S 86. *Studies with Radioactive Penicillin.*

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Penicillin labelled with ^{35}S has been prepared by growing the mould on a medium containing a low concentration of inorganic sulphate as the only source of sulphur. A high conversion of sulphur into penicillin was achieved, making it possible to detect as little as 0·002 International Unit by measuring the radioactivity. By this method penicillin can be detected in small concentrations at hitherto inaccessible sites.

The product has been used to measure the amount of penicillin taken up by sensitive bacteria and to trace the fate of penicillin when administered to experimental animals.

The study of the fundamental mode of action of penicillin on bacteria is beset with difficulties owing to the small amount of material involved. The uptake of penicillin by bacteria cannot be detected by biological methods since these depend on the penicillin being in solution. Attempts to observe a reduction in penicillin concentration caused by absorption by bacteria have been unsuccessful. As a result it is not known whether penicillin acts directly on the organisms themselves or whether it deprives them of some vital substance by reaction in the external environment. The incorporation of a radioactive tracer in molecules of penicillin has improved many-fold the accuracy of estimation and made possible the detection of penicillin in hitherto inaccessible places. This synthesis has made it feasible to study the interaction of the drug with bacteria themselves (Rowley, Miller, Rowlands, and Smith, *Nature*, 1948, **161**, 1009) and to trace the fate of penicillin in the body after different types of administration (Rowlands, Rowley, and Stewart, *Lancet*, 1948, II, 493).

The first suggestion for the preparation of the radioactive penicillin was to incorporate phenylacetic acid containing ^{14}C into the medium on which the mould was grown (Smith and Bide, *Biochem. J.*, 1948, **42**, xvii). The use of the carbon isotope was abandoned in favour of ^{35}S, mainly because the latter was available, but also because work at the Glaxo Laboratories had indicated that *Penicillium notatum* would grow on a medium containing a low concentration of inorganic sulphate as the only source of sulphur. It was necessary to use penicillin of the highest possible specific activity since preliminary biological experiments had shown that susceptible bacteria would take up only minute amounts of penicillin, if any at all. The mould was grown on a medium similar to that recommended by Jarvis and Johnson (*J. Amer. Chem. Soc.*, 1947, **69**, 3010), but with the sulphur content reduced by 75%. This had the effect of sacrificing total yield of penicillin in favour of high conversion of sulphur. For the first synthesis 100 microcuries of ^{35}S were available in 0·014 mg. of sulphur. To produce the minimum workable quantity of medium it was necessary to dilute this with inactive material, to give a total of 0·109 mg. of sulphur. The resultant 2 ml. of medium was diluted with 1 ml. of water to compensate for losses due to evaporation.

The medium containing the radioactive sulphate was autoclaved in a 50-ml. conical flask, inoculated with the mould, and shaken for four days in an incubator at 24°. After filtration the broth was cooled nearly to freezing point, acidified with 1 ml. of 0·1N-hydrochloric acid and extracted with ether. The ethereal extracts were then re-extracted into dilute phosphate buffer at pH 7·5 containing 0·1% of sodium hexametaphosphate to stabilise the penicillin (Smith, *Quart. J. Pharm.*, 1946, **19**, 309). The solution was finally adjusted to pH 6 and aerated to remove ether. The yield of this first synthesis was 250 I.U. of penicillin, representing a 12% conversion of sulphur in the broth into penicillin with an activity of 0·05 microcurie per I.U. Subsequent syntheses have produced penicillin with activities as high as 0·1

microcurie per I.U. and with sulphur conversions as high as 20%. It is interesting to note that the presence of ionising radiations did not adversely affect the growth of the mould. Control experiments showed that, if anything, production of penicillin was enhanced. An approximate calculation indicates that the exposure of the mould would amount to about 100 rep (roentgens equivalent physical).

The specific activity produced does not represent the highest attainable, since the radioactive sulphur had first to be diluted with inactive material. When stronger sources are available, the activity might be increased by a factor of five but at that concentration there might be deleterious effects of the radiations on the mould.

The radioactivity was measured by means of a Geiger–Müller counter with a thin mica window. The counter was filled with helium at atmospheric pressure, so that the 2·5-mg./sq.cm. window did not need support and the efficiency of detection was increased. With such soft β-radiations as from ^{35}S allowance has to be made for absorption of the electrons in the sample itself. The use of " infinitely thick " samples was rejected owing to the lower sensitivity for the very weak activities expected in the subsequent experiments. Instead, a curve was experimentally derived relating loss from self-absorption with weight per unit area of the source of radiation. It was possible in the majority of the experiments to use samples such that this correction was less than 10%. Taking into account statistical errors of counting and errors in making the absorption correction, it was found possible to measure 0·002 I.U. of penicillin by its radioactivity, with an accuracy of 5%, by counting for one hour.

With the best biological method it is possible to measure as little as 0·03 I.U. with an accuracy of 20%.

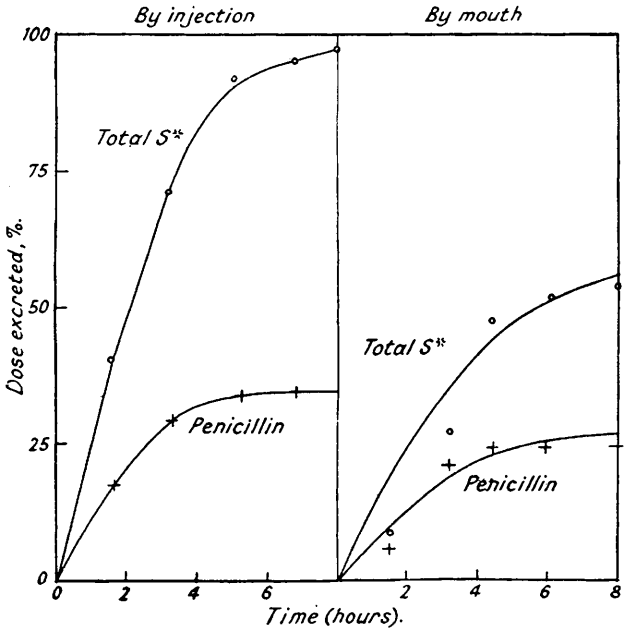
Experimental Work using Radioactive Penicillin.—The radioactive penicillin has so far been used in two types of investigation : one an attempt to detect an uptake of penicillin by susceptible bacteria (Rowley *et al.*, *loc. cit.*), and the other a study of the absorption and excretion of penicillin in experimental animals (Rowlands *et al.*, *loc. cit.*).

In the first investigation a measured amount of radioactive penicillin was added to a thick suspension of *Staphylococcus aureus* in broth. At definite intervals up to 24 hours small fractions of the suspension were removed, filtered through " Gradocol " membranes, and washed through with water. Aliquot portions of these filtrates were taken, dried on sample holders, weighed, and counted. Any uptake of penicillin by the bacteria should be shown by a decrease in the concentration of penicillin, in the filtrate. In spite of the killing of 95% of the bacteria in the suspension by the penicillin uptake could not be detected with certainty. From these results it was concluded that the absorption of penicillin probably amounted to less than ten molecules per bacterium. The objection to investigating the activity of the filtration residue was that, being bulky, it might cause considerable self-absorption of the radiations. By using penicillin of high specific activity and keeping a careful check on self-absorption, it has been possible recently to examine the bacteria themselves for radioactivity. With this direct approach a definite uptake could be detected. This is of the same order of magnitude as the lower limit expressed above, but it is less equivocal, in view of the difficulties of measuring the numbers and sizes of bacteria, to express the uptake in terms of units of penicillin per gram dry-weight of bacteria. The uptake was found to vary with the strain of organism and with its sensitivity to penicillin, amounting, on the average to 5 I.U./gm. dry-weight of bacteria (Cooper and Rowley, *Nature*, 1949, **163**, 480). Experiments are being continued in the Wright–Fleming Institute in an attempt to determine the actual site of absorption in the individual bacterium.

The second problem studied relates to the absorption and excretion of penicillin in experimental animals (Rowlands *et al.*, *loc. cit.*). Penicillin is rapidly excreted, and up to 70% is found unchanged in the urine. The fate of the remainder (whether it remained in the body or was excreted as breakdown products) was unknown. Radioactive penicillin was administered to female cats rendered docile by a mild anæsthetic. Samples of urine were examined for radioactivity and also for unchanged penicillin by means of a biological assay. Immediately the dose had been given, the bladder was emptied by a glass catheter, and this sample discarded. Thereafter the animals were catheterised at regular intervals for 8—10 hours.

The Figure shows the recovery plotted against time of both radioactive sulphur (S^*) and unchanged penicillin, for both intramuscular injection and oral administration. When the penicillin was given intramuscularly, all the radioactivity was recovered in the urine after 8 hours. The biological assay showed that only a fraction of the sulphur excreted was still in the form of penicillin. This made it clear that the penicillin breakdown products were excreted as rapidly as the penicillin itself and that there was no retention in the body.

When penicillin was given by mouth, by no means all the penicillin or breakdown products appeared in the urine. To investigate this further, one cat was killed 6 hours after oral administration of radioactive penicillin. Of the original radioactivity 60% still remained in the gut, whereas 25% had appeared in the urine. It is thus probable that the low efficiency of orally administered penicillin is due to non-absorption from the lumen of the gut and that, once it has crossed the intestinal wall, it behaves similarly to injected penicillin. It is suggested that the low absorption of orally administered penicillin is due to purely mechanical causes and that there is no fundamental difference in this respect between penicillin and other drugs. This question is to be investigated further.



Excretion of penicillin by cats.

The exact nature of the penicillin-breakdown products is of interest. Two of the possibilities are penicillamine and penicilloic acid. This question is being followed by tracer-dilution technique and by partition chromatography at the Wright–Fleming Institute. It may then be possible to throw some light on the site of decomposition of penicillin in the body, whether the breakdown occurs exclusively in some organ such as the liver or whether it occurs wherever penicillin is in contact with tissue fluids.

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