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A COMPARISON OF THE BIOLOGICAL PROPERTIES OF CEPHALOSPORIN N AND PENICILLIN

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Since cephalosporin N (abbreviated to "N" in this communication), which has been shown to be a new type of penicillin by Abraham, Newton, Crawford, Burton, and Hale (1953), differs strikingly from the previously known natural penicillins, it was decided to compare its behaviour towards bacteria and in the animal body with that of penicillin G.

MATERIALS AND METHODS

Materials.—We are indebted to the Antibiotics Research Station of the Medical Research Council for the preparation of the N. Nearly all the work was done with material having 25 units/mg., though for a few experiments samples having 29 and 32 units/mg. were taken. These arbitrary units are not in any way related to penicillin units, nor has the potency of N been expressed in terms of penicillin units or vice versa. It seems likely that pure N will have a potency of the order of 80 arbitrary units/mg.

For comparison, crystalline sodium penicillin G (Glaxo or Boots) of not less than 1,600 i.u./mg. was used. It was not weighed out, but the phials were assumed to contain the 100,000 or 200,000 units stated; inaccuracies which could thus result were partly eliminated by using as assay standard for any given experiment part of the solution actually used for that experiment.

Antibacterial Action.—Titrations were carried out in fluid media, using twofold dilutions throughout.

Assay of Antibiotics in Body Fluids.—In urine both antibiotics were assayed by the cylinder-plate method, using plates bulk-seeded with *Staph. aureus* NCTC 6571 for penicillin and with an avirulent *S. typhi* for N. Samples were diluted in phosphate buffer *p*H 6.8, and a standard curve was prepared for each set of assays from solutions of known strength made up in the same buffer.

For blood and cerebrospinal fluid assays, the cylinder-plate method required too much fluid and/or was insufficiently sensitive. Various micro-dilution methods with media containing fermentable sugars and an indicator (Florey *et al.*, 1949) were only moderately satisfactory, since the end-point was not sharp in the presence of serum. A micro vertical diffusion method using the same organisms as for the plate assays was much more satisfactory, and was carried out as follows:

Miniature test-tubes, 5–6 cm. long, were made from glass tubing 3.95 ± 0.05 mm. internal diameter, and were closed with glass caps made from wider tubing. After being dry sterilized they were placed in racks and filled with inoculated melted nutrient agar. A fixed volume (usually 8.7 μl.) of fluid to be assayed was layered on top of the agar, the tubes were incubated at 37° C. overnight, and the length of the zone of inhibition was then measured. With 8.7 μl. of solution and with *S. typhi* as test organism 10 μg./ml. of N or 9 μg./ml. of penicillin could be detected, while with *Staph. aureus* (a more sensitive test organism for penicillin) about 0.3 μg./ml. of penicillin was measurable. With *S. typhi* the length of the zone of inhibition could be measured to within 0.1 mm., but with *Staph. aureus* the growth boundary was less sharp, though still serviceable.

One drawback of the vertical diffusion method, especially with narrow tubes, is that the test solution is liable to leak between the solidified medium and the walls of the tube, thus giving spuriously high results. This possibility was eliminated by making the density of the medium, and thus of any fluid exuding from it, greater than that of the test solution by incorporating sucrose to a concentration of 10%, which apparently did not interfere with the growth of either test organism. Davis *et al.* (1950) reported, apropos a similar diffusion method with capillary tubes open at both ends, that if the glass were made water-repellent the solid medium was much less readily dislodged; such treatment of the glass tubes may well overcome the risk of test solution creeping between tube and solidified medium, but it was not tested, as the sucrose medium gave very satisfactory results.

The medium was made as follows: To a series of capped 1-oz. phials containing 100 mg. of agar powder and 10 ml. tryptic meat digest broth were added while warm 10 ml. of a solution containing 20% (w/v) sucrose and 2% glucose, sterilized by Seitz-filtration. Before use 2–4 drops of a broth culture of the test organism were added to the melted contents of a phial which were filled into the tubes with a pointed Pasteur pipette.

Blood samples were collected from rats after snipping the tip of the tail which, with the rest of the animal, was kept warm; the blood was taken into sterile capillaries which were sealed at both ends and placed in the refrigerator if not dealt with within one and a half hours. After centrifuging, the tube was cut just above the serum meniscus and the appropriate volume of serum, diluted if necessary with sterile normal rat serum, was transferred to the top of the seeded medium in an assay tube. Standards were set up at the same time with known amounts of antibiotic in the same volume of normal serum, and a standard curve was constructed from which the concentration of antibiotic in the unknowns could be read off. Two points should be mentioned:

(1) The curve was relatively steeper with just measurable amounts of antibiotic, possibly because oxygen is more available at the top of the tube. At the lowest level producing any effect, a few colonies appeared just below the surface of the agar, suggesting that better aeration had allowed them to develop although the concentration of antibiotic was slightly greater than that which inhibited the growth of the organisms just below them.

(2) When serum was assayed, a normal response was obtained with moderate or high concentrations of antibiotic. With low concentrations, however, the top 4–5 mm. of medium contained opaque material, usually in three distinct bands. This is presumably due to serum proteins which diffuse into the medium and are coagulated by acid generated by the bacteria in the lower parts of the tube. This opaque material obscures the position of the edge of the inhibition zone, but becomes transparent after half an hour or so if a drop of dilute alkali is added; if a little Indian ink is added to the alkali the upper boundary of the solid medium is more easily seen.

Cerebrospinal fluid was obtained by cisternal puncture of ether-anaesthetized rabbits, using the technique described by Markham *et al.* (1951). As normal cerebrospinal fluid free from antibiotic was not available, dilutions of the samples for assay and of standards were made in buffer.

Urine was obtained from female rats, catheterized with polythene tubing about 1 mm. in diameter, and was collected in tared phials and stored in the refrigerator.

RESULTS

Antibacterial Action

Potency and Range of Bacteria Inhibited.—The results of dilution tests in fluid medium with twofold dilution steps, carried out in parallel with N and penicillin, are summarized in Table I. Those for N broadly agree with the results of semi-quantitative streaking tests on solid medium done by Miss P. F. Boyd (unpublished). With both antibiotics the titre at 48 hours was either the same as that at 24 hours or was not more than one tube lower.

TABLE I

COMPARATIVE ANTIBACTERIAL TITRES OF CEPHALOSPORIN N AND PENICILLIN G

Organism	Inoculum. Broth Culture Diluted 1 in	Medium	Lowest Concn. (μg./ml.) Preventing Growth for 24 hr.	
			N	Penicillin
Staph. aureus NCTC 6571	1,000	HB	(*a*) 20 (*b*) 10	0·038 <0·095
Staph. aureus (penicillin resistant) ..	1,000	HB	1,000	13
M. tetragenus NCTC 7501	1,000	HB	10	<0·095
Sarcina lutea NCTC 248	1,000	HB	10	<0·095
Strep. pyogenes CN 10	10	HB+10% S	5	<0·095
Strep. viridans NCTC 3165	10	HB+10% S	10	0·19
Pneumococcus type I CN 33	10	HB+10% S	10	0·19
S. typhi avirulent ..	1,000	HB	(*a*) 10 (*b*) 5	4·8 6·0
S. typhi virulent ..	1,000	HB	(*a*) 5 (*b*) 6	4·8 >6·0
S. paratyphi B ..	1,000	HB	(*a*) 10 (*b*) 5	9·6 >6·0
S. paratyphi C ..	1,000	HB	(*a*) 5 (*b*) 5	0·3 0·75
S. typhimurium ..	1,000	HB	(*a*) 5 (*b*) 5	9·6 >6·0
S. enteritidis ..	1,000	HB	(*a*) 5 (*b*) 5	9·6 >6·0
Bact. friedländeri NCTC 5054 ..	1,000	HB	20	19·2
	100	HB+10% S	10	>6·0
Sh. shigae *Sh. sonnei* *Sh. flexneri* Z *Bact. coli*, type I	1,000	HB	>40	>6·0
Proteus vulgaris NCTC 3811 ..	1,000	HB	20	>6·0
Vibrio cholerae ..	100	HB	10	6·0
B. subtilis NCTC 1379	100	HB	5	0·19
B. anthracis avirulent	1,000	HB	5	<0·095
C. xerosis NCTC 7243	10	HB+10% S	20	0·38
C. diphtheriae gravis	10	HB+10% S	5	0·19
C. diphtheriae intermedius	10	HB+10% S	20	<0·095
C. diphtheriae mitis ..	10	HB+10% S	20	0·38
C. ovis	10	HB+10% S	10	0·38
L. monocytogenes NCTC 2167 ..	Undil.	Dextrose-phosphate Liver broth	5	0·19
Br. abortus	,,	,,	5	>6·0
P. pestis NCTC 144 ..	,,	HB+10% S	5	1·5
P. lepiseptica ..	,,	HB+10% S	10	1·5
P. muriseptica NCTC 948	,,	HB+10% S	10	3·0
N. meningitidis ..	,,	HB+20% S	5	<0·095
N. gonorrhoeae NCTC 8375	,,	HB+20% S	5	0·19
H. pertussis CN 134	,,	HB+20% S	5	0·75
H. influenzae (incubated 4 days) ..	,,	Fildes'	40	6·0
Cl. welchii NCTC 6125	100	HB+5% S	5	0·19
Cl. sporogenes NCTC 532	100	HB+5% S	5	0·19
Cl. tetani NCTC 279	100	HB+5% S	5	<0·095
Cl. septique NCTC 547	100	HB+5% S	5	0·19
Myco. tuberculosis H37Rv (human) (incubated 6 days)	*	Dubos	>40	>6·0
Myco. tuberculosis Branch (bovine) (incubated 6 days)	*	Dubos	>40	>6·0
Actinomyces graminis (Bostroem) ..	10	HB+10% S	>40	6·0

The inoculum was 0·02 ml. of a broth culture of the organism, diluted as indicated, per 2 ml. of medium.
HB=Ox heart infusion broth.
S=Rabbit serum, in percentage indicated.
* For *Myco. tuberculosis* the inoculum was 0·1 ml. of an undiluted 6-day culture in Dubos medium, per 5 ml.

The majority of the organisms were inhibited by 5–20 μg. of N/ml.; none of those tested were more sensitive than this, and a few were not affected by 40 μg./ml., the highest concentration used.

Nature of Antibacterial Action.—Viable counts showed that the action of N on *Staph. aureus* NCTC 6571 in tryptic meat digest broth was bactericidal, the count progressively falling to zero within 24 hours or so of the start of incubation. The rate of killing was the same with a just inhibitory concentration as with three times this concentration.

Combined Action of N and Cephalosporin P_1.—Since these two antibiotics are formed concurrently by the same cephalosporium (Burton and Abraham, 1951 ; Crawford *et al.*, 1952), it was of interest to study their combined action.

In the first experiment, with *Staph. aureus* NCTC 6571, tubes of tryptic meat digest broth were made up containing 2.25, 0.75, and 0.25 μg./ml. of crystalline cephalosporin P_1 (abbreviated here to "P"). These concentrations are above, approximately at, and below the minimum inhibitory concentration. A similar set of tubes contained in addition about one-fifth the minimum inhibitory concentration of N. Six more tubes contained three concentrations of N, with and without one-fifth the minimum inhibitory concentration of P. The warmed tubes were inoculated with approximately equal numbers of staphylococci and pour-plate viable counts were made at intervals.

The results were quite clear-cut and showed that the presence of a low concentration of P or N had a slightly antagonistic effect on the antibacterial action of the other.

An attempt was made to repeat the experiment with an avirulent strain of *S. typhi.* However, as the highest concentration of P that was used (0.1 mg./ml.) had no effect on the growth of the organism, the fact that it did not influence the killing effect of N may have little significance.

Cross-resistance.—Staph. aureus NCTC 6571 made more resistant to penicillin is more resistant to N, and vice versa.

Toxicity

Intravenous Injection (Mice).—The slow injection of as much as 100 mg. of N in 0.45 ml. distilled water into the tail vein in nine 20-g. mice failed to produce the slightest noticeable effect in seven of them, either immediately or during the subsequent seven days. Of the other two mice one showed slight waltzing movements and the other appeared slightly sick, but these effects lasted a few minutes only.

By contrast, mice were killed by lower doses of penicillin given in the same way and in the same strength (w/v) solution in distilled water (Table II).

TABLE II
TOXICITY OF CRYSTALLINE PENICILLIN G GIVEN INTRAVENOUSLY TO 20-G. MICE

Dose of Penicillin (mg.)	Mice Injected	Died	Survived
90	4	4	0
70	4	3	1
60	4	1	3
50	5	1	4

All the mice which died did so within 4–60 minutes of the injection, except one receiving 70 mg., which survived between 2½ and 15 hours. The solution, which was injected slowly into the tail vein, contained 20% (w/v) of penicillin in distilled water.

The toxicity of penicillin is said to be related to that of the cation, and it is quite possible that the three samples of N used here, having 32, 29, and 29 units/mg., contained less total cation than did the two batches of penicillin.

Intracisternal Injection (Rabbits).—Ten mg. of N in 0.5 ml. distilled water administered intracisternally to a 3 kg. rabbit caused mild disorientation and muscular weakness for several hours, but the animal appeared almost normal on the following day. Samples of cerebrospinal fluid withdrawn after 5 hr. 20 min. and after 23 hr. contained 0.24 and <0.012 mg./ml., the lowest concentration of N detectable, respectively. Both samples formed a tenuous clot and were cloudy from the presence of cells, mainly polymorphonuclear leucocytes with a few lymphocytes.

Another rabbit, weighing 2.8 kg., received 55 mg. N in 0.52 ml. water. As soon as the effect of the ether had worn off, the animal showed twitching and weakness, and made unsuccessful attempts to stand. After about an hour it became drowsy and nystagmus was evident. Three hours after the injection the animal was comatose, but thereafter it slowly recovered, though even after 24 hours it was weak. Samples of cerebrospinal fluid taken after 5 hr. 20 min. and after 24 hr. contained 1.8 and 0.065 mg. of N/ml. respectively. Both samples were cloudy.

Penicillin was injected into two rabbits of 2.7 kg. in the same way ; 5 mg. in 0.5 ml. of distilled water or normal saline caused fits leading to death after about 45 and 30 minutes respectively.

The rate of disappearance of N from the cerebrospinal fluid would appear to be of the same order as that reported for penicillin.

Absorption, Distribution in the Body, and Excretion

Distribution of N in the Blood.—Experiments in which N was added to heparinized rabbit blood, and then estimated in the plasma separated therefrom at intervals, showed that, like penicillin, N does not appreciably enter the red blood corpuscles.

Disappearance from Serum after Intravenous Injection.—The antibiotics, dissolved in 0.5–1.5 ml. of very dilute phosphate buffer or normal saline, were injected into the exposed saphenous vein of anaesthetized rats. Blood samples were taken at intervals, and the antibiotic content of the serum was plotted against time after injection. From the smoothed curves, serum levels of antibiotic at 10, 20, 30, 40, 60, 80, 100, 120, 150, and 180 minutes after injection were read off and replotted as a percentage of the theoretical serum level at zero time (plotted logarithmically), against time after injection. The results are shown in Fig. 1.

The theoretical level at zero time was calculated on the assumptions that the blood volume is 1/13th of the body weight, and that the volume of corpuscles is 40% of the volume of blood.

Concentration in Serum and Excretion After Subcutaneous Injection.—The results of two typical experiments in rats are shown in Fig. 2. The results of all the experiments are summarized in Fig. 3 (serum levels) and Fig. 4 (excretion data). As might be expected, the rate of excretion of both antibiotics was closely dependent on their concentration in the serum, but was not influenced by the rate of urine formation.

The general pattern of excretion was similar for the two substances, though less N was recovered than penicillin, and its peak rate of excretion occurred slightly later.

Concentration in Serum and Excretion after Gastro-intestinal Administration.—Three mice received 25 mg. of N by stomach tube ; three other mice received 7.5 mg. of penicillin by the same route. Four hours later the animals were killed, and the amounts of antibiotic remaining in the gut and present in the urine were measured. In all the mice well over 90% of the dose could not be accounted for, presumably partly because of the destruction by acid gastric juice. Of the residual material, less penicillin than N was found in the gut and more in the urine.

To avoid destruction by acid in the stomach, female rats were anaesthetized with ether, catheterized, and 1 ml. of solution of antibiotic in water was introduced through an abdominal incision into the duodenum. The abdominal cavity was closed, and urine and blood samples were collected at intervals. After about 4 hours the rat was killed and the contents of the small intestine were washed out with buffer and assayed. These results and the urine assays are summarized in Table III ; with regard to serum levels, that of penicillin rose to a low but detectable level after 30–60 min. and persisted for two hours before gradually falling. The peak level was from one-tenth to one-thirtieth of that after a comparable dose of penicillin given subcutaneously. N could not be detected in the serum at any time, because of the much lower sensitivity of test organisms to this antibiotic. Table III confirms the impression gained from the mouse experiments that N is less readily absorbed from the intestine than is penicillin.

Although distilled water was used for dissolving the antibiotics for this experiment the solutions probably approached the tonicity of normal saline. At any rate, pieces of duodenum taken at the end

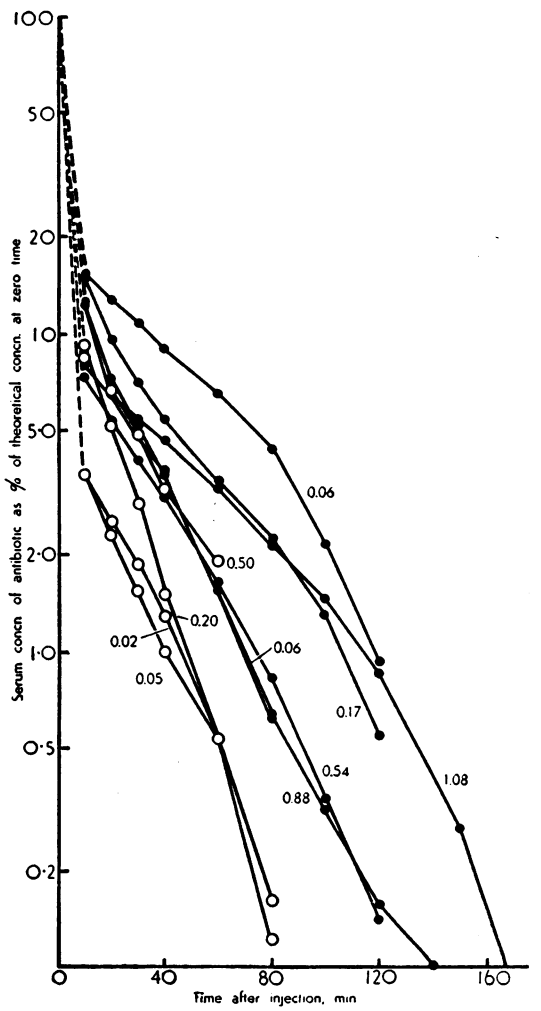

FIG. 1.—Concentration in serum of cephalosporin N (solid circles) and penicillin G (open circles) at different times after intravenous injection into rats of the doses (g./kg. body weight) indicated. The concentration is expressed on a logarithmic scale.

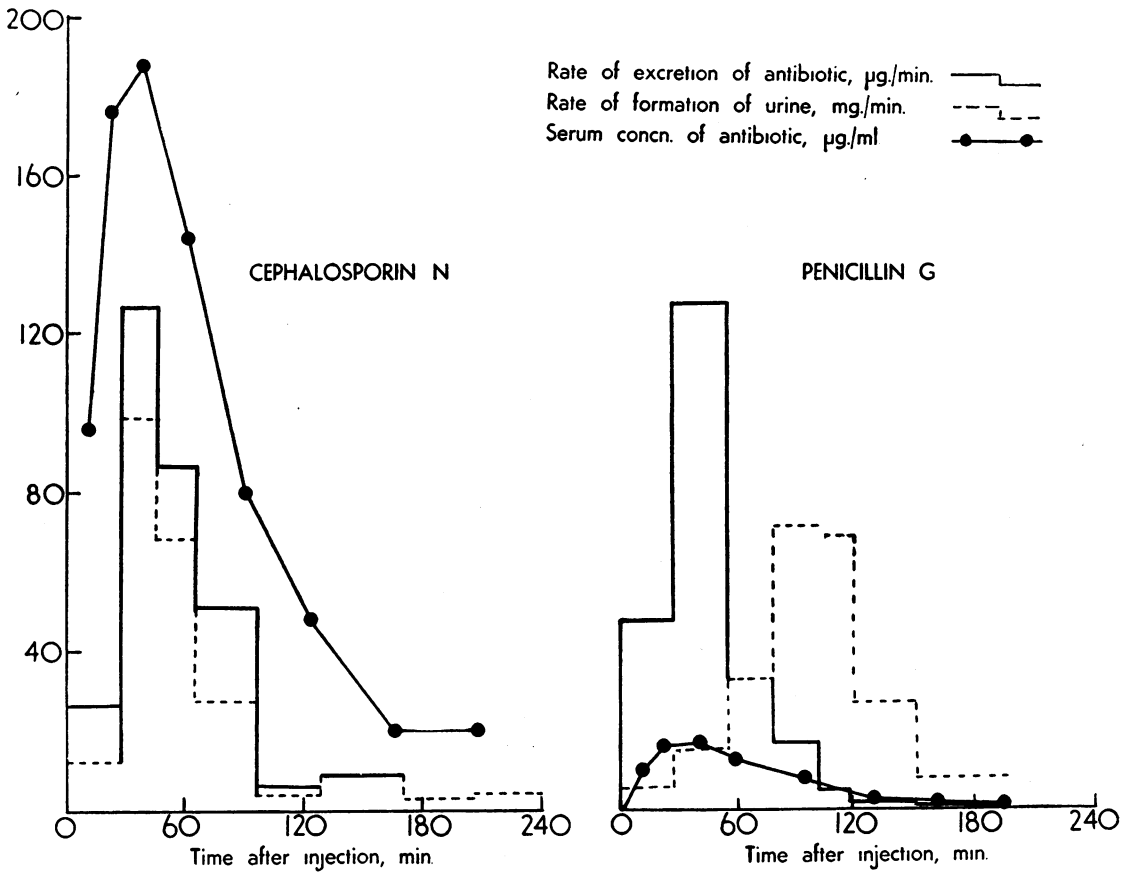

FIG. 2.—Typical results of subcutaneous injection of 30 mg. cephalosporin N (left) and 10 mg. penicillin G (right) into 200-g.rats. In the former the rate of excretion of N happens to coincide with the rate of formation of urine, but usually there is no correlation, as on the right.

of some of the experiments showed histologically no signs of irritation or of damage to the villi.

TABLE III

DISTRIBUTION OF PENICILLIN OR N AFTER DIRECT INTRODUCTION IN 1 ML. WATER INTO THE DUODENUM OF THE RAT

Dose (g./kg.)	Per Cent of Dose Recovered	
	In Urine	From Gut
Penicillin:		
0·06	11·1	13·8
0·11	8·0	33
0·15	20·7	11·5
0·23	11·1	25
0·26	4·1	24
Cephalosporin N:		
0·17	<2·3	41·3
0·30	Lost	30
0·38	<0·15	52
0·55	<0·5	38
0·86	<0·1	32

Experiments lasted about 4 hr. Rats weighed 165–200 g. Urine was collected in several fractions by catheter.

Effect of Animal Passage on Hydrophilic Character of N.—It seemed possible that N might be changed during its passage through the animal body. No information on this point was obtained other than that the active material in the urine of rats which had received N intravenously was, like N itself (but unlike penicillin G), not extracted preferentially into butyl acetate from watery solution at *p*H 2.

DISCUSSION

The investigations of Abraham *et al.* (1953) leave no doubt that cephalosporin N is a form of penicillin with physical and chemical properties distinguishing it sharply from the types of penicillin known hitherto. The experiments described in this paper were undertaken to see if these different properties conferred on N a distinctive behaviour in the animal body and towards bacteria. In

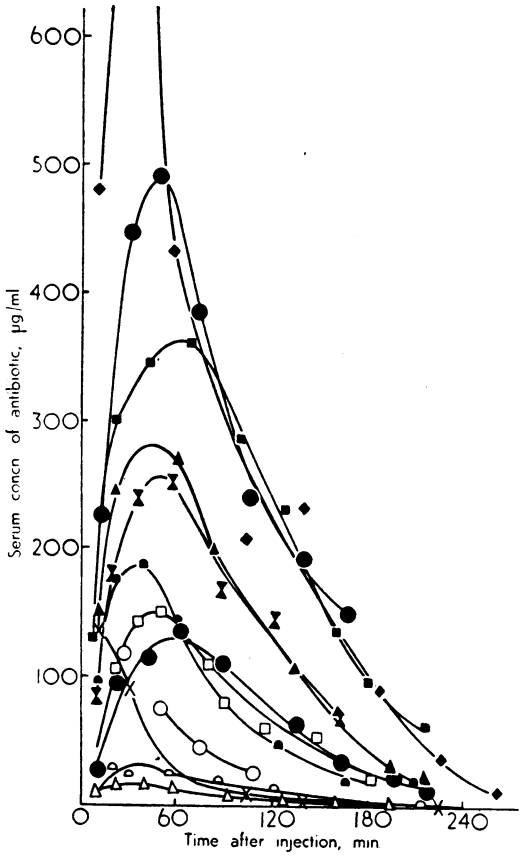

FIG. 3.—Serum concentration of cephalosporin N (solid points) and penicillin G (open points) at different times after subcutaneous injection of various doses into rats. Doses (g./kg. body weight) were as follows: Cephalosporin N: 0.54, ◆——◆, ●——● (upper); 0.49, ■——■, ▲——▲; 0.33, ⧗——⧗; 0.23, ●——● (lower); 0.15, ◗——◖. Penicillin G: 0.45, □——□; 0.15, ×——×; ○——○; ⌂——⌂; 0.05, △——△.

particular an answer was sought as to whether it might have therapeutic advantages over penicillin G and similar penicillins.

Impure N is certainly as little toxic to mice as "pure" penicillin G, possibly less so.

Against most bacteria—with certain exceptions important clinically, e.g., *Myco tuberculosis, Salmonella* spp., penicillin-resistant staphylococci, *Bact. friedländeri,* etc., against which it is less active—N seems to have a uniform minimum inhibitory concentration of the order of 10 µg./ml. Penicillin, while having a similar potency against several Gram negative organisms, including *S. typhi,* is over a hundred times more active against many Gram positive pathogens. Thus, in the most general terms it is only against certain organisms causing diseases not normally treated by penicillin that the potency of the two antibiotics is comparable, weight for weight.

In the experiments on absorption and excretion there was a wide variation of response from animal to animal with both drugs which could not be related to sex, body weight, rate of urine formation, or any other factor, but the following statements can be made with some certainty:

1. After intravenous administration N persists longer and gives a higher level in the serum at any one time than does an approximately equimolar dose of penicillin. Initially both drugs rapidly leave the circulation, more than 80% of the dose having disappeared during the first 10 minutes; thereafter the fall in the serum concentration of N tends to be more gradual than that of penicillin, though the relative rate of excretion of both increases slightly towards the end of the experiment. The slight concavity at the beginning of the N curves (Fig. 1) suggests that the initial escape of N from the blood stream is not as rapid as that of penicillin.

2. After subcutaneous administration the serum level of N is again higher than that of penicillin (Fig. 3), the peak levels occurring after 30–60 and 15–45 minutes respectively. Urinary excretion is substantially complete with both drugs after 100–150 minutes, and the peak occurs at 30–40 minutes.

3. For a given serum concentration penicillin is more rapidly excreted in the urine than is N. However, the latter persists in the serum only slightly longer than does penicillin, presumably due to its greater destruction or disappearance in the animal body, as shown by its lower total urinary recovery.

4. Though quantitative experiments on absorption from the gut were incomplete, owing to the concentration of N in the various body fluids being scarcely if at all detectable, it is clear that N is absorbed from the intestine more slowly than is penicillin.

The doses of penicillin given in the animal experiments were, from a therapeutic point of view, very large. This arose from the conviction that the substances should be compared on a roughly equimolar basis. Large amounts of N, and therefore of penicillin also, had to be used in order that readily measurable amounts of N might be present in the body fluids. (The molecular weight of N is not known. In this work it was assumed to be twice or three times that of penicillin G.)

Because the differences in behaviour between penicillin G and N in the animal body are not striking, and the advantages in antibacterial action

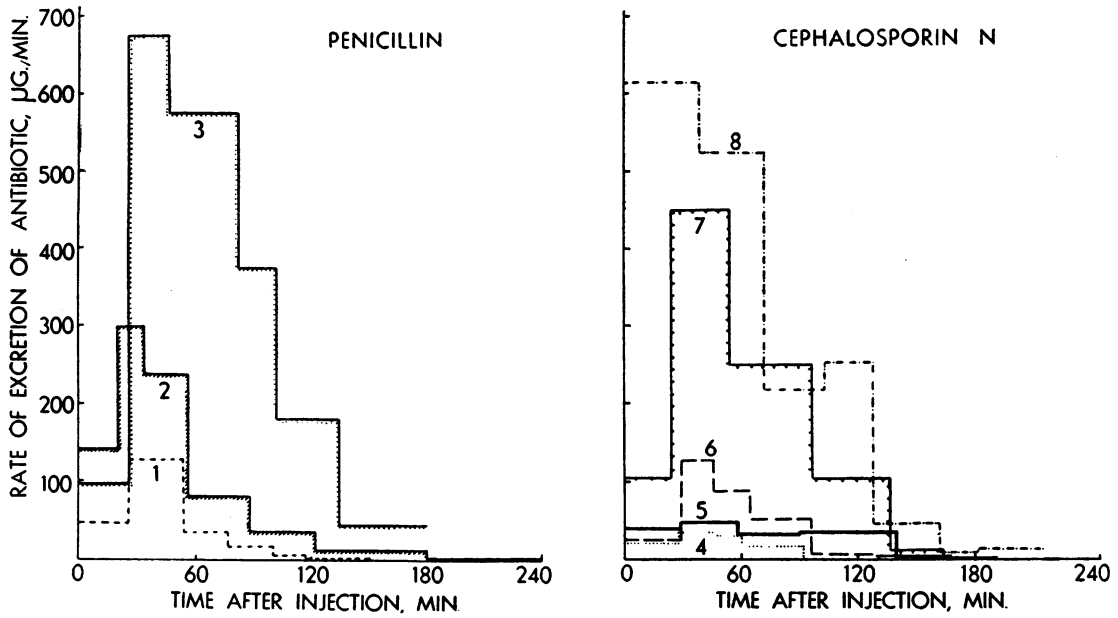

FIG. 4.—Rate of excretion of penicillin G (left) and cephalosporin N (right) after subcutaneous injection of various doses in rats. Dosages (g./kg. body weight) and per cent of dose recovered in urine (in brackets) were as follows:—Penicillin: Exp. 1, 0.05 (61%); Exp. 2, 0.15 (57%); Exp. 3, 0.45 (58%). Cephalosporin N: Exp. 4, 0.30 (4%); Exp. 5, 0.23 (12.7%); Exp. 6, 0.15 (23%); Exp. 7, 0.49 (35%); Exp. 8, 0.49 (63%). In Exp. 8 the high recovery and the earliness of the peak of excretion suggest that inadvertently some of the dose may have been given intravenously.

are either nil or more or less strongly in favour of penicillin (depending on the organism under consideration), cephalosporin N would not appear to be so good a therapeutic agent as penicillin G, in spite of the possible greater toxicity of the latter, except perhaps in one respect. Now that there is considerable fear of using chloromycetin because of its effect on the bone marrow, it might be worth exploring anew the possibility of using penicillin for the treatment of typhoid fever. Since N appears to be less readily absorbed from the gut than penicillin, it could conceivably be more effective than the latter if it could be brought into the lower small intestine. Unfortunately N, like penicillin, is subject to destruction in the stomach by the acid gastric juice, and in the intestine by penicillinase-producing bacteria.

On present knowledge it seems safe to conclude that N has no immediate therapeutic future.

SUMMARY

1. The minimum inhibitory concentrations of cephalosporin N and penicillin G against a number of micro-organisms have been measured.

2. The two substances have been compared in respect of their absorption from subcutaneous tissue and intestine, the maintenance of serum levels, and excretion by the kidneys. While their general behaviour is the same, N appears to be absorbed more slowly from the intestine, and to be excreted at a lower rate and in smaller amount than penicillin.

3. The toxicity of impure N, given intravenously to mice or intracisternally to rabbits, is less than that of penicillin G.

We are greatly indebted to Mr. N. Smith, who carried out all the antibacterial titrations and performed the viable counts; to Dr. A. G. Sanders, who assisted with the intracisternal punctures; and to Mr. J. Kent and Miss E. J. Page, who helped with the animal and other work.

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