VI° CONGRESSO INTERNAZIONALE DI MICROBIOLOGIA
VIth INTERNATIONAL CONGRESS OF MICROBIOLOGY

SYMPOSIUM

INIBITORI DI CRESCITA E CHEMIOTERAPIA

GROWTH INHIBITION AND CHEMOTHERAPY

SUPPLEMENTO AI RENDICONTI
DELL'ISTITUTO SUPERIORE DI SANITÀ

ROMA: FONDAZIONE EMANUELE PATERNÒ
VIALE REGINA MARGHERITA, 299 - ANNO 1953

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HARRY EAGLE

SECTION ON EXPERIMENTAL THERAPEUTICS, LABORATORY OF INFECTIOUS DISEASES, NATIONAL MICROBIOLOGICAL INSTITUTE, NATIONAL INSTITUTES OF HEALTH,* BETHESDA 14, MARYLAND, U.S.A.

THE BINDING OF PENICILLIN IN RELATION TO ITS CYTOTOXIC ACTION.

There is a considerable body of experimental evidence that the cytotoxic effect of a wide variety of organic arsenicals rests on the inactivation of cellular enzymes essential to the viability of the cell, and which contain -SH groups reactive with the arsenical. In a series of aryl arsenoso compounds, the relative cytotoxic activity paralleled, and was probably determined by, the degree to which they were concentrated by the test organism. Further, the development of resistance to certain of these compounds was associated with a loss in the ability of the cells to bind and concentrate the drug. The resistant organisms bound and concentrated precisely those arsenicals to which were still susceptible, and failed to concentrate those to which they had become resistant (1). The toxicity to the host was similarly related to the amounts of arsenic taken up by the host cells.

Given this clear and quantitative relationship between the binding of a series of cytotoxic agents, and their biological effects, particular interest attaches to experiments designed to establish (a) whether antibiotics are similarly bound and concentrated by susceptible bacteria; (b) whether there is any correlation between the degree to which they are bound by a given strain and its sensitivity to the antibiotic; and finally, (c) whether the emergence of resistance is associated with the fact that the resistant celle no longer binds and concentrates the drug. Experiments along these lines, using S^{35}-labelled penicillin in order to measure the degree of binding, have been carried out by three groups of workers [Rowley, et al. (2, 6); Maass and Johnson (7, 8); and Pollock and Perret (9, 10)].

* United States Public Health Service, Department of Health, Education and Welfare.

A) The susceptibility of Bacterial strains to Penicillin as a function of their Reactivity with the Antibiotic.

Preliminary experiments were carried out with iodinated penicillin X; but the present experiments were done with S^{35}-labelled antibiotic, prepared by using labelled Na_2SO_4 in the growth medium. The bacterial species used in most of the present experiments were *Streptococcus pyogenes* (C203 strain); *Diplococcus pneumoniae* III; *Micrococcus pyogenes* (Smith); *Streptococcus fecalis;* and *E. coli* (K12). The $LD_{99.9}$ concentrations of penicillin for these five strains were 0.006, 0.018, 0.024, 2.0, and 30 micrograms/ml, respectively. When these five strains were exposed to the same concentration of penicillin (0.01 micrograms/ml) for the same time period (2 hours at 37°), the concentration of firmly bound « penicillin » inside the cell varied in approximately the same order, and to the same degree, as their sensitivity to penicillin (cf. Table 1). Further, despite the 5,000-fold difference in the bactericidal con-

TABLE I

Correlation Between the Amount of Penicillin Bound by Various Bacterial Strains Under Standard Conditions and Their Susceptibility to the Antibiotic

All the organisms were exposed to S35-labelled penicillin at 0.01 micrograms/ml for 2 hours at 37°. The values given in the table represent the « penicillin » remaining in the cell after at least 2 washings.

Bacterial Species	Penicillin $LD_{99.9}$ µg/ml	Relative Susceptibility	Intracellular Bound «Penicillin» (µg gm) after Exposure to 0.01 µg ml
Streptococcus pyogenes .	.006	165	3.05
Diplococcus pneumoniae	.018	55	1.5
Micrococcus pyogenes (Smith)	.024	42	.96
Streptococcus mitis . .	.1	10	1.22
Micrococcus pyogenes (MII)	.25	4	.07
Streptococcus fecalis .	2.0	0.5	.054
E. coli	30.0	0.033	.0014

centrations, at those equi-effective $LD_{99.9}$ levels the concentration of bound « penicillin » inside the cell was remarkably uniform, varying only between 1 to 3 micrograms/gm dry weight. The bactericidal activity of penicillin was thus related to, and perhaps determined by, the concentration bound by the cell.

In order to determine whether the large differences in the amounts of penicillin bound by these various species reflected actual differences in the reactivity of cellular components, rather than differences in the permeability of the cells, bacterial extracts were prepared by sonic vibration, clarified by centrifugation at 27,000 g, and the sedimentable components (largely protein) in those extracts collected in an ultracentrifuge at 144,000 g. The reactivity of that sedimented material with penicillin varied in the same order and to the same degree as (a) the capacity of the intact organism to react with the penicillin, and (b) the susceptibility of the cells to penicillin. The primary determinant of sensitivity to penicillin therefore appears to be the reactivity with the antibiotic of one or more cellular components of large molecular weight.

A number of experiments were undertaken to delimit the effect of various environmental factors on the binding of penicillin by intact bacteria. At low concentrations of antibiotic, the strains of penicillin-sensitive *Micrococcus pyogenes* and *Streptococcus pyogenes* used in these experiments concentrated the drug as much as 50- to 100-fold. At high concentrations of antibiotic, the intracellular concentration of the unwashed cells varied from one to two times that in the outside fluid. In every experiment with these two species, as well as with *Diplococcus pneumoniae* and *Streptococcus fecalis*, there was significantly more labelled sulphur inside the bacteria than outside. However, penicilloic acid, penillic acid or penicillamine were not similarly concentrated. No matter whether the concentration in the medium was 0.01 or 100 micrograms/ml, with these degradation products the ratio of intracellular S^{35}: extracellular S^{35} varied only between 0.3 and 0.6, and the intracellular concentration did not exceed that which could be explained on the basis of a diffusion equilibrium. These data thus confirm and extend the findings of Pollock and Perret with *B. cereus*. At least with the compounds so far tested, only the actively antibacterial penicillin has been concentrated by susceptible cells; its inactive degradation products are not concentrated.

The total intracellular penicillin obviously includes a certain amount of free antibiotic in diffusion equilibrium with the surrounding fluid. If one assumes the entire cellular fluid to participate in such a diffusion equilibrium, and subtracts those values from the total bacterial « peni-

cillin », one obtains minimum values for the excess « penicillin » in the cell, i.e. antibiotic which had reacted with cell components. Contrary to previous reports, the concentration of this excess « penicillin » increased progressively with the concentration to which the bacteria had been exposed, and there was no indication that the bacteria had been saturated even at the highest concentration used in these experiments. To a certain extent, varying with the conditions of exposure, some of this excess « penicillin » could be removed by washing. Of that bound at low concentrations, a relatively small amount was dissociated from the cell by 2-4 washings in several thousand volumes of broth. Of that bound at high concentrations, a much larger proportion could be so removed. However, the residual concentration of firmly bound penicillin was found to increase progressively with the concentration to which the cells had been exposed. There was no indication that the cells had been saturated even by 1000 micrograms/ml in the outside fluid. At this concentration, the « penicillin » firmly bound by *Micrococcus pyogenes* was 140 micrograms/ml, 500 micrograms/gm, and on the order of 6000 molecules/cell. Qualitatively similar relationships have been obtained with *Streptococcus pyogenes*, *Diplococcus pneumoniae*, *Streptococcus fecalis*, and *E. coli*.

The firmness of the combination between penicillin and the bacteria was further indicated by experiments in which the organisms which had bound penicillin at relatively low concentrations were then allowed to grow out in a penicillin-free medium. Under these conditions some of the bound penicillin leached out of the cell; but the residual material remained firmly attached and persisted in the bacteria through 6-7 generations and a 100-fold increase in cell mass (cf. Pollock and Perret). In confirmation of the results obtained by Maass and Johnson, this bound penicillin does not equilibrate with freshly added antibiotic. If, instead of being placed in a medium containing no penicillin, the organisms were placed in a medium containing unlabelled antibiotic, the presence of the unlabelled drug in the outside fluid had no demonstrable effect on the dissociation of the bound material.

There are several reports that the degree to which penicillin is bound is profoundly influenced by the metabolic state of the bacteria, and that from 3 to 10 times as much is bound by bacteria in the active stage of growth as is bound by resting cells. This we have not been able to confirm. In our hands comparable amounts of penicillin were bound whether the organisms were in the logarithmic stage of growth, in a static fully grown culture, or suspended in salt solution. This is in keeping with the observation that cell-free bacterial extracts also

bind penicillin, and to approximately the same degree per unit weight as the intact cells.

B) The failure of mammalian cells to bind Penicillin.

One of the most striking properties of penicillin is its relative non-toxicity for the host. In keeping with the thesis that the degree of binding by the cell determines, and is a measure of, the cytotoxic activity of penicillin, mammalian cells failed to bind detectable amounts of the drug. These experiments were carried out with a mouse fibroblast (the L strain of Earle) and a human carcinoma cell (HeLa), both grown in tissue culture on glass. The concentration inside the cell did not exceed that which could be explained on a simple diffusion equilibrium, and actively antibacterial penicillin was not bound to any greater extent than was e.g. penicilloic acid.

C) The Binding of Penicillin by Penicillin-resistant variants of normally sensitive cells.

The foregoing data relate to the penicillin-binding capacity of various bacterial strains as they occur in nature. With these, penicillin susceptibility appears to parallel, and is probably determined by, their reacting affinity with penicillin. This is not true, however, of resistant variants derived from those strains. In our hands, when strains of *Diplococcus pneumoniae*, *Micrococcus pyogenes*, or *Streptococcus fecalis* were rendered highly resistant to penicillin by selective propagation in increasing concentrations of the antibiotic, those resistant variants reacted with and concentrated the antibiotic fully to the same degree as their much more sensitive precursors. If the bacterial action of penicillin is normally conditioned by its combination with vulnerable cell components, and if the natural resistance of such organisms as *E. coli* and *Streptococcus fecalis* is a function of the lower reactivity of certain vulnerable cell components with penicillin, one must conclude that a quite different mechanism is involved in the resistance of variants of e.g. *Diplococcus pneumoniae* or *Streptococcus pyogenes*: their decreased susceptibility to penicillin is not due to a decreased reactivity with the antibiotic. Such resistant variants may contain, or produce, larger amounts of the hypothetical compound or compounds which are inactivated by combination with penicillin. Alternatively, in the resistant organism the cell component which is inactivated by penicillin is no

longer essential to the cellular economy. These, and other possible explanations remain to be explored.

Discussion.

A number of observations therefore suggest that the binding of penicillin by bacteria may be the primary determinant of its cytotoxic action, and that the affinity of as yet unidentified cell components for penicillin may be a measure of the concentrations necessary for an antibacterial effect.

1) There was a highly suggestive correlation between the degree to which bacterial species concentrated « penicillin » and their sensitivity to the antibiotic. The greater that combining affinity, the less penicillin was necessary in the outside fluid in order to exert an antibacterial action. Cell free bacterial extracts reacted with penicillin in the same order, and to essentially the same degree, as the intact cells.

2) In the species here studied, the bactericidal concentration (LD99.9) varied 5,000-fold (from 0.006 to 30 micrograms/ml); nevertheless, the concentrations bound by the organisms at those $LD_{99\cdot9}$ levels were of the same order of magnitude. In general, in order to effect the death of the cell, the intracellular concentration of bound « penicillin » had to attain approximately 1-3 micrograms/gm, and on the order of 1000-3000 molecules/cell. The difference between species highly susceptible to penicillin (e. g. group A streptococci) and other relatively resistant bacterial species (e.g. *Streptococcus fecalis*, *E. coli*) did not lie in the presence or absence of cell components capable of combining with the antibiotic, but rather in the concentrations which had to be present in the outside fluid in order to effect that combination.

3) Only active penicillin was bound and concentrated by susceptible organisms. With degradation products which were not antibacterial, the bacterial concentration was regularly lower than that in the surrounding fluid, and consistent with a diffusion equilibrium.

4) Mammalian cells, not killed by penicillin, failed to bind demonstrable amounts even when the concentration in the outside fluid was 1000 micrograms/ml.

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ADRIEN ALBERT

PROFESSOR OF MEDICAL CHEMISTRY
THE AUSTRALIAN NATIONAL UNIVERSITY

MODE OF ACTION OF ANTIBACTERIAL SUBSTANCES

A convenient division of this subject is to distinguish between those substances which are chemotherapeutic and those which are not. Here « chemotherapeutic » is used, as in Ehrlich's original definition, to mean substances harmful to bacteria in the presence of living mammalian cells. The chemotherapeutic antibacterials are further subdivided into two classes, the general and the local, that is to say, those that are active in the blood stream and those that are active only in wounds. This classification is not quite rigid, and it is realized that some substances were the first to be discovered, and then the local chemotherapeutic substances. The general chemotherapeutic agents have all been discovered since 1934 (the special case of arsenicals for spirochetes excepted). It is proposed to deal with the three classes of substances in this historical order.

1. Non-Chemotherapeutic Substances

The use of *mercuric salts* as antibacterials is even older than the discovery of bacteria. Ehrlich showed that these salts had no chemotherapeutic properties. There was a tendency to speak of them as « general protoplasmic poisons », but Chick (1908) showed that bacteria are easily revived from mercurial poisoning by treatment with -SH substances ,thiols), such as thioglycollic acid, or even plain hydrogen sulphide. Fildes (1940) published a series of experiments which seemed to indicate that mercuric chloride (and organic mercurials such as phenyl mercuric nitrate) act upon bacteria by combining with essential mercapto-groups (-SH). Voegtlin (1925) had already shown that arsenicals act upon trypanosomes by combining with these groups, and are effective only after conversion to a particular one of the three possible levels of oxidation, viz. the arsenoxide level. Hence Albert, Falk and Rubbo (1944) stated that it was a necessary corollary of Fildes's hypothesis, that arsenicals should be highly active againts the common

bacteria, just as mercurials are. The literature gave non indication that such an activity of arsenicals existed, but simple tests soon showed that they are powerfully antibacterial (although only when present at the arsenoxide level, just as would be expected).

Mercurial salts, whether organic or inorganic, are not affected by serum, but plasma inactivates them and also the -SH substances present in healing wounds. The most interesting substance in this class is merthiosal (« Merthiolate »), which does not contain ionic mercury but slowly liberates it by hydrolysis. There appears to be no evidence that any mercury compound can distinguish between human cells and bacteria. The organic mercurials enjoyed tremendous popularity in the 1930s, for dressing wounds, but they are now comparatively little used.

The antibacterial properties of *phenols* have long been known. The destructive effect of phenol itself on the human skin has been ameliorated by the introduction of alkyl- or chloro-groups, the latter change being initiated by Ehrlich and Bechhold. Following the work of Vermast (1921), who found that benzoic acid is most strongly antibacterial when least ionized, Ordal (1941, 1943) showed that this was also true for phenols (it had already been shown to be true for the action of phenols on some other kinds of organism). Fairly recent work done in Cambridge with the electron microscope has tended to show that phenols act on the cell walls of bacteria in such a way that the cells burst and discharge their contents. Those phenols which are least harmful to the skin are the most strongly antagonized by serum. *p*-Chloro-*m*-xylenol is one of the best disinfectants for the intact skin and mucous membranes but its use in wounds in questionable, a remark which applies to all phenols. In both pure and crude forms, phenols enjoy a well-deserved popularity in the disinfection of bed-pans and surgical instruments. They are cheap, rapid, persistent, irreversible, non-staining and pleasant to use, a combination of properties that is most uncommon.

The mode of action of *halogens* has been extensively explored in America by Wyss and his colleagues. The relative activities of the free (elementary) halogen and the hypohalite molecules to which they hydrolyse in water have been carefully worked out. It has been claimed that the N-halogenation of proteins is the metabolic site of attack; crude oxidizing action also takes place. Iodine is one of the most effective agents for sterlizing catgut and is effective in the sterilization of the unbroken skin. The chlorination of water is one of the greatest safeguards of health in our cities. Swimming-baths are commonly chlorinated, but there is an unfortunate tendency to under-chlorinate, result-

in in the accumulation of nitrogen trichloride which is much more irritating than chlorine to the eyes, and not so antibacterial. The use of hypochlorous acid in wounds (by a drip technique to overcome the disadvantage of its rapid destruction by organic matter) was popular during the first World War, but it was later found that war-wounds ware healed much more rapidly by amino-acridines (Poate, 1944).

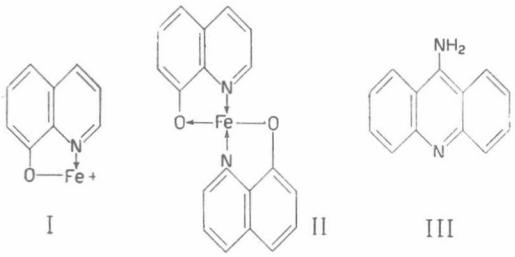
As far as is known, *formaldehyde* acts on bacteria by combining with the amino-groups of proteins which are thereby completely changed in nature and function. It combines even with insoluble protein, as in the intact skin. Potassium permanganate and hydrogen peroxide act just as frank *oxidizing agents*, and are themselves destroyed in the process.

2. Local Chemotherapeutic Substances

Under this heading, we should consider those antibacterial substances which are innocuous to human cells (e.g. leucocytes), but which are not effective when given by mouth, or injected into the blood-stream. The most intensely studied examples of this group are 8-hydroxyquinoline and the aminoacridine family. Both of these examples turn out to be simple representatives of much larger families, the chelators and the cationics, respectively.

The mode of action of 8-*hydroxyquinoline* has been shown to be connected with its ability to form complexes with the ions of heavy metals (Albert, 1944; Albert, Rubbo, Goldacre and Balfour, 1947). These authors found that any change in the structure preventing this chelation abolished the antibacterial action completely. It was then found that 8-hydroxyquinoline is only antibacterial when traces of iron or copper were present, as is always the case in bacteriological media: when these traces of metallic ions were removed, the substance was no longer toxic to bacteria (Rubbo, Albert and Gibson, 1950). Evidently the metal-complex is the toxic agent, and of the two known complexes, the evidence favours (I) (Albert, Gibson and Rubbo, 1953). In the presence of excess 8-hydroxyquinoline, complex (II) is formed instead. In (II), the iron is saturated with 8-hydroxyquinoline and hence (II), unlike (I), cannot combine with other metal-avid groups (see below). It would be expected that such a substance should be non-toxic, and in fact it has been found that highly concentrated solutions, e.g. 1 in 10,000, of 8-hydroxyquinoline will not kill staphylococci, although dilute solutions (e.g. 1 in 500,000) are bactericidal in one hour, or less. This must be one of the most paradoxical effects in the whole literature of antisepsis. It is evidently caused by the large amount of the drug (in the stronger

solution) relative to the trace of iron in the medium, because the addition of one equivalent of ferrous sulphate makes the stronger solution rapidly bactericidal (Albert, Gibson and Rubbo, *loc. cit.*). Needless to say, this amount of iron is, by itself, quite innocuous.



I II III

The action of 8-hydroxyquinoline appears to take place *inside* the cell, as the following evidence indicates. In general, substances with high oil/water partition-coefficients penetrate cells better than those which are less liposoluble (c.f. Davson and Danielli, 1943). Accordingly, analogues of 8-hydroxyquinoline with lower partition-coefficients were synthesized. It was found that this effect could best be obtained by incorporating an extra ring-nitrogen atom. All the six possible isomerides were prepared (Albert and Hampton, 1952). All these substances naturally enough retained their chelating properties, but they had little or no antibacterial activity. Hence a further change was made in these molecules: alkyl groups were inserted so that the partition-coefficients were restored to their original values. The new substances were then found to be powerfully bactericidal (Albert, Hampton, Selbie and Simon, 1953). Not all substances which form complexes with iron are antibacterial. Work in hand suggests that inactivity can, in general, be correlated with too low a partition-coefficient.

Excellent as 8-hydroxyquinoline and its derivatives are in sterilizing a blood-free wound, they are rapidly inactivated by intact red cells (but not by lysed cells). Here is a worth while problem, to investigate how this inactivation takes place. Is the drug merely mechanically removed by the cells or, as seems more likely, is it metabolised, e.g. by sulphation (a common fate this for phenols elsewhere in the body). The solving of this problem may lead to the synthesis of simple analogues which would be active parenterally (in this connexion, see aureomycin, below).

The actual site of action of the 8-hydroxyquinoline complex inside the cell is unknown, but it appears to catalyse the oxidation of a meta-

bolically important metal binding group (apparently -SH), and this action is prevented by the presence of a trace of cobalt (Albert, Rubbo, Goldacre and Balfour, 1947; Rubbo, Albert and Gibson, 1950; Albert, Gibson and Rubbo, 1953).

The mode of action of *aminoacridines* has been shown to be a cationic effect (Albert, Rubbo, Goldacre, Davey and Stone, 1945). Thus, in a series of over one hundred acridines, those members which were highly ionized (as cation) at the pH of the test, were highly bacteriostatic, whereas those members which were poorly ionized were almost inactive. This sharp division applies even to groups of isomers. For example, 1-, 3- and 4-mono-aminoacridines are feebly ionized at pH 7 and feebly active at this pH, whereas 2- and 5-mono-aminoacridines are completely ionized and highly active.

This 5-aminoacridine (III) proved to be the best member of the series in clinical trials. Nevertheless the simple aminoacridines (including proflavine, « Rivanol » and « Trypaflavin ») are not highly active against bacteria when given parenterally. Even when pushed to their toxic limits, they are too rapidly eliminated from the body. This is a little surprising, because a somewhat more complex aminocridine (« Atebrin », also known as quinacrine or mepacrine) persists for some time in the blood and tissues and is a very effective antimalarial (it is only feebly antibacterial). The discovery of sulphonamides and of penicillin halted the search for an acridine, effective against bacteria in the bloodstream. However, there is every likelihood that the molecule of aminacrine could be altered to produce a drug better retained in the body, without sacrifice of antibacterial action.

5-Aminoacridine (Aminacrine of the British Pharmacopoeia) is used clinically in the sterilization of grossly infected wounds. It is not toxic when used in this way, and some clinicians consider that it is preferable to penicillin and sulphonamides, because it prevents any chance of the patient's acquiring a sensitivity which might be disastrous if these drugs had to be used later for general chemotherapy. Moreover it prevents the acquisition of penicillin- and sulphonamide-resistant organisms.

The aminoacridines appear to act upon a metabolic process taking place on the outside of cells (Albert, 1951). Unlike 8-hydroxyquinoline, they are not toxic to fungi, and they are not rapidly bactericidal. Where these qualities are not important, the aminoacridines are to be preferred to the hydroxyquinolines because the acridines are far less inhibited by red blood cells.

Further work established that the aminoacridines are not the only substances which are highly antibacterial when fully ionized as cations.