus from the mouth (1)	12	...
,, ,, ,, (2)	0	...
B. coli	0	...
B. influenzæ (Pfeiffer) 6 strains	0	...

TABLE III.—*Inhibitory Power of Penicillin on Different Bacteria.*

	Dilution of penicillin in broth.											
	1/5.	1/10.	1/20.	1/40.	1/80.	1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	Control.
Staphylococcus aureus	0	0	0	0	0	0	0	0	±	+ +	+ +	+ +
,, *epidermidis*	0	0	0	0	0	0	0	0	±	+ +	+ +	+ +
Pneumococcus	0	0	0	0	0	0	0	0	0	+ +	+ +	+ +
Streptococcus (hæmolytic)	0	0	0	0	0	0	0	0	0	±	+ +	+ +
,, *viridans* (mouth)	0	0	0	0	0	0	±	+ +	+ +	+ +	+ +	+ +
,, *fæcalis*	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
B. anthracis	0	0	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
B. pseudo-tuberculosis rodentium	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
B. pullorum	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
B. dysenteriæ	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
B. coli	+ +	+ +	+ +	...	...	...	...	...	...	...	...	+ +
B. typhosus	+ +	+ +	+ +	...	...	...	...	...	...	...	...	+ +
B. pyocyaneus	+ +	+ +	+ +	...	...	...	...	...	...	...	...	+ +
B. proteus	+ +	+ +	+ +	...	...	...	...	...	...	...	...	+ +
V. choleræ	+ +	+ +	+ +	...	...	...	...	...	...	...	...	+ +

	1/60.	1/120.	1/300.	1/600.	Control.
B. diphtheriæ (3 strains)	0	±	+ +	+ +	+ +
Streptococcus pyogenes (13 strains)	0	0	0	+ +	+ +
,, ,, (1 ,,)	0	0	±	+ +	+ +
,, *fæcalis* (11 ,,)	+ +	+ +	+ +	+ +	+ +
,, *viridans* at random from fæces (1 strain)	0	0	0	+ +	+ +
,, ,, ,, ,, (2 strains)	0	0	±	+ +	+ +
,, ,, ,, ,, (1 strain)	0	±	+ +	+ +	+ +
,, ,, ,, ,, (1 ,,)	+	+ +	+ +	+ +	+ +
,, ,, ,, ,, (1 ,,)	+ +	+ +	+ +	+ +	+ +
,, ,, at random from mouth (1 ,,)	0	±	+ +	+ +	+ +
,, ,, ,, ,, (2 strains)	0	0	+ +	+ +	+ +
,, ,, ,, ,, (1 strain)	0	0	0	+ +	+ +

0 = no growth; ± = trace of growth; + = poor growth; + + = normal growth.

extracted in normal salt solution for 24 hours it is found that the extract has bacteriolytic properties.

If this extract is mixed with a thick suspension of staphylococcus suspension and incubated for 2 hours at 45° C. it will be found that the opacity of the suspension has markedly diminished and after 24 hours the previously opaque suspension will have become almost clear.

Influence of the medium on the antibacterial titre of the mould culture. — So far as has been ascertained nutrient broth is the most suitable medium for the production of penicillin. The addition of glucose or saccharose, which are fermented by the mould with the production of acid, delays or prevents the appearance of the antibacterial substance. Dilution of the broth with water delays the formation of the antibacterial substance and diminishes the concentration which is ultimately reached.

INHIBITORY POWER OF PENICILLIN ON THE GROWTH OF BACTERIA.

Tables II and III show the extent to which various microbes, pathogenic and non-pathogenic, are inhibited by penicillin. The first table shows the inhibition by the agar plate method and the second shows the inhibitory power when diluted in nutrient broth.

Certain interesting facts emerge from these Tables. It is clear that penicillin contains bacterio-inhibitory substance which is very active towards some microbes while not affecting others. The members of the coli-typhoid group are unaffected as are other intestinal bacilli such as *B. pyocyaneus*, *B. proteus* and *V. cholerae*. Other bacteria which are insensitive to penicillin are the enterococcus, some of the Gram-negative cocci of the mouth, Friedländer's pneumobacillus, and *B. influenzae* (Pfeiffer), while the action on *B. dysenteriae* (Flexner), and *B. pseudo-tuberculosis rodentium* is almost negligible. The anthrax bacillus is completely inhibited in a 1 in 10 dilution but in this case the inhibitory influence is trifling when compared with the effect on the pyogenic cocci.

It is on the pyogenic cocci and on bacilli of the diphtheria group that the action is most manifest.

Staphylococci are very sensitive, and the inhibitory effect is practically the same on all strains, whatever the colour or type of the staphylococcus.

Streptococcus pyogenes is also very sensitive. There were small differences in the titre with different strains, but it may be said generally that it is slightly more sensitive than staphylococcus.

Pneumococci are equally sensitive with *Streptococcus pyogenes*.

The green streptococci vary very considerably, a few strains being almost unaffected while others are as sensitive as *S. pyogenes*. Gonococci, meningococci, and some of the Gram-negative cocci found in nasal catarrhal conditions are about as sensitive as are staphylococci. Many of the Gram-negative cocci found in the mouth and throat are, however, quite insensitive.

B. diphtheriæ is less affected than staphylococcus but is yet completely inhibited by a 1% dilution of a fair sample of penicillin.

It may be noted here that penicillin, which is strongly inhibitory to many bacteria, does not inhibit the growth of the original penicillium which was used in its preparation.

The Rate of Killing of Staphylococci by Penicillin.

Some bactericidal agents like the hypochlorites are extremely rapid in their action, others like flavine or novarsenobillon are slow. Experiments were made to find into which category penicillin fell.

To 1 c.c. volumes of dilutions in broth of penicillin were added 10 c.mm. volumes of a 1 in 1000 dilution of a staphylococcus broth culture. The tubes were then incubated at 37°C. and at intervals 10 c.mm. volumes were removed and plated with the following result :

	Number of colonies developing after sojourn in penicillin in concentrations as under:				
	Control.	1/80.	1/40.	1/20.	1/10.
Before . . .	27	27	27	27	27
After 2 hours . .	116	73	51	48	23
,, 4½ ,, . .	∝	13	1	2	5
,, 8 ,, . .	∝	0	0	0	0
,, 12 ,, . .	∝	0	0	0	0

It appears, therefore, that penicillin belongs to the group of slow acting antiseptics, and the staphylococci are only completely killed after an interval of over 4½ hours even in a concentration 30 or 40 times stronger than is necessary to inhibit completely the culture in broth. In the weaker concentrations it will be seen that at first there is growth of the staphylococci and only after some hours are the cocci killed off. The same thing can be seen if a series of dilutions of penicillin in broth are heavily infected with staphylococcus and incubated. If the cultures are examined after four hours it may be seen that growth has taken place apparently equally in all the tubes but when examined after being incubated overnight, the tubes containing penicillin in concentrations greater than 1 in 300 or 1 in 400 are perfectly clear while the control tube shows a heavy growth. This is a clear illustration of the bacteriolytic action of penicillin.

TOXICITY OF PENICILLIN.

The toxicity to animals of powerfully antibacterial mould broth filtrates appears to be very low. Twenty c.c. injected intravenously into a rabbit were not more toxic than the same quantity of broth. Half a c.c. injected intraperitoneally into a mouse weighing about 20 gm. induced no toxic symptoms. Constant irrigation of large infected surfaces in man was not accompanied by any toxic symptoms, while irrigation of the human conjunctiva every hour for a day had no irritant effect.

In vitro penicillin which completely inhibits the growth of staphylococci in a dilution of 1 in 600 does not interfere with leucocytic function to a greater extent than does ordinary broth.

USE OF PENICILLIN TO DEMONSTRATE OTHER BACTERIAL INHIBITIONS.

When materials like saliva or sputum are plated it is not uncommon to see, where the implant is thick, an almost pure culture of streptococci and

pneumococci, and where the implant is thinner and the streptococcal colonies are more widely separated, other colonies appear, especially those of Gram-negative cocci. These Gram-negative cocci are inhibited by the streptococci (probably by the peroxide they produce in their growth) and it is only when the mass effect of the streptococci is reduced that they appear in the culture.

Penicillin may be used to give a striking demonstration of this inhibition of bacteria by streptococci and pneumococci. Sputum is spread thickly on a culture plate, and then 5 or 6 drops of penicillin is spread over one half of it. After incubation it may be seen that on the half untreated with penicillin there is a confluent growth of streptococci and pneumococci and nothing else, while on the penicillin-treated half many Gram-negative cocci appear which were inhibited by the streptococci and pneumococci, and can only flourish when these are themselves inhibited by the penicillin.

If some active penicillin is embedded in a streak across an agar plate planted with saliva an interesting growth sometimes results. On the portion most distal from the penicillin there are many streptococci, but these are obscured by coarsely growing cocci, so that the resultant growth is a copious confluent rough mass. These coarse growing cocci are extremely penicillin sensitive and stop growing about 25 mm. from the embedded penicillin. Then there is a zone of about 1 cm. wide of pure streptococci, then they are inhibited by the penicillin, and as soon as that happens Gram-negative cocci appear and grow right up to the embedded penicillin. The three zones of growth produced in this way are very striking.

USE OF PENICILLIN IN THE ISOLATION OF *B. INFLUENZÆ* (PFEIFFER) AND OTHER ORGANISMS.

It sometimes happens that in the human body a pathogenic microbe may be difficult to isolate because it occurs in association with others which grow more profusely and which mask it. If in such a case the first microbe is insensitive to penicillin and the obscuring microbes are sensitive, then by the use of this substance these latter can be inhibited while the former are allowed to develop normally. Such an example occurs in the body, certainly with *B. influenzæ* (Pfeiffer) and probably with Bordet's whooping-cough bacillus and other organisms. Pfeiffer's bacillus, occurring as it does in the respiratory tract, is usually associated with streptococci, pneumococci, staphylococci and Gram-negative cocci. All of these, with the exception of some of the Gram-negative cocci, are highly sensitive to penicillin and by the addition of some of this to the medium they can be completely inhibited while *B. influenzæ* is unaffected. A definite quantity of the penicillin may be incorporated with the molten culture medium before the plates are made, but an easier and very satisfactory method is to spread the infected material, sputum, nasal mucus, etc., on the plate in the usual way and then over one half of the plate spread 2 to 6 drops (according to potency) of the penicillin. This small amount of fluid soaks into the agar and after cultivation for 24 hours it will be found that the half of the plate without the penicillin will show the normal growth while on the penicillin treated half there will be nothing but *B. influenzæ* with Gram-negative cocci and occasionally some other microbe. This makes it infinitely easier to isolate these penicillin-insensitive organisms, and repeatedly

B. influenzæ has been isolated in this way when they have not been seen in films of sputum and when it has not been possible to detect them in plates not treated with penicillin. Of course if this method is adopted then a medium favourable for the growth of *B. influenzæ* must be used, e.g. boiled blood agar, as by the repression of the pneumococci and the staphylococci the symbiotic effect of these, so familiar in cultures of sputum on blood agar, is lost and if blood agar alone is used the colonies of *B. influenzæ* may be so minute as to be easily missed.

Figs. 3 and 4 are photographs of culture-plates made after the method described above. On the plate shown in Fig. 3 a mixture of staphylococci and *B. influenzæ* was spread over the whole plate of Fildes medium (Fildes, 1921), then 6 drops of penicillin were spread over the lower half of the plate. The upper half shows the mixed culture while the lower half gives a pure culture of *B. influenzæ*. Fig. 4 represents a culture of nasal mucus from a "cold" made on the same medium. Here, on the upper half (untreated with penicillin) staphylococci and diphtheroid bacilli grow abundantly, while on the treated (lower) half only some three or four colonies of *B. influenzæ* appear.

In conjunction with my colleague, Dr. McLean, a series of cultures were made from the throats of 25 nurses warded for "influenza." The swabs were planted on boiled blood agar and over half of each plate was spread 3 or 4 drops of penicillin. The results are set forth in Table IV.

TABLE IV.—*Summary of Results obtained from Post-Nasal Swabs in 25 Consecutive Cases of "Influenza."*

	Without penicillin.			With penicillin.				Without penicillin.			With penicillin.		
	Pneumococcus or Streptococcus.	*B. influenzæ*	Gram-negative cocci.	Pneumococcus or Streptococcus.	*B. influenzæ*.	Gram-negative cocci.		Pneumococcus or Streptococcus.	*B. influenzæ*.	Gram-negative cocci.	Pneumococcus or Streptococcus.	*B. influenzæ*.	Gram-negative cocci.
1.	+ +	+	+	−	+ +	+	14.	+ +	−	−	−	+	−
2.	+ +	+ +	+ +	−	+ +	+	15.	+ +	−	−	−	+	+
3.	+ +	+ +	+	−	+	−	16.	+ +	−	−	−	+	+
4.	+	−	−	−	+	+	17.	+ +	−	−	−	+	+
5.	+ +	−	−	−	+ +	−	18.	+ +	−	−	−	+	+
6.	+ +	−	−	−	+ +	+ +	19.	+ +	+	−	−	+	+ +
7.	+ +	−	+ +	−	+	+	20.	+ +	−	−	−	−	+
8.	+	+	−	−	+	−	21.	+ +	−	−	−	+	+
9.	+ +	−	−	−	+	+	22.	+ +	−	−	−	+	−
10.	+ +	−	−	−	+	−	23.	+	−	+	−	+	+
11.	+ +	−	−	−	+	−	24.	+ +	+ +	−	−	+ +	−
12.	+ +	+	+ +	−	+	+	25.	+ +	−	+ +	−	−	−
13.	+	+ +	+ +	−	+	+ +							

In the above Table account has only been taken of the common microbes found in these cultures. In some there were a few diphtheroid bacilli which were always penicillin sensitive, and in others there were Gram-negative bacilli which were penicillin insensitive, although they were inhibited by streptococci or pneumococci. Pneumococci and streptococci were classed together, as complete tests were not made to differentiate one from the other.

(From the appearance of the colonies and the morphological characters pneumococci were evidently present in most cases in much larger numbers than were streptococci.)

The swabs were generally planted thickly and in some cases where the growth on the portion of the plate without penicillin was almost confluent, the cultures were sampled by taking smears from thick portions of the growth. In these cases it is possible that the results given do not give a quite complete picture of the cultures. This, however, does not affect the present argument that by the addition of penicillin to the culture medium, and the consequent inhibition of the pyogenic cocci, the isolation of *B. influenzæ* is very much easier. And in a number of cases it was isolated when it was completely missed in the cultures without penicillin.

It is quite immaterial how many pneumococci and streptococci are present in a specimen—they are completely inhibited—and even a few *B. influenzæ* can be isolated from a mixture with an enormous number of these cocci.

From a number of observations which have been made on sputum, post-nasal and throat swabs it seems likely that by the use of penicillin, organisms of the *B. influenzæ* group will be isolated from a great variety of pathological conditions as well as from individuals who are apparently healthy.

DISCUSSION.

It has been demonstrated that a species of penicillium produces in culture a very powerful antibacterial substance which affects different bacteria in different degrees. Speaking generally it may be said that the least sensitive bacteria are the Gram-negative bacilli, and the most susceptible are the pyogenic cocci. Inhibitory substances have been described in old cultures of many organisms; generally the inhibition is more or less specific to the microbe which has been used for the culture, and the inhibitory substances are seldom strong enough to withstand even slight dilution with fresh nutrient material. Penicillin is not inhibitory to the original penicillium used in its preparation.

Emmerich and other workers have shown that old cultures of *B. pyocyaneus* acquire a marked bacteriolytic power. The bacteriolytic agent, pyocyanase, possesses properties similar to penicillin in that its heat resistance is the same and it exists in the filtrate of a fluid culture. It resembles penicillin also in that it acts only on certain microbes. It differs however in being relatively extremely weak in its action and in acting on quite different types of bacteria. The bacilli of anthrax, diphtheria, cholera and typhoid are those most sensitive to pyocyanase, while the pyogenic cocci are unaffected, but the percentages of pyocyaneus filtrate necessary for the inhibition of these organisms was 40, 33, 40 and 60 respectively (Bocchia, 1909). This degree of inhibition is hardly comparable with 0·2% or less of penicillin which is necessary to completely inhibit the pyogenic cocci or the 1% necessary for *B. diphtheriæ*.

Penicillin, in regard to infections with sensitive microbes, appears to have some advantages over the well-known chemical antiseptics. A good sample will completely inhibit staphylococci, *Streptococcus pyogenes* and pneumococcus in a dilution of 1 in 800. It is therefore a more powerful inhibitory agent than

is carbolic acid and it can be applied to an infected surface undiluted as it is non-irritant and non-toxic. If applied, therefore, on a dressing, it will still be effective even when diluted 800 times which is more than can be said of the chemical antiseptics in use. Experiments in connection with its value in the treatment of pyogenic infections are in progress.

In addition to its possible use in the treatment of bacterial infections penicillin is certainly useful to the bacteriologist for its power of inhibiting unwanted microbes in bacterial cultures so that penicillin insensitive bacteria can readily be isolated. A notable instance of this is the very easy isolation of Pfeiffers bacillus of influenza when penicillin is used.

In conclusion my thanks are due to my colleagues, Mr. Ridley and Mr. Craddock, for their help in carrying out some of the experiments described in this paper, and to our mycologist, Mr. la Touche, for his suggestions as to the identity of the penicillium.

SUMMARY.

1. A certain type of penicillium produces in culture a powerful antibacterial substance. The antibacterial power of the culture reaches its maximum in about 7 days at 20° C. and after 10 days diminishes until it has almost disappeared in 4 weeks.

2. The best medium found for the production of the antibacterial substance has been ordinary nutrient broth.

3. The active agent is readily filterable and the name " penicillin " has been given to filtrates of broth cultures of the mould.

4. Penicillin loses most of its power after 10 to 14 days at room temperature but can be preserved longer by neutralization.

5. The active agent is not destroyed by boiling for a few minutes but in alkaline solution boiling for 1 hour markedly reduces the power. Autoclaving for 20 minutes at 115° C. practically destroys it. It is soluble in alcohol but insoluble in ether or chloroform.

6. The action is very marked on the pyogenic cocci and the diphtheria group of bacilli. Many bacteria are quite insensitive, *e.g.* the coli-typhoid group, the influenza-bacillus group, and the enterococcus.

7. Penicillin is non-toxic to animals in enormous doses and is non-irritant. It doses not interfere with leucocytic function to a greater degree than does ordinary broth.

8. It is suggested that it may be an efficient antiseptic for application to, or injection into, areas infected with penicillin-sensitive microbes.

9. The use of penicillin on culture plates renders obvious many bacterial inhibitions which are not very evident in ordinary cultures.

10. Its value as an aid to the isolation of *B. influenzæ* has been demonstrated.

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PENICILLIN AS A CHEMOTHERAPEUTIC AGENT

BY

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IN recent years interest in chemotherapeutic effects has been almost exclusively focused on the sulphonamides and their derivatives. There are, however, other possibilities, notably those connected with naturally occurring substances. It has been known for a long time that a number of bacteria and moulds inhibit the growth of pathogenic micro-organisms. Little, however, has been done to purify or to determine the properties of any of these substances. The antibacterial substances produced by *Pseudomonas pyocyanea* have been investigated in some detail, but without the isolation of any purified product of therapeutic value.

Recently, Dubos and collaborators (1939, 1940) have published interesting studies on the acquired bacterial antagonism of a soil bacterium which have led to the isolation from its culture medium of bactericidal substances active against a number of gram-positive micro-organisms.[1] Pneumococcal infections in mice were successfully treated with one of these substances, which, however, proved to be highly toxic to mice (Hotchkiss and Dubos 1940) and dogs (McLeod et al. 1940).

Following the work on lysozyme in this laboratory it occurred to two of us (E. C. and H. W. F.) that it would be profitable to conduct a systematic investigation of the chemical and biological properties of the antibacterial

1. See *Lancet*, 1940, 1, 1172.

RESULTS OF THERAPEUTIC TESTS ON MICE INFECTED WITH *Strep. pyogenes*, *Staph. aureus* AND *Cl. septique*

Expt.	—	Dose of infecting culture (c.cm.)	Interval before starting treatment (hrs.)	Duration of treatment	Single dose (mg.)	Total dose (mg.)	No. of mice	Survivors at end of— hours 6	12	24	days 2	3	4	5	6	7	8	9	10
Strep. pyogenes Lancefield, Gp. A.																			
1	Controls	0·5	..	..	..	..	25	..	15	9	8	6	..	5	..	4	..	..	4[1]
	Treated	0·5	1	12 hrs.	2	10·0	50	..	..	..	49	42	..	34	30	28	..	26	25
2	Controls	0·5[2]	..	..	..	..	25	24	3	0[3]	..	..	..	..	..	..	..	..	0
	Treated	0·5	2	45 hrs.	0·5	7·5	25	24	..	..	..	..	..	..	..	..	..	..	24
Staph. aureus[4]																			
1	Controls	1·0	..	..	..	..	24	21	1	0[5]	..	..	..	..	..	..	..	..	0
	Treated	1·0	1	55 hrs.	0·5	9·0	25	25	12	..	11	..	10	..	..	..	..	..	8
2	Controls	0·2[2]	..	..	..	..	24	23	15	5	0	..	..	..	..	..	..	..	0
	Treated	0·2	1	4 days	0·5	11·5	24	24	23	22	..	..	21	..	..	..	..	..	21
Cl. septique																			
1	Controls	see text	..	..	..	..	25	..	21	0[6]	..	..	..	..	..	..	..	..	0
	Treated	..	1	10 days	0·5	19	25	..	..	24	21	..	18	..	..	..	..	..	18
		..	1	10 ,,	1·0	38	25	..	..	..	..	..	24	..	..	..	..	..	24

1. A control mouse which was killed by mistake at 24 hrs. is counted as a survivor. Heart-blood culture strongly positive.
2. Between experiments 1 and 2 the virulence of the organism was raised by passage.
3. Controls all dead within 16 hrs.
4. A bovine strain exceptionally virulent to mice kindly supplied by Dr. H. J. Parish of the Wellcome Laboratories.
5. Controls all dead within 17 hrs.

substances produced by bacteria and moulds. This investigation was begun with a study of a substance with promising antibacterial properties, produced by a mould and described by Fleming (1929). The present preliminary report is the result of a coöperative investigation on the chemical, pharmacological and chemotherapeutic properties of this substance.

Fleming noted that a mould produced a substance which inhibited the growth, in particular, of staphylococci, streptococci, gonococci, meningococci and *Corynebacterium diphtheriæ*, but not of *Bacillus coli*, *Hæmophilus influenzæ*, *Salmonella typhi*, *P. pyocyanea*, *Bacillus proteus* or *Vibrio choleræ*. He suggested its use as an inhibitor in the isolation of certain types of bacteria, especially *H. influenzæ*. He also noted that the injection into animals of broth containing the substance, which he called "penicillin," was no more toxic than plain broth, and he suggested that the substance might be a useful antiseptic for application to infected wounds. The mould is believed to be closely related to *Penicillium notatum*. Clutterbuck, Lovell and Raistrick (1932) grew the mould in a medium containing inorganic salts only and isolated a pigment—chrysogenin—which had no antibacterial action. Their culture media contained penicillin but this was not isolated. Reid (1935) reported work on the inhibitory substance produced by Fleming's mould. He did not isolate it but noted some of its properties.

During the last year methods have been devised here for obtaining a considerable yield of penicillin, and for rapid assay of its inhibitory power. From the culture medium a brown powder has been obtained which is freely soluble in water. It and its solution are stable for a considerable time and though it is not a pure substance, its anti-bacterial activity is very great. Full details will, it is hoped, be published later.

EFFECTS ON NORMAL ANIMALS

Various tests were done on mice, rats and cats. There is some œdema at the site of subcutaneous injection of strong solutions (e.g. 10 mg. in 0·3 c.cm.). This may well be due to the hypertonicity of the solution. No sloughing of skin or suggestion of serious damage has ever been encountered even with the strongest solutions or after repeated injections into the same area.

Intravenous injections showed that the penicillin preparation was only slightly, if at all, toxic for mice. An intravenous injection of as much as 10 mg. (dissolved in 0·3 c.cm. distilled water) of the preparation we have used for the curative experiments did not produce any observable toxic reactions in a 23 g. mouse. It was subsequently found that 10 mg. of a preparation having twice the penicillin content of the above was apparently innocuous to a 20 g. mouse.

Subcutaneous injections of 10 mg. into two rats at 3-hourly intervals for 56 hours did not cause any obvious change in their behaviour. They were perhaps slightly less lively than normal rats but they continued to eat their food. Their blood showed a fall of total leucocytes after 24 hours, but after 48 hours the count had risen again to about the original total. There was, however, a relative decrease in the number of polymorphs, but the normal number was restored 24 hours after stopping the administration of the substance. One of these two rats was killed for histological examination; there was some evidence that the tubule cells of the kidney were damaged. The other has remained perfectly well, and its weight increased from 76 to 110 g. in 23 days. It is to be noted that these rats received, weight for weight, about five times the dose of penicillin used in the curative experiments in mice. No evidence of toxic effects was obtained from the treated mice, which received penicillin for many days.

Other pharmacological effects.—On the blood-pressure, heart-beat and respiration of cats no effects have been observed after intravenous injection of 40 mg.—enough to bring up the concentration in the blood just after injection to 1/5000. *Perfusion* of the isolated cat's heart, with Ringer-Locke solution containing 1/5000 penicillin produced progressive slowing during 15 minutes and at the end of that time the heart looked as though it would stop beating; however, it was quickly revived by perfusing with Ringer-Locke solution alone. The same depressant action was seen at 1 10,000 dilution but the effect was less than at 1 5000. Solutions are absorbed from the intestine in the rat without causing any observable damage to the mucosa. They are also readily absorbed after subcutaneous injection and the substance can be detected in the blood. It is excreted by the kidneys, the urine becoming bright yellow. At least 40–50% appears in the urine in a still active form. *Human leucocytes* remain active in a 1 1000 solution for at least 3 hours.

It must be emphasised that the results of these preliminary tests have been obtained with an impure

substance and such slight toxic effects as have been noted may possibly be due, in part at least, to these impurities.

EFFECTS ON BACTERIA IN VITRO

In view of this slight evidence of tissue toxicity it is all the more striking that the substance in a dilution of one in several hundred thousand inhibits in vitro the growth of many micro-organisms, including anaerobes. Of those so far tested in this laboratory the following are sensitive to the inhibiting action of our preparation: *Clostridium welchii* (2 strains); *Cl. septique* (1 strain, Nat. coll. type-cultures No. 458); *Cl. œdematiens* (1 strain, N.C.T.C. No. 277); *C. diphtheriæ* (1 strain, mitis type); *Streptococcus pyogenes* (Lancefield group A); *Str. viridans* (1 strain from tooth); *Str. pneumoniæ* (type 8); staphylococci (3 strains). Penicillin is not immediately bactericidal but seems to interfere with multiplication.

THERAPEUTIC EFFECTS

From all the above tests it was clear that this substance possessed qualities which made it suitable for trial as a chemotherapeutic agent. Therapeutic tests were therefore done on mice infected with streptococci, staphylococci and *Cl. septique*; the results are summarised in the accompanying table, the preliminary trials on small numbers of mice being omitted.

Bacteriological methods: streptococcus and staphylococcus.—The staphylococcus was kept in culture in meat extract broth and the streptococcus in the same with serum. Both organisms were repeatedly passed through mice and were used for experiment 2–4 days after the last passage. The experimental infection was induced with 20–24 hour broth cultures injected intraperitoneally. According to opacity measurements with Brown's tubes, doses of 450 and 350 million cocci, living and dead, were given respectively in the two streptococcal experiments, and doses of 760 and 200 million in the staphylococcal experiments.

Pathogenic anaerobes.—The therapeutic effects were tried in mice infected with spores of *Cl. septique* in the manner described by Henderson and Gorer (1940). Attempts to carry out similar tests with *Cl. welchii* were temporarily abandoned owing to the difficulty of establishing in mice a type of infection which is both certainly fatal and allows adequate time before death for treatment to take effect. A spore suspension of *Cl. septique* was made by anaerobic growth at 37° C. for 48 hours on Dorset's egg slopes, suspension of the growth of each slope (surface about 15 sq. cm.) in about 1 c.cm. of sterile distilled water, centrifugalisation, once washing with distilled water, recentrifugalisation and resuspension in the original volume of distilled water. The suspension was then heated to 75° C. for one hour. The virulence of the suspension, when injected with an equal volume of 5% calcium chloride was roughly titrated by injection into the thigh muscle of mice, and a dose was chosen for the therapeutic experiment such as would kill for certain without being unnecessarily severe, viz., 0·05 c.cm. of the suspension diluted one in three, mixed with 0·05 c.cm. of the calcium chloride solution. This was injected into the thigh muscles of the 75 mice whose subsequent fate is recorded in the table.

Treatment.—The general principle has been to keep up an inhibitory concentration of the substance in the tissues of the body throughout the period of treatment by repeated subcutaneous injections. No extended tests have been made to determine the minimum effective quantities or the longest intervals possible between injections. The doses employed have been effective and not toxic; they may have been excessive. The solution contained 10 mg. per c.cm. of substance.

In the first streptococcal experiment the treatment was continued for 12 hours only. That this was inadequate was shown by deaths occurring during the arbitrarily chosen 10-day period. In the second experiment the time of treatment was lengthened, with improved results. In this and the two staphylococcal experiments injections were given 3-hourly for the first 32–37 hours, then at longer intervals.

In preliminary experiments with *Cl. septique* it was found that the infection could be satisfactorily held in check so long as penicillin was being given (i.e., for 2 days) but when the administration was stopped the infection developed. In the experiment quoted, therefore, the injections were given for 10 days, 3-hourly for 41 hours, then at longer intervals, and twice daily for the last 2 days of the period. No deaths have subsequently occurred (22 days after beginning of experiment).

The behaviour of the mice infected with streptococci and staphylococci was interesting. For some hours after the start of treatment they looked sick—some even appeared to be dying—but as the experiment went on they progressively improved till at the end of 24 hours in the case of the streptococci and about 36 to 48 hours with the staphylococci it was difficult or impossible to distinguish them from normal mice. The survivors of the *Cl. septique* infection on the other hand remained well throughout, except for a few in which leg lesions appeared near the site of injection and cleared up in a few hours.

Summarising the data given in the table we see that in the final streptococcus experiment (no. 2) whereas 25/25 controls died, 24/25 treated animals survived. With *Staphylococcus aureus* the final experiment (no. 2) shows 24/24 deaths of the controls and 21/24 survivals among the treated. Lastly, with *Cl. septique* when the larger doses of penicillin were given (bottom line) the figures are 25/25 control deaths and 24/25 treatment survivals.

CONCLUSIONS

The results are clear cut, and show that penicillin is active in vivo against at least three of the organisms inhibited in vitro. It would seem a reasonable hope that all organisms inhibited in high dilution in vitro will be found to be dealt with in vivo. Penicillin does not appear to be related to any chemotherapeutic substance at present in use and is particularly remarkable for its activity against the anaerobic organisms associated with gas gangrene.

In addition to the facilities provided by the university we have had financial assistance from the Rockefeller Foundation, the Medical Research Council and the Nuffield Trust. N. G. Heatley has held a Rockefeller fellowship. To all of these we wish to express our thanks. We also wish to acknowledge the technical assistance of D. S. Callow, G. Glister, S. A. Creswell, J. A. Kent, and E. Vincent.

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LETTERS TO THE EDITORS

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IN THE PRESENT CIRCUMSTANCES, PROOFS OF "LETTERS" WILL NOT BE SUBMITTED TO CORRESPONDENTS OUTSIDE GREAT BRITAIN.

An Enzyme from Bacteria able to Destroy Penicillin

FLEMING[1] noted that the growth of *B. coli* and a number of other bacteria belonging to the coli-typhoid group was not inhibited by penicillin. This observation has been confirmed. Further work has been done to find the cause of the resistance of these organisms to the action of penicillin.

An extract of *B. coli* was made by crushing a suspension of the organisms in the bacterial crushing mill of Booth and Green[2]. This extract was found to contain a substance destroying the growth-inhibiting property of penicillin. The destruction took place on incubating the penicillin preparation with the bacterial extract at 37°, or at room temperature for a longer time. The following is a typical experiment showing the penicillin-destroying effect of *B. coli* extracts. A solution of 1 mgm. penicillin in 0·8 c.c. of water was incubated with 0·2 c.c. of centrifuged and dialysed bacterial extract at 37° for 3 hours, in the presence of ether, and a control solution of penicillin of equal concentration was incubated without enzyme for the same time. (The penicillin used was extracted from cultures of *Penicillium notatum* by a method to be described in detail later. It possessed a degree of purity similar to that of the samples used in the chemotherapeutic experiments recorded in a preliminary report[3].) The growth-inhibiting activity of the solutions was then tested quantitatively on agar plates against *Staphylococcus aureus*. The penicillin solution incubated with the enzyme had entirely lost its growth-inhibiting activity, whereas the control solution had retained its full strength.

The conclusion that the active substance is an enzyme is drawn from the fact that it is destroyed by heating at 90° for 5 minutes and by incubation with papain activated with potassium cyanide at *p*H 6, and that it is non-dialysable through 'Cellophane' membranes. It can be precipitated by 2 volumes of alcohol, but much of its activity is lost during this operation. The activity of the enzyme, which we term penicillinase, is slight at *p*H 5, but increases considerably towards the alkaline range of *p*H. It is very active at *p*H 8 and 9. Higher *p*H's could not be tested as penicillin is unstable above *p*H 9.

The mechanism of the enzymatic inactivation of penicillin is being studied. No oxygen uptake occurs during the reaction, and the inactivation proceeds with equal facility under aerobic and anaerobic conditions. No appearance of acid groups could be detected by *p*H measurement with the hydrogen electrode. Extracts of a number of other micro-organisms, made by crushing the bacteria in the bacterial grinding mill, were tested for penicillinase. The enzyme was absent from extracts of the penicillin-sensitive *Staphylococcus aureus*, of yeast and of *Penicillium notatum*. It was present in a Gram-negative rod, insensitive to penicillin, found as a contaminant of some Penicillium cultures. Unlike *B. coli*, it was not necessary to crush the organism in the bacterial mill in order to obtain the enzyme from it; the latter appeared in the culture fluid. The enzyme was also found in *M. lysodeikticus*, an organism sensitive to the action of penicillin, though less so than *Staphylococcus aureus*. Thus, the presence or absence of the enzyme in a bacterium may not be the sole factor determining its insensitivity or sensitivity to penicillin.

The tissue extracts and tissue autolysates that have been tested were found to be without action on the growth-inhibiting power of penicillin. Prof. A. D. Gardner has found staphylococcal pus to be devoid of inhibiting action, but has demonstrated a slight inhibition by the pus from a case of *B. coli* cystitis. The bacteriostatic action of the sulphonamide drugs is known to be inhibited in the presence of tissue constituents and pus.[4] That the anti-bacterial activity of penicillin is not affected under these conditions gives this substance a definite advantage over the sulphonamide drugs from the chemotherapeutic point of view. The fact that a number of bacteria contain an enzyme acting on penicillin points to the possibility that this substance may have a function in their metabolism.

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Dec. 5.

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