FURTHER OBSERVATIONS ON PENICILLIN

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THE work on penicillin briefly reported by Chain and others (1940) is here presented in greater detail, and its further development to the stage of human therapy is described.

Growth of Penicillin-producing Mould

The mould will grow and produce penicillin on a variety of different media, but that used by Clutterbuck, Lovell and Raistrick (1932) is easy to prepare and gives as high a yield of penicillin as others containing peptone, horse-muscle digests, &c. This modified Clapek-Döth medium consists of NaNO₃ 3%, KH₂PO₄ 1 g., KCl 0·5 g., MgSO₄·7H₂O 0·5 g., FeSO₄·7H₂O 0·01 g., glucose 40 g., with water to 1 litre. Oxford tap-water has proved as good as distilled water for this purpose. Yeast-extract has usually been added to speed up the growth of the mould (details later).

The medium, sterilised by autoclaving, is sown with a spore suspension made by shaking up sterile water in a screw-capped bottle containing a slope on which the mould has grown and spored freely. Twenty-four hours after sowing a very delicate fluffy gauze-like growth can be seen, which is at its best focus of the vessel (at 24° C.). The growth becomes more voluminous during the next day and on the 3rd day, if the liquid layer is not more than 1 cm. thick, this reaches the surface of the medium and is visible. Usually around the centre of the vessel are a few tiny foci of white mycelium, but this rapidly spreads outwards and by the 6th or 7th day the growth consists of a continuous, compact, often corrugated, dark greenish-blue felt whose upper surface cannot be wetted by water. The surface is of course freely wetted, and is brownish yellow and slimy. If on the 1st or 2nd day the culture-vessel is disturbed or rocked the gauze-like growth tends to conglomeration into ropes and balls, and those parts of the surface of the medium which do not lie over these will be covered several days later by secondary growths; for this reason once they have been sown the vessels should not be touched for some days. Droplets, usually but not invariably yellow, may or may not appear on the surface of the mould, but the mould is never wetted by them. As incubation is prolonged the colour of the mycelium becomes more faded and grey.

The changes in the appearance of the mould are accompanied by changes in the pH of the medium, which begins between 6 and 7 and may fall as low as 3 by the 5th day. On adjusting the pH to about 6·5 development ceases. On adjusting the pH to about 6·5 development ceases. On adjusting the pH to about 6·5 development ceases.

The serial dilution method used by Fleming (1929), _A. B. C._

of development may be greater or less than that described, depending largely on the depth of the medium. A systematic study of the factors influencing penicillin-production was begun, but it could not be completed owing to the very numerous and often interdependent variables, and to the fact that the assay-method then in use could only detect large differences of titre. The following conclusions, however, could be drawn:

1. Penicillin production seems to take place over a wide range of oxygen tension. (The mould will not grow anaerobically.)

2. The mould grows satisfactorily at 24°C. At lower temperatures growth is delayed and as harvesting of the medium is carried out in the incubator higher temperatures have not been studied, 24°C. being about the upper limit of growth. Fleming (1929) in his original description stated that the mould would not grow at 37°C. and this has been confirmed.

3. Crude attempts to change the pH of the medium or to maintain it at a constant value have not resulted in a noticeable increase in yield of penicillin, nor has the incorporation of ten times the normal amount of phosphate buffer.

4. The medium should not have a depth greater than 1–2 cm. If deeper than 2 cm. diffusion is visibly inadequate, for two distinct layers can be seen in it, the upper being yellow, the lower colourless.

5. When the medium is fit to be harvested it can be drawn off from under the mycelium and replaced with fresh medium in which more penicillin will form in about half the time required for the initial production. The medium can be changed several times in this way; with one batch it was changed 14 times.

6. The mould must be grown and the medium harvested and replaced under strictly sterile conditions since penicillin is destroyed by certain bacteria (Abraham and Chain 1940).

7. The addition of yeast-extract (Gladstone and Edles 1940) accelerates the growth of the mould but does not affect the yield of penicillin. In large-scale growth we have always added yeast-extract; in starting a batch the medium is made up to contain 10% of it, but the medium used for replacement cultures only 2%. The lowest effective temperature of the yeast is not impaired by prolonged autoclaving at a high temperature. Marmite or malt extract have no effect on the rate of growth or on the yield of penicillin.

8. The yield is not greatly affected either by doubling or by halving the strength of the medium.

9. If the sodium nitrate is replaced by ammonium lactate the pH of the medium falls to below 3 and development almost ceases. On adjusting the pH to about 6·5 development proceeds and the medium ultimately becomes very alkaline. Yet penicillin production remains little affected. The sodium nitrate can be wholly or partly replaced by peptone or by peptic and tryptic digests of muscle, and sucrone or maltose can be used in place of glucose, without materially altering the yield of penicillin.

10. Occasionally a batch produces little or no penicillin. Although this can generally be traced to bacterial contamination in some instances contamination could not be proved, and the suspicion arose that penicillin production might be a variable and easily disturbed function of the mould. To test this, pure-line cultures were made from single cells of two batches, one of which had given a yield of penicillin and the other none. Five pure lines of the former and nine of the latter were inoculated in triplicate into bottles of medium; only the former yielded penicillin. The difference could be detected between any of the lines, proving that the mould had lost its power to form penicillin. The possibility that the failure had been due to contamination by another mould was investigated, but no evidence of this could be found.

Method of Assay

The serial dilution method used by Fleming (1929), which measures the lowest concentration of penicillin that will prevent growth of the test-organism in broth, is laborious and can only be applied to sterile material. This at once reduces its usefulness for chemical investigations, for the material to be tested would have to be sterilised by filtration and it seems that under some conditions penicillin may be partially adsorbed on Seitz filter pads. The method of estimation finally adopted is
as follows. Ordinary nutrient agar plates are seeded with the test organism—Staphylococcus aureus has been used in this work—by allowing a broth culture of the organism to flow over the surface of the agar and draining off the excess broth. The plates are then dried for an hour in the incubator at 37° C. in a special rack which supports the lid of the Petri dish half an inch above the lower part. When dry the seeded plates are removed from the rack and the Petri dishes are then placed in a thermostat at 37° C. for one or two days. Cylinders made from short lengths of glass tubing, the dimensions of which will be seen from the inset of fig. 1, are placed on the agar. The lower edge of the cylinder is carefully ground level and has an internal bevel so that the thin edge tends to sink into the agar and make a water- and bacteria-tight seal. This is a convenient way in which they can be stacked for autoclaving, sowing and so on; each plug is well separated from the other but no bench space is lost, and should the medium boil in the autoclave the plugs are unlikely to be wetted. One litre of medium containing 10% yeast-extract is well sterilised at 17° C. When a batch of vessels is first set up the medium (containing 10% of yeast-extract) is sterilised in the vessels, which are then inoculated with a few drops of a spore-suspension and incubated at 24° C. Apart from order to hasten the production of penicillin we attempted in order to hasten the production of penicillin we attempted to reduce these precautions to a minimum, and at first obtained satisfactory results; but lately contamination has become so frequent that substantial modifications have had to be introduced, some of which are still on trial and therefore cannot usefully be described.

The withdrawal of culture-medium is done by suction, the medium being replaced with sterile air. In the ordinary method the refilling was done by simple pouring, but safer methods (not yet finally determined) have now been introduced, and precautions at all stages are being tightened up.

The operations can conveniently be carried out on the "changing-trolley" (fig. 3), made by replacing the top of a small all-metal table by a wooden plank A, to which are hinged the four wooden slabs B on which the vessels are placed. The raised rim C is prolonged into a handle D, by which the slabs (and the vessel on it) can be tilted forward. The slabs are not hinged square with the edge of A, but at a slight angle to it, so that on depressing the handle the fluid in the vessel drains not merely to the front edge but to the corner nearest the spout. The medium is drawn off by suction with the help of a special pistol-shaped holder E, carrying two detachable sterilised tubes F and G. F is plugged with cotton-wool before sterilising, and through it is blown into the vessel a current of (sterile) air at a somewhat faster rate than

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1. Made for us by J. Macintyre and Co., who also made the porcelain assay cylinders.

2. The strains of penicillium used in this work have been obtained from Prof. Alexander Fleming of St. Mary's Hospital, London.
the fluid is drawn out by tube G. Suction and pressure are controlled by the single trigger H of the pistol. A fresh tube is used for every batch of vessels and F is changed after every two batches. Both tubes are sterilised wrapped in paper and only one end of the tube is unwrapped when being fitted into the pistol: the tubes are unsheathed from the remaining paper wrappings immediately before use. From the pistol the harvested medium passes to the inverted bottle K, in which it is filtered through a silk bag. The filtered liquid is passed to the collecting bottle L which is changed after every batch of four vessels. When not in use the pistol is hung on the pin M. For refilling the vessels are tilted at about 45°. A fitting on each wooden slab enables the vessel to be wedged in this position (as shown by vessel N in fig. 3), so that both hands are free to manipulate plug and bottle of medium.

**Extraction from culture medium**

**Principle.**—Penicillin can be extracted by ether, amyl acetate and certain other organic solvents from an aqueous solution whose pH has been adjusted to 2. From the organic solvent the penicillin may be recovered by shaking with phosphate-buffer or with water the pH of which is kept at 6-7. Penicillin is quickly destroyed at pH 2 at room temperature, so the first extraction must be carried out rapidly or at a low temperature. Once it has been extracted into solvent the penicillin is stable for some days.

**Laboratory large-scale extraction.**—It was planned to produce a weekly output of about 500 litres of the crude medium containing penicillin, the working up of which by hand would obviously have been out of the question. A continuous countercurrent extraction apparatus was devised, an adequate description of which cannot be given in this paper. The crude penicillin, having been filtered and acidified, is passed through special jets which have it up into droplets of water. These are allowed to fall through a column of amyl acetate, to which the penicillin is given up. The crude solution is acidified immediately before entering the jets, so that the aqueous solution is at a pH at which penicillin is unstable. For only a few seconds before it has been extracted. As the crude solution is passed through a cooling coil surrounded by circulating tap-water probably very little destruction of penicillin takes place. Phosphoric acid is used for the acidification as the pH of the first stage of dissociation is approximately 2; it will therefore act as its own buffer and inaccurate correlation of the rates of flow of the acid and of the crude solution will not cause big fluctuations in the final pH. Fresh solvent is continuously fed in to the bottom of the column, from the top of which an equal amount of penicillin-rich solvent is collected for further working up. This solvent has 1 / 10 - 1/ 8 of the volume of the crude solution from which the penicillin has been extracted, and many impurities, notably those forming emulsions, have been eliminated.

The preparation used in the mouse-experiments previously reported (Chain et al. 1940) was purified only to the extent of removing the sodium salt from a first ether extract (ether was at that time used instead of amyl acetate). Though strongly active this product probably had less than 1 / 10 of the activity of our present "therapeutic penicillin." A batch intended for injection into man was therefore prepared on a smaller scale by the intravenous injection of 10 mg. in 0.3 c.cm. of water but became very ill after receiving 20 mg., though it recovered in about 1 ½ hours. When given intravenously to an afebrile human subject 100 mg. of the same material caused a fever lasting several hours with a rise in temperature of about 2 °F. in each rabbit, but "middle fraction" from the adsorption column (see below) had no pyrogenic effect. The further purification here described secures a pyrogen-free product suitable for intravenous use.

**Further purification.**—Batches of 3 litres of the penicillin-containing solvent as delivered from the extraction-apparatus are extracted with five successive amounts of 300 ml. each of water, using baryta to adjust the pH to 0.5-7. The five watery solutions have already become virtually free of unextracted organisms and are very nearly free of decreasing concentration of penicillin. The first extract, which is the strongest, is set aside for further working up, but a sixth extract is made with fresh water so that the number of watery solutions remains the same. The amyl acetate or ether extract of a small amount of penicillin, is recirculated in the extraction apparatus. In the course of time pigment accumulates in the solvent and is periodically removed by extraction with 1% sodium hydroxide followed by thorough washing.

The strongest aqueous extract is partially decolorised by shaking with about 8% of animal charcoal and filtering. The charcoal residue is washed twice, the washings being put through the extraction apparatus or used in other ways. The partially decolorised solution is cooled, acidified and extracted into successive amounts of ether: the strongest of the ether extracts is then passed through an adsorption column of Brockmann alumina. The spent ether contains solid matter but no penicillin, which remains behind in the column. Though the chromatograms vary, four main zones can be seen, their boundaries overlapping to some extent. These are, starting from the top:

1. A dark brownish-orange layer whose depth is inversely proportional to the amount of charcoal used for the decolorisation and which may be absent altogether. This layer contains some penicillin.
2. A light yellow layer containing most of the penicillin but none of the pyrogen.
3. An orange layer which contains some penicillin and some or all of the pyrogen.
4. A brownish or reddish-violet layer which contains practically no penicillin. The violet pigment disappears on exposure to light.

The column is washed by passing successive small amounts of ether through it and is then divided into the four fractions, of which the first is discarded. The others are eluted with successive amounts of M/15 phosphate buffer (pH 7.2), from the strongest of which the penicillin is again extracted into ether, while the more dilute solutions are used for the elutions of the next more dilute fractions. Finally is run through an adsorption column of baryta into water using sodium hydroxide to adjust the pH. (As the solution is not buffered the greatest care must be taken in adding the alkali, for penicillin is rapidly destroyed in alkaline solution.)

The "non-pyrogenic" and the "therapeutic" fraction, which contains perhaps 80% of the penicillin put through the column, is extracted into pyrogen-free water, all glassware having been rinsed with the latter. It is a deep reddish-orange fluid, yellow in dilute solution, with a faint but characteristic smell and a bitter taste.
Yields and losses.—The crude medium, as harvested, usually contains between 1 and 2 units per c.cm. and the dried purified therapeutic material has an activity of 40–50 units per mg. A hundred litres of the medium, containing, say, 150,000 units, gives a yield of about 1 g. of therapeutic material containing, say, 45,000 units, so that about a third of the penicillin present is actually extracted in a form suitable for intravenous injection. There are many stages in the preparation at which the loss may occur and as far as possible these have been checked individually, but owing to overlapping of different batches during the working up (e.g., in serial extraction) and insufficient accuracy in the assay method, it has been difficult to analyse the losses accurately. No big loss, however, occurs at any one stage.

Storage and dispensing.—The strong aqueous extract saturated with ether is quite stable. It is stored either as it is in the refrigerator, or is dried by the lyophilic method and kept in a desiccator, as the voluminous yellow powder is hygroscopic. A solution of the sodium salt of penicillin in water kept in the ice-chest for 3 months did not lose any activity. The barium salt kept dry at room temperature in the desiccator retained its activity for at least 8 months. In dispensing the material for intravenous injection it is assumed that the ether-containing solution is sterile, but most of the ether must be removed by suction before use. The ether-free solution is stored in the ice-chest in measured doses.

BACTERIOSTATIC ACTION OF PENICILLIN

Using purified penicillin more than a thousand times stronger than the crude material employed by Fleming in 1929, we have been able to obtain more precise information about the bacterial species to which penicillin is effective and about a number of other important pathogenic organisms.

Method.—To 4·5 c.cm. quantities of fluid culture medium 0·5 c.cm. of graded dilutions of a strong, filtered penicillin solution were added. Each tube of a series was then inoculated with a standard drop (0·04 c.cm.) of a 24 hours fluid culture of the microbe under investigation. The control culture showing good growth; partial inhibition was recorded when the growth was clearly less than that of the control. All tests were done in duplicate. Complete inhibition was shown by the absence of turbidity after 24 hours at 37° C., the control culture showing good growth; partial inhibition was recorded when the growth was clearly less than that of the control.

Results.—In table 1 the bacterial species are arranged as far as possible in their order of sensitivity, but both the order and the actual figures must be taken with reserve. As the work progressed the penicillin was continually becoming purer and stronger, so that some of the figures obtained in the early stages may well be too low. Moreover, there has sometimes seemed to be some loss in potency of penicillin solutions when sterilised by Seitz-filtration, a point that needs further investigation. Finally, only a few species have been tested in more than one medium and with a minimal inoculum, both of which factors influence the observed titre.

Certain species, such as pneumococci and Strep. viridans, cannot be put in a single place in the order of sensitiveness because they show great strain differences, and it will be noted that in the pneumococci these cut across the type distinctions. Maclean, Rogers and Fleming (1939) have recorded a similar variability in sensitiveness to sulphapyridine of individual strains of the pneumococcal types. It seems likely that an experimental comparison of the effects of the two inhibitory agents on a wide range of pneumococci would throw light on their modes of action.

The testing of Myc. tuberculosis was not easy. Owing to the imperfect stability of penicillin at 37° C. and the slow growth of the microbe it was inadmissible merely to inoculate the inhibitor with the pneumococcal type distinctions. The microbe was therefore cultivated in glycerol-broth, and penicillin solution was added every 2 days in such a way as to maintain at least the stated concentration for 14 days. The microbe grew well in all dilutions. A similar experiment had already been done by Dr. R. L. Vollum, with similar results.

It should be noted that complete, or even partial inhibition of microscopic growth after 24 hours of incubation is not the most delicate criterion of antibacterial action. In somewhat higher dilutions the growth is retarded, and, as has already been recorded (Gardner 1940), a microscopical effect on the bacteria, indicative of defective fission, may often be seen far beyond the macroscopically inhibitory dilution. We cannot say whether the virulence of the bacteria is thereby reduced; but if it is the effect may be of therapeutical importance. For example, in one experiment with S. typhi (Bact. typhosum), whereas the last visible, partial inhibition was at 1/10,000, an elongation of the cells was microscopically detectable only at 1/60,000, a concentration which may be a therapeutic possibility.

Adaptation of Staph. aureus to penicillin.—In order to find an answer to the questions whether bacteria will

<table>
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<th>Table I—Dilutions of penicillin at which various inhibitory effects have been observed</th>
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<td>Bacterial species</td>
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<tr>
<td>N. gonorrhoea</td>
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<td>N. meningitidis</td>
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<td>Strep. pyogenes</td>
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<td>B. anthracis</td>
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<td>A. bovis (hominis)</td>
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<td>Cl. tetani</td>
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<td>Cl. welchii</td>
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<td>Cl. septique</td>
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<td>Cl. edema nanceum</td>
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<tr>
<td>Pneumococcus</td>
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<td>P. aerogenes</td>
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<td>C. diptheria (mitis)</td>
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<td>S. garreri</td>
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<td>S. typhi</td>
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<td>Pneumococcus</td>
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<td>Proteus</td>
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<tr>
<td>Strep. viridans</td>
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<td>Paste. pestis</td>
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<td>S. pyogenes</td>
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<td>S. parathophii</td>
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<tr>
<td>Act. diphteriae Shiga</td>
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<tr>
<td>Br. abortus</td>
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<td>Br. melitensis</td>
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<tr>
<td>Anaerobic streptococcus</td>
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<td>Y. cholera</td>
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<tr>
<td>Strep. pyogenes</td>
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<tr>
<td>Bac. actinomycetem</td>
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<tr>
<td>Bac. pyocyanin</td>
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<tr>
<td>Bac. tuberculosi</td>
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<td>L. terebromarginis</td>
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* Another strain was only inhibited up to 32,000. † Grown in Lemo broch. In beef broch complete inhibition only reached 100,000. ‡ In Pneumococcus, Strep. viridans and anaerobic streptococcus different strains appear at different levels in the table.
acclimatise themselves to inhibitory concentrations of penicillin, and if so whether they do so by producing the penicillin-destroying enzyme penicillinase, which has been demonstrated in certain saprophytes and commensals (Abraham and Chain 1940), a strain of Staph. aureus was cultivated for some months in broth in the presence of increasing quantities of comparatively crude penicillin. Even after a few daily subcultures the culture was capable of producing a sufficient amount of bacterial bodies to be used in the assay of an amount of bacterial bodies to be used in the assay of penicillin-destroying enzyme by the organism.

In serial dilutions on Staph. aureus, complete inhibition of the parent strain was observed. However, after a further 7 weeks the microbe was able to multiply in a concentration of penicillin a thousand times greater than that which inhibited the parent strain in a parallel test—while the parent was almost completely inhibited by 1 in 1000. After due incubation the supernatant fluid was diluted 1 in 2 with nutrient broth and filtered through a Seitz filter. To 2-25 c.c.m. of this 50% pus-broth 0-25 c.c.m. of a solution of 0-2 mg. of (impure) penicillin per c.c.m. was added, making a dilution of penicillin of 1/50,000. As a control, 2-3 c.c.m. of the pus broth with no added penicillin was inoculated with one standard drop (0-04 c.c.m.) of a 1/100 dilution of a 24-hour broth culture of Staph. aureus. The number of cocci in the inoculum was probably of the order of 40,000. After 24 hours' incubation at 37° C. the control tube showed a rich growth of the parent strain, while the test tube showed none. Platings from this tube and cultivation of 20, 5 and 1 drops in 5 c.c.m. broth tubes showed no growth, indicating that at least most of the cocci had been killed, since the amount of penicillin carried over with 1 drop (0-04 c.c.m.) was sufficient to inhibit growth of the test tube.

A similar experiment was done with a sample of urine loaded with pus due to infection with Bact. coli and Strep. fecalis. In this case several dilutions of penicillin were made in the filtered 50% pus-broth—1/50,000, 1/150,000, 1/450,000 and 1/1,350,000. The inoculum of Staph. aureus was the same as in the previous experiment. After due incubation the two stronger concentrations of penicillin showed complete inhibition of staphylococcal growth, while in the two weaker ones, as in the control of 50% pus-broth alone, full growth occurred. Since the full staphylococcal titre of the sample of penicillin used was only about 2,200,000, the experiment gives no evidence of any antagonism by the pus.

**Tissue autolysates.**—The possibly antagonistic effect of tissue autolysates was tested as follows.

A liver taken aseptically from a rat was minced in the homogeniser of Potter and Elvehjem (1936) and allowed to stand for 2 hours in distilled water at 37° C. There was no gas evolution during the incubation and no putrefactive smell at the end of the experiment. The liver suspension had partly liquefied and when it was centrifuged about 2 c.c.m. of a clear, dark yellow solution were obtained. After due incubation the two stronger concentrations of penicillin showed complete inhibition of staphylococcal growth, while in the two weaker ones, as in the control of 50% pus-broth alone, full growth occurred. Since the full staphylococcal titre of the sample of penicillin used was only about 2,200,000, the experiment gives no evidence of any antagonism by the pus.

**Peptones** are equally devoid of antagonistic action; in fact in the usual assay method agar containing peptone is used as the culture medium for the staphylococci.

**Characteristics of antibacterial action**

The bacteriostatic power of penicillin against streptococci and staphylococci is as great as, or greater than, that of the most powerful antiseptics known, such as the heavy metal compounds, the acridine derivatives, &c. The results of all tests show the penicillin to have no direct immediate bactericidal action. This was borne out by two observations. First, the oxygen uptake of staphylococcal suspensions was not inhibited to any measurable degree by the addition of penicillin to a final concentration of 1/1000, over a period of 3 hours. Secondly, after incubation at 37° C, for 24 hours a staphylococcal suspension in broth containing 1/1000 penicillin grew in the presence of 1 mg. of (impure) penicillin per c.c.m. This fluid (0-9 c.c.m.) was incubated at 37° C. for 16 hours and then filtered, when about 2 c.c.m. of a clear, dark yellow solution were obtained. In water, both in duplicate, the depth of the zones of inhibition measured in millimetres:

<table>
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<tr>
<th>Medium</th>
<th>Inhibition (mm)</th>
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<tr>
<td>Serum</td>
<td>23</td>
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<tr>
<td>Water</td>
<td>22</td>
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**When penicillin diluted 1/10,000 with serum was thus tested in comparison with the same dilution in distilled water, both in duplicate, the depth of the zones of inhibition measured in millimetres:**

- In serum: 23-24
- In water: 22

Serum, therefore, in no way interferes with the action and diffusibility of the active substance.
large numbers of colonies on subculture. The only trace of lethal effect that we observed was an acceleration of the death-rate of small numbers of streptococci in Ringer's fluid and neutrophilic leucocytes in Ringer's fluid.

In its mode of action, penicillin resembles the bacteriostatic sulphonamide drugs, but on comparing their actions one becomes aware of the following important and significant differences.

1. The bacteriostatic power of penicillin against streptococci and staphylococci is much greater than that of the sulphonamides, even when these substances are tested under optimal conditions (small inoculum, peptone-free media, &c.). Saturated watery solutions of sulphanilamide and sulphathiazole show no complete inhibition on an assay plate, whereas penicillin at 1/500,000 gives an appreciable clear zone.

2. The action of penicillin against streptococci and staphylococci, unlike that of the sulphonamides, is only influenced to a minor extent by the number of bacteria to be inhibited. Even when the culture media are inoculated with several millions of staphylococci or streptococci per c.cm. of medium, the multiplication of these organisms may be completely inhibited by penicillin in a concentration as low as 1/1,000,000. With smaller inocula the inhibition will occur in even higher dilutions. This property of penicillin is of great importance for the treatment of heavily infected wounds, on which the sulphonamide drugs seem to have but little beneficial action.

3. The bacteriostatic power of penicillin against streptococci and staphylococci is not antagonised to any appreciable extent by protein breakdown products or products of tissue autolysis or pus, substances which annul completely the bacteriostatic action in vitro of the sulphonamide drugs. This is again of great importance in the treatment of suppurating wounds and makes possible the successful treatment of infections in which abundant production of pus takes place.

**EFFECT OF PENICILLIN ON CELLS**

**LEUCOCYTES**

There is little doubt that substances which are not bactericidal but only bacteriostatic depend to a considerable extent for their curative properties on the activity of the leucocytes which deal with the organisms while the latter are prevented from multiplying. In tests on leucocytes "therapeutic penicillin" has been compared with some other bacteriostatic substances which are at present being used in clinical treatment and with sodium hypochlorite.

**Technique.—We are indebted to Dr. C. G. Paine of The Jessop Hospital, Sheffield, for sending details of his technique for examining leucocytes. His solution has been used, though his method for examining the leucocytes has been altered.

Human leucocytes survive well in a solution containing urea 0·6 g., glucose 1·610 g., 0·025 g., NaCl 4·85 g., KC1 0·625 g., MgCl2 0·54 g., CaCl2 0·93 g. in a litre of distilled water, with 10% of serum added at the time of the experiment. The test substance was dissolved in the salt solution and diluted serially, each dilution allowing for the addition of the serum. The control consisted of salt solution and serum. The leucocytes came from the same donor as the serum. A drop of blood was allowed to clot on a coverslip and the coverslip incubated at 37° C. for about 30 min. in a Petri dish containing moist blotting-paper. The clot was then washed in cold 10% dilution of the test solution with 1% saline solution and the coverslip reversed over a well-slide containing the test solution. Many leucocytes stuck to the coverslip and could be examined under all powers of the microscope. The slide and microscope stage were kept at 30°–37° C. in a thermostatically controlled box.

Penicillin, the flavines and the sulphonamides were obtained as dry powders and dissolved in the salt solution. Hypochlorite was obtained as the concentrated stock solution of "electrolytic sodium hypochlorite " dispensed for clinical use, which contains 1% NaOCl. Dilutions were given in terms of this solution, not of hypochlorite. Albucid was provided as a 30% solution of the sodium salt (solvent not stated); this solution was diluted as usual, and the concentrations given are those of the pure hypochlorite. Sulphapyridine and sulphonamide solutions were shaken with the salt solution for 2 or 3 hours at 37° C. and the excess centrifuged off.

The pH of the salt solution and of the final preparations was checked with brom-thymol-blue. Albucid was found to be slightly acid even when buffered by serum. Each substance was diluted with 9 parts of Ringer's fluid before the results were tested, as it was found that the results were not consistent. A wide range of dilutions was used in most experiments but only the significant results are reported.

**Results.—**Penicillin at a dilution of 1/100 killed leucocytes immediately; at 1/250 more than 50% lived for 4 hours but the greatest time for which they were watched—but the survivors were sluggish towards the end. The preparation at 1/500 was indistinguishable from the control.

Proflavine (2:8-diaminoacridine hemisulphate) killed the leucocytes immediately at 1/20,000 and within 2 hours at 1/50,000. In 1/100,000 the leucocytes were sluggish after 2 hours, but in 1/200,000 they were active for at least the same length of time. In the lower dilutions the leucocytes became stained yellow immediately, at 1/100,000 by the end of an hour and in 1/500,000 by the end of 2 hours. Leucocytes were killed immediately by 2:7-diaminoacridine monohydrochloride at 1/10,000, but at 1/20,000 they were active. Albert, Francis, Garrod and Linnell (1938) found leucocytes fully active in both substances at 1/8400 after 2 hours. They mixed whole blood with the test solution on the coverslip and lowered a slide over to form a thin film. Though the method was not the same as ours it is difficult to see where the discrepancy arises, though the large amount of serum present inactivates the proflavine.

The stock solution of hypochlorite diluted to 1/100 killed immediately. After 2 hours the leucocytes in 1/500 were motile but sluggish compared with 1/1000, which halved the greatest time for which they were watched. Sulphapyridine and sulphathiazole, being relatively insoluble, were used in saturated solution. At dilutions of 1/1:1 and 1/10 the saturated solution neither inhibited nor killed immediately. After 2 hours some of the leucocytes were dead in both the sulphapyridine preparations; in the sulphathiazole preparations all were alive though some were inactive at 1/1-1.

It appears therefore that the flavines are more toxic than were previously supposed (this finding is in agreement with recent tissue-culture studies) and are not suitable for repeated use in high concentration on such lesions as burns. The hypochlorite solution, which is better than 5% solutions, is dead in both the flavines and the sulphonamides and should make an admirable local application for infected tissue surfaces. Its great solubility might lead to rapid removal by the bloodstream, but only further tests can show whether this is so. Continuous irrigation or repeated application would in any case overcome the difficulty.

The fact that penicillin at 1/500 does not appear to embarrass leucocyte activity in vitro contrasts with its complete bacteriostatic effect on staphylococci and streptococci, but in vivo at dilutions of at least 1/100,000. It is clear that leucocytes will remain completely active in any concentration of penicillin likely to be reached after intravenous injection.

**Tissue Culture**

Complementary to these experiments on leucocytes are those done on tissue cultures by Dr. P. B. Medawar of the Zoology Department, who has kindly allowed us to include his results here.

**Technique.—**The method employed had been devised for the determination of the inhibitory power of drugs (Medawar 1940, Jacoby, Medawar and Willmer 1941). It takes advantage of the fact that tissues freshly explanted into a medium of the same as ours it is difficult to see where the discrepancy arises, though the large amount of serum present inactivates the proflavine.

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Their actions in vitro proved to be identical, and the figures quoted may be taken to refer to either.

**Local application**

The effect of local application has been tried on the relatively delicate tissue of the central nervous system. In two rabbits under Nembutal anaesthesia a needle was inserted into the cisternal space and when a few drops of clear cerebrospinal fluid had escaped a burette containing 1/1600 penicillin in normal saline was attached to the needle. A slow intravenous injection of 11 c.c.m. of 25% sodium chloride was then made, which caused rapid absorption of cerebrospinal fluid into the blood-stream with consequent indrawing of fluid from the burette into the cisternal space. Weed (1922) showed that under the influence of this powerful osmosis the fluid introduced passed along the perivascular sheaths and even into the perineuronal spaces of the cerebral cortex. In this instance 2 c.c.m. of penicillin solution was drawn into the needle in 23 minutes and 1-3 c.c.m. in 32 minutes in the other. After the cisternal needle had been withdrawn and the anaesthetic had passed off neither rabbit showed the slightest functional disturbance. Both were killed 6 days later, when histological examination of the brain by Dr. Dorothy Russell showed that there was no meningitis or damage to the marginal glia and that the choroid plexuses and ependyma were everywhere normal. Dr. Russell investigated the effect on the brain in 3 other rabbits by methods she had previously described (Russell and Falconer 1941) and again no lesions attributable to the penicillin were discovered. These results, taken in conjunction with those on leucocytes and on tissue cultures, strongly support the view that local application of quite strong solutions should prove innocuous to tissue cells.

**Absorption and excretion of penicillin**

It has previously been shown that penicillin is absorbed after subcutaneous injection in mice, and from the intestine in rats, and that antibacterial activity appears in the urine secreted subsequently. Absorption and excretion have now been further investigated on rabbits, cats and man, always using the sodium salt of penicillin. The blood and urine levels have been compared after intravenous, subcutaneous and intestinal administration, often on the same animal. Although the substance loses activity quickly in acid solution it was not considered worth while at this stage to study the results of direct administration into the stomach, except for two tentative observations on normal subjects and in Case 6. Absorption from the gastro-intestinal tract has mainly been studied by the direct introduction of penicillin into the duodenum through a small fistula. In man a duodenal tube was used. The standard dose in all experiments on the rabbit and cat was 400 units. Unless otherwise stated the urine was collected by catheter, the bladder was washed out and the washings were added to the urine for assay. When necessary a short ether anaesthetic was given to facilitate the injection and the collection of the urine.

In rabbits.—Rabbit 1 received two doses by each route. After duodenal administration no detectable activity was
observed in the blood after \(\frac{1}{2}\) hr. (one experiment) or 1 hr. (2 experiments); 14% and 5-3% respectively of the active substance were excreted in the urine. Similarly after intravenous injection there was no activity in the blood at 1 hr., 2 hr., and 3 hr. The results were: 62% the second hour and 48% the third hour. There was an excretion in the urine of 14% and 5-3% respectively.

One rabbit after a duodenal injection showed no activity in the blood at 1 and 2 hr. and none in the urine, but since the urine was passed naturally in a metabolism cage during the night activity may have been lost by bacterial action, in spite of the presence of chloroform.

In a third rabbit the standard dose was injected into the intestine in an acute experiment. On the supposition that penicillin might be absorbed from the gut and then inactivated in the liver, samples were drawn from the portal vein and the vena cava at 10, and later 20, minute intervals for 1 hr. In no blood sample could activity be detected, but the urine at the end of 2\(\frac{1}{2}\) hr. contained 12% of the injected penicillin. Bile from a cannula in the common duct (the cystic duct having been tied) consistently showed a trace of activity up to 2 hr., the end of the experiment. In a fourth rabbit, also under nembutal, the penicillin was injected intravenously and blood taken from the ear vein. There was no activity in 4 half-hourly blood samples and a trace in the first hour, but the results are not significant. The repetition of this experiment on a fifth rabbit gave essentially the same result.

The rabbit, therefore, gives a good return in the urine after intravenous injection (20-50%), but a return of less than 20% after administration into the intestine.

Penicillin injected into the bile at 37° C. of penicillin with blood and with slices of liver, there was no detectable destruction. Nor was there any diminution of activity after incubation with rabbit bile, though there was perhaps a slight fall with cat bile. It is impossible to say whether the material recovered from the urine has been changed in any way during its passage through the body. It has a high antibacterial titre and has been used again for administering penicillin.

The cat differs from the rabbit in maintaining an antibacterial concentration of penicillin in the blood for at least \(\frac{1}{2}\) hr. after subcutaneous or intravenous injection and at least 3 hr. after intestinal administration. The inhibitory power of the blood was followed in one subject at frequent intervals from 5 min. until 2 hr. 5 min. after the injection. The high initial value gradually fell to a just discernible trace in the last sample. Urinary excretion was still occurring at 6 hr. 15 min. and there was still activity in the urine collected from the 6th to the 14th hour. After a dose of 100 mg. inhibition was just detectable in the blood at the end of an hour. After repeated 100 mg. doses a trace of inhibition was similarly found in the blood one hour after an injection (case 3, who had had five initial hourly doses of 200 mg.). The best experiments from the point of view of estimating the urinary recovery after intravenous injection were those on patients No. 3 and 5, whose urine output was accurately measured during the course of treatment. In case 3 about 30% of the total active substance was injected in the urine and 30% was actually recovered in purified form (the rest being lost in the re-extraction process). In case 5, in which penicillin dissolved in saline was administered containing a considerable antibacterial activity reappeared in the urine and 54% was recovered for further use.

For duodenal administration a tube was passed its location in the duodenum confirmed by fluoroscopy. Sixteen thousand units (about 400 mg. of the solid material) were given as 25 c.c. water was injected down the tube, followed by 60 c.c. more of water. After this injection the blood inhibited bacterial growth for 3 hr. and urinary excretion continued for 6 hr. The inhibitory level in the blood was steadier and persisted for a longer time after intravenous injection. The delay was twice the maximum so far given intravenously and it is quite possible that larger doses will always be needed for treatment by the intestinal route.

Nevertheless the indications are that this is a possible way of administering penicillin.

A contrast thus emerges between the behaviour towards penicillin of the rabbit on the one hand and of the cat and man on the other. After intravenously to a rabbit no activity could be detected in the blood at the end of half an hour, while in the cat after the same dose the blood was active for at least \(\frac{1}{2}\) hr. Similarly after duodenal injection there was no activity in the blood and little in the urine of the rabbit, whereas in the cat activity could be detected in the blood and there was a considerable excretion in the urine. Man appears to be in both instances more like the cat than the rabbit. The apparent more effective mechanism for the inactivation of penicillin than either of the other two species.

By whatever route penicillin is administered not all the active substance appears in the urine. Where this is true the patient soon becomes infected, and it is essential to note that material partially purified but containing pyrogen is freed from that impurity by passage through the body.

**Therapeutic Trial of Penicillin**

**Methods of administration.**—To avoid the uncertainties of intestinal absorption, the first cases were treated by penicillin intravenously. Since it is rapidly eliminated by the kidneys and probably partially
inactivated in the body, it was clear that frequent doses would be necessary. At the outset it was felt that intravenous administration might give the best diffusion into the tissues as a result of the repeated temporary raising of the concentration of the drug in the blood above the highest level that could be attained by continuous administration of the same total quantity. The attempt to give frequent injections using a cannula was tried. At first a dilution of 1/1000 in normal saline was used, and as this was found to be no more harmful to the vein than normal saline alone the concentration was raised to 1/500. The vein tolerated this concentration only in the urinary tract, and not in the blood. It may however be possible to carry the penicillin through the stomach by raising the pH of the gastric contents. Experiments with such substances as magnesium trisilicate or the recently described aluminium phosphate together with sodium bicarbonate. In this case however the stomach by raising the pH of the gastric contents. Experiments with such substances as magnesium trisilicate or the recently described aluminium phosphate together with sodium bicarbonate. In this case however we were aiming at keeping up an antibacterial concentration only in the urinary tract, and not in the blood. The possibility of administration in capsules which will pass through the stomach has not been overlooked, but some tentative experiments with salol-coated capsules were discouraging. Moreover, Cook and LaWall (1936) stated that only small quantities of such drugs were absorbed satisfactorily; we have therefore not thought it worth while to entrust a scarce and valuable substance to such unreliable vehicles. Rectal administration was tried in one subject, but very little active substance was found in the urine afterwards found that feces inactivate penicillin, probably by bacterial action. It was thought undesirable to introduce very strong solutions of penicillin subcutaneously or intramuscularly owing to uncertainty about the local effects. More dilute solutions, for example 1 in 500, which have been shown to be innocuous to leucocytes, would involve the use of excessive amounts of fluid. In case 1 the injection of 100 mg. in 2 c.cm. of water intramuscularly caused some tenderness, though this cleared up quickly.

**INTRA VENOUS ADMINISTRATION IN STAPHYLOCOCCAL AND STREPTOCOCCAL INFECTIONS**

**Case 1.—Policeman, aged 43.** Admitted Oct. 12, 1940. Suppuration of face, scalp and both orbits, starting from a sore at the corner of the mouth a month earlier. Primary infection *Staph. aureus*; secondary, *Strep. pyogenes*. Sulphapyridine 19 g. given from Dec. 12 to 19; no improvement; drug-rash. Jan. 19: incision of multiple abscesses on face and scalp. Osteomyelitis of right humeral head, proved by X-rays, showed on Jan. 31, 1941, after 3 weeks of pain; a fragment of right humeral head removed. At noon 100 mg. penicillin injected into intravenous apparatus (Marriott and Kekwick 1940) which delivered a steady flow of 500 c.cm. of 1-05% sodium citrate or 0-9% sodium chloride in 24 hr. Each dose of penicillin dilute solution was 100 mg., and the total amount of non-pyrogenic water, injected into the rubber tubing of the drip apparatus and flushed in with a little of the citrate-saline solution. This method is admittedly inconvenient owing to the well-known difficulties of maintaining an intravenous infusion for more than two or three days, but it was thought that if the therapeutic efficacy of penicillin could be established by this method other ways of giving the drug could then be explored.

In case 5 the effect of running in a continuous supply of penicillin solution was tried. At first a dilution of 1/1000 in normal saline was used, and as this was found to be no more harmful to the vein than normal saline alone the concentration was raised to 1/500. The vein tolerated this concentration only in the urinary tract, and not in the blood. The possibility of administration in capsules which will pass through the stomach has not been overlooked, but some tentative experiments with salol-coated capsules were discouraging. Moreover, Cook and LaWall (1936) stated that only small quantities of such drugs were absorbed satisfactorily; we have therefore not thought it worth while to entrust a scarce and valuable substance to such unreliable vehicles. Rectal administration was tried in one subject, but very little active substance was found in the urine afterwards found that feces inactivate penicillin, probably by bacterial action. It was thought undesirable to introduce very strong solutions of penicillin subcutaneously or intramuscularly owing to uncertainty about the local effects. More dilute solutions, for example 1 in 500, which have been shown to be innocuous to leucocytes, would involve the use of excessive amounts of fluid. In case 1 the injection of 100 mg. in 2 c.cm. of water intramuscularly caused some tenderness, though this cleared up quickly.
slight rigor; then 75 mg. 3-hourly. Feb. 24: general condition better, discharge less. By 9 p.m. had received 1’4 g.

Several different samples of penicillin were used in this case. The first five doses had been recovered from the urine and blood of case 1 and caused no reaction. The next samples, which caused some shivers, were "third fraction," and although they had been passed through an absorption column again they still contained the pyrogen. The later doses were "second fraction" (our present "therapeutic penicillin"). Here there was a local infection by a hemolytic streptococcus which had proved resistant to sulphanilamide in large doses and to moderate doses of sulphapyridine. Penicillin therapy was followed by a great improvement in the patient's general condition, under the dose being insufficient to maintain a detectable concentration of penicillin continuously in the blood.

Case 3.—Labourer, aged 48, of poor physique. Admitted May 2, 1941. Carbuncle 4 in. across over left scapula for 5 days; now discharging; pus grew pure Staph. aureus.

Historical; chronic bronchial and nasal catarrh for 4 months. Left axillary adenitis. Hb. 106%; red cells 4,970,000; white cells 23,000. Blood-urea 31 mg. per 100 c.cm. Urine normal. White cells 8300 (polymorphs 74%).

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Staph. aureus. X-ray: opacity of left antrum, ethmoids clear. May 15: blood sample an hour after dose of penicillin showed no antibacterial activity; dose increased to 50 mg. hourly. General improvement. May 16: obviously better; swelling largely subsided. May 17: carbuncle almost normal and patient generally well; right eye treated with penicillin 1 in 5000, which caused no discomfort. May 22: improvement maintained, patient talking and playing with toys. Chest clinically normal. Slight pyrexia still thought to be due to pyrogen in small doses. On reaction from thromboses in veins used for injections (see fig. 7). Penicillin stopped. May 26: progress good. Temperature normal. General condition excellent. Eye movements returning. X-ray of sinuses: only slight clouding left antrum; chest: patch of consolidation left apex and left hilar shadow right mid-zone. These thought to be embolic signs but general condition so good that no further penicillin needed. May 27: 1 A.M. vomited and had general convulsions. Lumbar puncture gave uniformly blood-stained fluid under high pressure. Became comatose with neck rigidity, positive Kernig's sign and spastic limbs. May 28: temperature began to rise again. May 29: appearance much as on admission. Penicillin 2 g. given in next 36 hours, but died May 31.

Autopsy (Dr. A. H. T. Robb-Smith).—Brain showed no tissue due to main venous sinuses; adhesions and old haemorrhage in hypophyseal region. Considerable old and recent haemorrhage in region of pons and cerebellum due to rupture of aneurysm on left vertebral artery. Cavernous-sinus region and left orbit occupied by edematous granulation tissue; left carotid artery partially occluded by thrombus in its cavernous course and completely occluded in its carotid siphon; brain showed scattered abscess cavities, larger ones in cerebellum, smaller ones containing cysts lined by yellowish membrane; surrounding this is a granulation tissue formed largely of histiocytes containing lipoid and blood-pigment, lymphocytes and plasma cells with a very occasional neutrophil. Histologically granulation tissue is essentially similar whether in lung abscesses, orbital tissues or cavernous regions (fig. 8). There is a small central area of necrosis sometimes containing a few gram-positive cocci; around this is an edematous exudate with lipoid-containing histiocytes; surrounding this is a granulation tissue formed largely of histiocytes containing lipoid and blood-pigment, lymphocytes and plasma cells with a very occasional neutrophil.
leucocyte; this tissue is well vascularised and there is some fibroplastic proliferation, particularly in the periphery. In the cavernous region the thrombi contain organizing thrombi; the left carotid and vertebral arteries show organizing thrombi which do not appear to be infected, but as there are large breaks in the media and elastica of the walls of both these vessels it must be presumed that they are the late results of an acute arteritis probably of bacterial origin. The other organs showed no significant change.

The autopsy showed that the infection in the cavernous sinus, orbits and in the lungs had been almost entirely overcome, and that healing processes were well advanced. Death was due to the ruptured mycotic aneurysm and that healing processes were well advanced. The left carotid and vertebral arteries show organising thrombi; the left carotid and vertebral arteries show organising thrombi in the left femoral and pelvic vessels it must be presumed that they are the late results of an acute arteritis possibly of bacterial origin. The other organs showed no significant change.

The author also mentions a case of staphylococcal septicaemia, localising in the left hip-joint, which had been uninfluenced by infection by Staph. aureus, and the administration of penicillin. They are the late results of an acute arteritis possibly of bacterial origin. The other organs showed no significant change.

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In case 4, a 1/5000 solution of penicillin in normal saline was applied to a corneal ulcer. It caused no irritation and the condition of the eye improved. The local application of penicillin in other eye infections was therefore tried on patients in the Oxford Eye Hospital.

CASE 7.—Married woman, aged 52. Had a corneal ulcer of the left eye 4 months previously, treated successively by instillation of Collosal Argentum, boracic lotion and mercurochrome, and ultraviolet light. In 6 weeks ulcer resolved completely, but 3 weeks later similar ulcer developed in the right eye. Treated on similar lines, for 6 weeks as outpatient and then for fortnight as inpatient, but without improvement. May 26, 1941: infiltrating ulcers in inner and upper quadrants of limbus, gross injection of conjunctiva and considerable corneal opacity. Swab from eye grew Staph. aureus.

May 30: treatment with penicillin begun; 1/5000 solution in normal saline dropped into eye hourly by day and 2-hourly
by night. After 2 days little progress had been made and continuous application considered necessary. 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Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burns of Burn...
To soak infected hands in hot baths and apply fomentations "as hot as can be borne" are methods of preoperative and postoperative treatment which are so ingrained in the medical and nursing professions that it will take time for them to be supplanted. Let us review the disadvantages of the "wet" method:—

(a) It is extremely painful. I still remember dredging the time coming round for my own fomentations, which were applied at two-hourly intervals by a conscientious sister who believed in fomentations being really hot.*

(b) General swelling and tenderness of the hand, due to the hot applications, mask localised swelling and tenderness due to extension of the infected process; consequently the accurate diagnosis of the location of pus is often delayed.

(c) The hot-water treatment produces scalding, which, in addition to causing pain, predisposes to superficial infection and even paronychia of uninfected digits.*

(d) Sodden, swollen tissues tend to narrow exits through which pus can escape.

TECHNIQUE OF THE DRY METHOD

During the past three or four years I have been increasingly impressed by the manifold advantages of the dry method of treating seriously infected hands. The best way of expounding its principles will be to quote the case of a surgical staff-nurse with suppurative tenosynovitis of the index finger. As I have suffered from suppurative tenosynovitis myself, and as this patient was a trained surgical staff-nurse with suppurative tenosynovitis of the index finger, it was possible to contrast and compare the wet and dry treatments with more than ordinary enlightenment.

I was called to see the nurse four days after the symptoms and signs had begun. She had scratched the back of her index finger while doing a dressing; the facts that the original lesion was on the dorsum of the finger and that the patient could flex the finger without much pain had been the cause of diagnostic difficulty to several observers. (How often the all-important diagnosis of the dry after-treatment for seriously infected hands is greater than mine, informs me that both he and Sister Wray, of Sheffield Royal Infirmary, find that in a small percentage of cases progress is unsatisfactory. I think it unlikely that in this instance suppurative therapy played any part. In nearly all the cases in which I have employed dry after-treatment for seriously infected hands the infecting organism has been a streptococcus. As suppurative therapy came into general use about the same time that I changed from the wet to the dry treatment, it might be argued that it was the sulphonamide which was playing the major part in the improved results. I have no wish to detract from the great benefits which have accrued from sulphonamide therapy in this branch of surgery, but the case reported here demonstrates that, with adequate drainage, dry after-treatment and elevation are in themselves an advance.)

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In my experience, the dry after-treatment has proved uniformly satisfactory. T. B. Mouat, whose experience is greater than mine, informs me that both he and Sister Wray, of Sheffield Royal Infirmary, find that in a small percentage of cases progress is unsatisfactory. In such cases Sister Wray changes to what are known as 'rotation' dressings for about 48 hours. The hand remain immobiled, but every 4 hours the following moist dressings are applied in turn: eusol, normal strength;