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The Site of Action of Penicillin

1. UPTAKE OF PENICILLIN ON BACTERIA

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Since the discovery of penicillin considerable data have been published about its mode of action. Among the findings which have a real bearing on the primary action of the drug on bacterial metabolism is the early observation that penicillin does not affect resting cells, but gradually inhibits the respiration of rapidly growing cells (Chain & Duthie, 1945). This would indicate that the killing effect is associated with the act of division, and for this reason studies with isolated enzyme systems are unlikely to progress far unless the enzymes studied are closely connected with cell division.

Gale & Rodwell (1949) have shown that by training a sensitive organism to dispense with certain amino-acids it becomes more resistant to penicillin without any contact with the drug. It appears that if an organism requires energy to transport preformed amino-acids from the external environment it will be sensitive to penicillin. In the case of one such amino-acid (glutamic acid, Gale & Taylor, 1947) it was found that penicillin prevented active transport across the cell wall. Active growth of the organism for several hours in the presence of penicillin is required for the prevention of amino-acid transport. This prevention may, therefore, be an end result of a series of reactions instituted by the penicillin, and is very probably still a secondary effect of the drug, although it may occur earlier than many other effects noticed. It remains to be seen whether this phenomenon is actually the cause of death of the organism.

The present studies aimed at examining the primary action of penicillin on the bacterial cell. The penicillin molecules which affect bacteria may

become attached to them. Hobby, Meyer & Chaffee (1942) were unable to show any definite removal of the drug by bacteria when using biological assay methods, and so a more sensitive method would be required to detect the minute amounts of penicillin which may be involved. The incorporation of a radioactive element in the antibiotic seemed a likely method of affording a high sensitivity, and had the added advantage that the fate of the penicillin could be followed after reaction with the organism. Radioactive ^{35}S was added to a synthetic penicillin medium, and the crude labelled penicillin extracted from the culture filtrate was utilized in our experiments. Initially some difficulties were encountered owing to the very small amount of penicillin removed by the bacteria, and to the very soft radiations of ^{35}S (Rowley, Miller, Rowlands & Lester Smith, 1948). However, by using penicillin of the highest specific activity and by directly measuring the radioactivity on thin layers of dried bacteria, consistent results have been obtained (Cooper & Rowley 1949).

EXPERIMENTAL

Preparation of radioactive penicillin

The chemically defined medium of Jarvis & Johnson (1947) for the growth of the mould has been successfully modified by decreasing the S content to about a quarter, thereby deliberately sacrificing the total yield of penicillin in favour of a higher yield calculated on the S. The radioactive S in most instances was supplied as Na_2S , which was converted to sulphate by boiling with ammonia and redistilled H_2O_2 . After inoculation with a loopful of vegetative mould suspension, the medium was shaken for about 4 days at 24° and

filtered free from mycelium. The broth and washings were cooled in ice, acidified to pH 2 with HCl, and rapidly extracted several times with ice-cold ether. The penicillin was extracted from this with dilute phosphate buffer at pH 7.5 containing 0.1% of sodium hexametaphosphate to stabilize the penicillin.

Table 1 shows the quantities used and yields obtained. The penicillin was dispensed in ampoules (40 units), freeze dried and stored in the refrigerator. The biological and radioactivities were redetermined at weekly intervals.

Table 1. *Yields of radio penicillin obtained in five fermentations*

Vol. medium (ml.)	³⁵ S concn. (μ c./ml.)	Penicillin yield (units)	Conversion of ³⁵ S (%)	Specific activity	
				(μ c./unit)	(Counts/unit)
2	50	330	20	0.060	12,100
10	50	2500	18	0.037	7,500
10	88	1500	16	0.095	19,000
2	250	220	8.0	0.183	37,000
5	100	420	5.3	0.063	12,800

Uptake of penicillin by bacteria

At least 50 mg. of dry bacteria were required for each experiment, and the organisms were harvested from 16 hr. cultures on agar plates and employed in concentrations of 0.1–10 mg. dry wt./ml. of medium. None of the organisms employed produced penicillinase.

To measure uptake, a bacterial suspension in broth was divided into several equal portions (generally 2 ml.) and incubated for 1 hr. Radio penicillin was added in varying amounts, and the suspensions were incubated for a further hour. The organisms were then centrifuged at about 1500 rev./min. for 20 min., washed once and resuspended in distilled water.

Samples of these suspensions (0.1 ml.) were immediately transferred to 9.9 ml. of formol saline for turbidimetric assay on a Hilger absorptiometer, calibrated in terms of bacterial dry wt./ml. Similar portions placed on 1 cm. nickel planchettes were dried at 120° for 20 min. and kept in a desiccator until counted. Spreading of the samples was readily achieved by diluting with distilled water until the suspension covered all the surface of the planchette, when the bacteria settled evenly and were not disturbed by drying. Under these conditions the sample layer was never thicker than 0.3–0.6 mg./sq.cm., when self-absorption effects were found to be negligible.

When they had been sampled for turbidimetric and total radioactive assay, the bacterial suspensions in distilled water were centrifuged and the clear supernatant was counted. This usually contained negligible radioactivity. The difference between this count and that of the suspension as a whole was taken to be the radioactivity attached to the bacteria. This difference method would tend to reduce possible errors from background variation, and is equivalent to counting background for a time equal to that taken over each bacterial suspension.

The activity was low, about 10 counts/min. above background, which was reasonably constant at 14 counts/min. Either nine or five samples of each suspension were counted for 5 or 10 min. respectively, giving a total of about 500 counts above background from which the probable statistical error to be expected is $\pm 7\%$. This, with an error of $\pm 3\%$ from the absorptiometer readings and an unknown error in

taking samples, produced results which varied within the approximate limits of $\pm 20\%$.

Uptake has been expressed as International penicillin units/g. dry wt. of bacteria.

Assay of radio penicillin. The radioactive assays were performed with standard equipment and using well-known techniques. The equipment consists of a thin mica window Geiger Müller counter (approximately 2 mg./sq.cm.; General Electric Co. Ltd) operating into a T.R.E. type 1014 probe unit, the counts being registered by a type 200A. scaler. The

resolving time of the probe unit was set at 400 μ sec., the counting rate being sufficiently low (below 600 counts/min.) to neglect any correction from this source of error.

Total counts were of the order of twice the background. Although the use of 'infinitely thick' layers of material on the planchette would have increased the counting rate, the measurements were confined to thicknesses below that at which self-absorption corrections became necessary. This choice was mainly dictated by the small quantity of radio penicillin available, together with the difficulty of placing sufficient material on the planchette. An experimental curve was derived relating loss of counts from self-absorption with wt./unit area of material on the planchette. It was found that a thickness of 1.0 mg./sq.cm. gave an error of approximately 5%. The layer thickness used in the experiments never exceeded 0.6 mg./sq.cm. and were generally much less, thus giving a negligible correction.

All counts were comparative, and the radioactivity of the penicillin was assayed at weekly intervals. A ³⁵S standard, giving approximately 500 counts/min., was counted at regular intervals together with a background count, enabling a decay curve of ³⁵S to be plotted. This with a U standard count provided a rough check on the operation of the equipment. The geometrical arrangement below the window of the counter was identical in all the experiments, thus ensuring constant efficiency and scatter. The planchettes employed were sufficiently thick for the back scatter to be saturated.

RESULTS

Variation in uptake with concentration of penicillin in medium

Bacteria are unable to divide when incubated in broth above a certain bacterial concentration (the 'M concentration', Bail, 1929). Under these 'resting' conditions (e.g. 3 mg. dry wt./ml.) and in saline, the penicillin taken up on the bacteria was found to increase with the concentration of penicillin both with sensitive and with resistant organisms. Four sensitive staphylococci tested differed in shape of uptake curve from two resistant staphylococci, and

from *Escherichia coli* and *Klebsiella pneumoniae*, at penicillin concentrations less than 0.1 unit/ml. (Fig. 1 shows two typical curves).

Fig. 1. Penicillin uptake on resting sensitive (—□—□—) and resistant (—△—△—) staphylococci on suspension in solutions of the drug. The theoretical uptake is calculated on the assumption that all the water removed by drying the cells at 120° is available for solution of the penicillin.

Fig. 2. Relation between penicillin uptake and penicillin sensitivity of rapidly growing bacteria.

The penicillin concentration attained inside the cell at these low titres was much greater than that in the external environment with sensitive organisms, but much less in the case of resistant ones.

Under conditions of rapid division the uptake was greater, and even quite resistant organisms could concentrate penicillin from the medium to some extent. When 0.04 unit/ml. of radioactive penicillin was incubated for 1 hr. with a rapidly growing culture, the uptake was found to increase with the sensitivity of the strain (Fig. 2).

Uptake with time

If radio penicillin is added to a 'resting' culture of a sensitive staphylococcus, which is then immediately centrifuged, the uptake is found to be essentially the same after 3 min. as after longer contact times.

Fig. 3. Rate of penicillin uptake (—△—△—) on rapidly growing sensitive staphylococci, compared with rate of growth in presence (—×—×—) and absence (—○—○—) of the drug. At the point ↓ radio penicillin was added up to a final concentration of 0.04 unit/ml.

If, on the other hand, the cells are rapidly dividing the initial uptake is greater, and then increases at a measurable rate to a value over tenfold that of the 'resting value' (Fig. 3), reaching a maximum shortly before growth is stopped. Thus it can be seen that a rapid initial uptake occurs under all conditions of contact, and this uptake is augmented when the cell is allowed to divide in the presence of penicillin. The initial rapid uptake is as complete at 0° as at 37°, within the 3 min. required to carry out a determination.

Attempts to prevent penicillin uptake

Penicillin-sensitive staphylococci in saline were treated with various agents for 30 min. at 37°, washed twice with saline and resuspended in broth at a concentration of 3 mg. of bacterial dry wt./ml. After 1 hr. incubation, radioactive penicillin was added to a concentration of 0.1 unit/ml. and after a further hour at 37° the organisms were centrifuged and washed prior to counting.

Results presented in Table 2 show that mere death of the cell was not sufficient to prevent its 'resting uptake', neither was the centre reacting with penicillin blocked by pretreatment with formaldehyde, CTAB, euflavin or hydrochloric acid.

Table 2. *Effect of some agents on the ability of sensitive staphylococci to take up penicillin from a 0.1 unit/ml. solution*

(Bacteria were allowed 0.5 hr. contact with agent and washed twice before the uptake was determined.)

Agent	Concn. (%, w/v)	Uptake on control (units/g.)	Uptake after treatment (units/g.)	Viability of cells after treatment with agent
Formalin	3 (v/v)	3.8	3.7	—
CTAB	0.05	4.4	2.3	—
Crystalline penicillin	200 unit/ml.	4.4	0	+
Eufavin	0.03	4.4	3.1	+
Acetic anhydride	10	11.5	0.5	Some survivors
Buffered acetic anhydride	10	11.5	0.4	+
Buffered acetyl chloride	10	11.5	1.8	+
Hydrochloric acid	N	11.5	9.1	Some survivors

Table 3. *Non-removal of penicillin from sensitive staphylococci by repeated washing in distilled water*

(Suspensions were allowed to stand for 20 min. between each centrifuging.)

Penicillin concn. from which uptake took place (unit/ml.)	Penicillin remaining on bacteria after 4 washings (units/g.)				Mean uptake (unit/g.) (average of columns 2-5)
	1st	2nd	3rd	4th	
0.25	1.8	2.2	2.4	2.9	2.3
1.5	3.2	2.9	3.1	3.9	3.3
3.0	2.7	4.5	3.3	3.4	3.5
7.2	4.0	5.5	6.2	4.8	5.1

Table 4. *Inability of several agents to remove penicillin from sensitive staphylococci*

Agent	Concn. (%, w/v)	Living cells after 1 hr. contact with penicillin (units/g.)		HCHO-treated cells after 1 hr. contact with penicillin (units/g.)		Living cells after 5 min. contact with penicillin (units/g.)		Cells rapidly grown with penicillin for 1 hr. (units/g.)	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated
Aerosol OT	0.1	3.0	3.0	3.4	1.7	4.3	3.1	5.2	6.6
CTAB	0.1	3.0	2.6	3.4	5.3	4.3	2.5	—	—
Penicillin	0.1	3.0	1.3	3.4	4.6	4.3	3.2	5.2	4.7
Sodium taurocholate	0.7	3.0	2.7	3.4	2.2	4.3	3.7	—	—
Cysteine HCl	1	—	—	—	—	4.3	4.2	—	—
Glucose	1	3.0	5.4	—	—	—	—	—	—
Phenol	5	4.5	3.2	—	—	—	—	—	—
NaOH	N	3.0	3.1	—	—	—	—	—	—
HCl	N	3.0	3.4	—	—	—	—	—	—

However, pretreatment with ordinary penicillin, or buffered acetic anhydride, completely prevented uptake within experimental error.

In an attempt to remove the 'active centre' from the cells along with the Gram-positive complex (Henry & Stacey, 1948), it was found that sensitive staphylococci which had been heated at 60° for 5 days in saline alone as well as with 1 % taurocholate, were unable to take up penicillin in 0.1 unit/ml.

Attempts to remove attached penicillin from staphylococci

Sensitive staphylococci (3 mg. dry wt./ml. in broth) were incubated with various concentrations of radio penicillin for 1 hr., centrifuged and washed. After radioactive and turbidimetric assay, the suspension was rewashed with distilled water and recounted four times. No change in attached radio-

activity was found (Table 3). Washing was similarly ineffective in removing radioactivity from resistant bacteria. Other samples of sensitive staphylococci were similarly treated with radioactive penicillin at 0.1 unit/ml., washed and incubated for 30 min. in saline with various agents to see if they removed the radioactivity. Samples were assayed after a further washing. None of the agents tested were effective in removing radioactivity from living or formaldehyde-killed staphylococci after 1 hr. contact or from cells rapidly grown for 1 hr. in presence of penicillin (Table 4).

However, it was found that within experimental error the bacteria completely lost any attached radioactivity after heating for several days in saline at 60°; furthermore, the organisms were unable to reabsorb any more penicillin from a 0.1 unit/ml. solution after this treatment.

DISCUSSION

All bacteria tested could take up penicillin to some extent. Resting, penicillin-sensitive bacteria could concentrate the drug from the external into the internal environment only at low external penicillin concentrations. With resting sensitive organisms at higher concentrations and with resistant organisms at all concentrations, the penicillin was always at a lower level inside than outside the cells.

This ability of sensitive bacteria to concentrate the drug from the medium cannot be explained as a simple diffusion across the cell wall. Furthermore, this high uptake is not a general property of all bacteria, so that it must be assumed that sensitive organisms possess a special centre which has a fairly high affinity for the drug.

That this concentration of radioactivity into the cell is directly related to the antibacterial action of penicillin is borne out by the following points. (1) It occurs only from 0.0-1 unit/ml., i.e. over the minimal concentration range in which penicillin-sensitive organisms are killed; the centre is apparently saturated at higher concentrations. (2) With a fixed concentration of penicillin in the medium, the uptake increases with the sensitivity of the strain. (3) Pre-treatment with ordinary crystalline penicillin prevents attachment of radioactivity from low concentrations, while CTAB and other compounds do not. This uptake is not, therefore, a general surface property, but is specific to the penicillin molecule. (4) Much more radioactivity becomes attached when the organisms are grown in presence of the drug. It is known that active growth in presence of penicillin is necessary for it to exert its antibacterial action.

The radioactivity is not removed by repeated washings, by detergents or by ordinary crystalline penicillin. As penicillin itself is known to possess only feeble surface activity, any adsorbed penicillin should be removed by these treatments. It therefore

seems unlikely that the reaction of penicillin with the cell is anything but a direct chemical combination with a cellular component; it does not seem to be an equilibrium reaction. This component is present in minute amounts in resting cells, but more appears to be produced during growth.

Some attempts were made to discover the chemical nature of this component by pretreatment of the cell with various reagents. Such experiments are inconclusive, as it is uncertain that the reagent will necessarily be able to affect the centre, or that any blockage noticed will not be due to a steric effect following reaction with a nearby group, but it is interesting to note that acetylating agents prevent uptake, while formaldehyde does not. Maas & Johnson (1949) have recently been able to demonstrate an uptake of firmly bound penicillin of a similar order to that reported above, using both biological and radioactive methods of assay. They were unable to correlate the uptake of the drug with sensitivity of the organism to penicillin and they say that it is entirely possible that this uptake may not be connected with the action of penicillin.

SUMMARY

1. The preparation of radioactive penicillin is described.

2. The amount of penicillin which becomes attached to bacteria under various conditions has been measured, and the uptake most probably responsible for its antibacterial activity has been distinguished.

3. This uptake appears to be due to a direct chemical reaction with a cellular component present in minute amounts in resting bacteria, more of which becomes available to the penicillin during growth.

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