SOME CHEMOTHERAPEUTIC AND PHARMACOLOGICAL ASPECTS OF THE NEW SYNTHETIC PENICILLINS

by

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(Presented at the Xth General Meeting of Japan Society of Chemotherapy, on June 8, 1962, in Tokyo, Japan)

The concept of chemotherapy as advocated by EHRLICH received considerable impetus by FLEMING's discovery of penicillin in 1929. However, the full significance of FLEMING's work was not fully appreciated until ABRAHAM, CHAIN, FLOREY and their colleagues in 1941[1] isolated natural penicillin in sufficient quantities for clinical evaluation.

The remarkable clinical effectiveness of penicillin stimulated a flood of research into the development of new natural antibiotics. Many of these, however, proved to be toxic and their side effects restricted their application. Nevertheless, this work diverted attention from penicillin, and research work in the penicillin field was mainly confined to studying possible modifications produced by the addition of different precursors to fermentation brews.

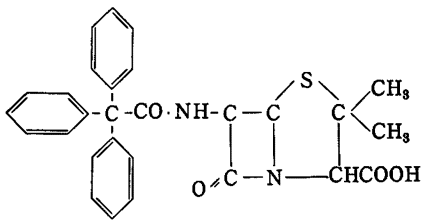
However, the research team at Beecham Research Laboratories in collaboration with Professor CHAIN, commenced an investigational programme with a view to the chemical modification of p-aminobenzyl penicillin. During the course of this work a marked discrepancy between the corresponding biological assays and the chemical assays was found. The cause of the discrepancy was investigated and this resulted in the recognition and subsequent isolation of 6-APA by my colleagues[2] (BATCHELOR, DOYLE, NAYLER and ROLINSON). We were now in a position to prepare numerous new penicillins by semi-synthetic routes which were hitherto unaccessible by the previous fermentation methods. It was hoped that by a systematic study of the new compounds it would provide in the first instance a lead to the development of a new penicillin which would be effective against staphylococci previously insensitive to the natural penicillins and which would still retain the character of non-toxicity. Our second objective was the development of another penicillin which would be effective against a wider range of organisms than penicillin G, particularly in the gram negative field.

In our approach to these studies we went on the assumption that *in vivo* activity might differ from *in vitro* activity. For example, a more active metabolite may be formed in the body or the activity of the antibiotic may be potentiated *in vivo* by some other mechanism. As far as was possible, therefore, all compounds which by *in vitro* tests were more stable to penicillinase than penicillin G were tested in the animal for activity against a penicillin-resistant and penicillin-sensitive staphylococcus.

The activity of the penicillins against penicillin-sensitive *Staphylococcus aureus* (SMITH) was assessed by determining the dose of penicillin needed to cure 50% (CD_{50}) of a group of mice infected intraperitoneally with a large dose of the organism. Activity against infections due to penicillin resistant *Staphylococcus pyogenes* (RUSSELL) was determined by the thigh lesion method described by SELBIE[3] and as adapted by BROWN and ACRED[4] for evaluating antibiotics.

In the first stages of our investigation we prepared a large number of different penicillins. Amongst these was tri-phenylacetyl penicillin :

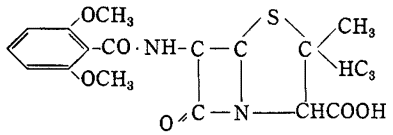
Fig. 1 Tri-phenylacetyl penicillin



This penicillin proved *in vitro* to be stable to Staphylococcal penicillinase and was active against the majority of resistant staphylococci at minimal inhibitory concentrations of 1~2 μg/ml. However, *in vivo* the compound proved to be completely inactive. No blood levels could be obtained as it was highly protein bound and was held firmly to the tissue proteins at the site of injection. In addition, after intravenous administration it proved to be toxic and therefore of no value clinically. This work, however, gave us a major pointer to the production of new penicillins which would be stable to penicillinase. It would appear that the steric configura-

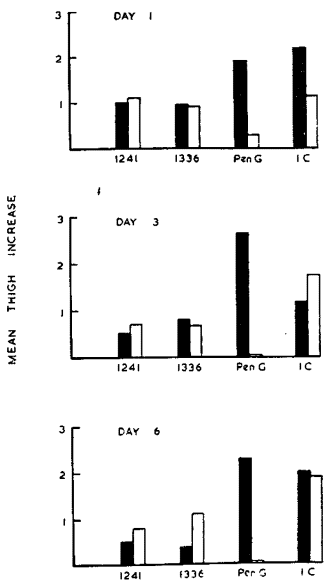
tion of the side chain prevents attachment of the enzyme and therefore prevents the disruption of the nucleus. A number of penicillins were now prepared which by their structural configuration would prevent the access of the enzyme to its points of attachment. This work led very quickly to the synthesis of 2:6-dimethoxybenzyl penicillin which has proved to be highly effective clinically and free from side effects.

Fig. 2 2:6-dimethoxybenzyl penicillin



A large number of analogous compounds to methicillin have also been prepared, but only a few have been shown to possess activity of the same order against both sensitive and resistant staphylococci. Derivatives of 1-naphthoic acid were among the more active of the new compounds. We therefore assessed these compounds by the 'double lesion'. In this procedure we injected the sensitive strain into one hind limb and the resistant strain into the other hind limb of a mouse. Groups of 10 animals were used and these were treated subcutaneously with the antibiotic for 3 days, and the mean increase in the thigh diameter was estimated and compared with the non-treated and non-infected control group.

Fig. 3 Mean increase compared with non-infected controls in thigh diameters of mice (10 per group) infected in the right thighs with *Staphylococcus pyogenes* (Russell)-a penicillin resistant staphylococcus, and infected in the left thighs with *Staphylococcus pyogenes* 2187-a penicillin sensitive staphylococcus. The mice were treated daily for three days with 100 mg/kg subcutaneously with each penicillin. I. C. - infected non-treated control group.

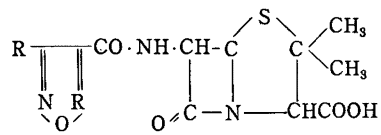


A comparison of 2-methoxynaphthyl penicillin and 2:6-dimethoxybenzyl penicillin is shown in Fig. 3. The effectiveness of both compounds against both the resistant and sensitive strains is demonstrated whereas penicillin G is only effective against the sensitive strain.

The penicillin derivatives of the following acids had little or no activity: Quinoline-4-carboxylic acid, Pyrimidine-5-carboxylic acid, Pyrazole-4-carboxylic acid, Indole-2-carboxylic acid, Benzofuran-2-carboxylic acid, Furan-3-carboxylic acid, Thiophene-2-carboxylic acid and Thiazole-4-carboxylic acid.

All of the preceding penicillins, however, have proved to be inactive orally, but a significant advance has been made recently with the synthesis in our laboratories of a series of isoxazolyl penicillins[5]. The general formula of this series is shown in Fig. 4.

Fig. 4 Isoxazolyl penicillins



R=alkyl or aryl radical

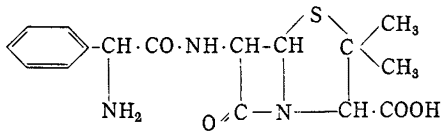
A number of these compounds have proved to be considerably active against both resistant and sensitive staphylococci. Among the more active of the compounds, however, the results *in vivo* show that there is little to choose between any of these compounds by the oral or intramuscular routes. One of these compounds, BRL. 1400 (5-methyl-3-phenyl-4-¡soxazolyl penicillin), has been examined extensively by Bristol Laboratories[6] and this compound is now in clinical use. We are continuing our examination of the series and have found that some have given superior blood levels in humans to BRL. 1400 (KNUDSEN, personal communication). Unfortunately detailed toxicity studies and clinical assessment have still to be completed, and it is too early at this stage to elaborate further.

Broad-spectrum Penicillin

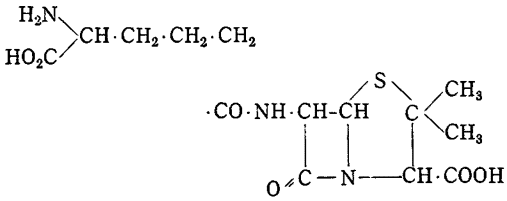
Penicillin G has a slight but significant activity against certain gram negative organisms *in vitro* but in general most of the new synthetic penicillins do not possess activity in this respect. However, we noted that cephalosporin N, which has an amino group in the side chain, showed a slight enhancement in activity. We felt, therefore, that the amino group might play a significant part in promoting activity against gram negative organisms.

Consequently, we prepared a number of penicillins

Fig. 5



Ampicillin-BRL. 1341



Cephalosporin N

with amino acid side chains and of these, α-aminobenzyl penicillin proved to be very active, and from the *in vitro* investigations by ROLINSON and STEVENS[7] it was shown to be slightly but significantly more active than chloramphenicol. We therefore carried out a comparison of the effect of ampicillin with chloramphenicol, tetracycline and streptomycin[8] in mice infected with both gram negative and gram positive organisms. The results *in vivo* clearly confirmed the *in vitro* results.

In these experimental infections, ampicillin was shown to be excedingly effective against both types of organisms. Clinically, particularly against the coliform group of organisms associated with urinary tract infections[9], the experimental expectations have been fully realised although the effects against active typhoid have been somewhat disappointing, but there are indications with increased and more frequent dosing better clinical responses can be obtained. Additional clinical tests show that ampicillin is also very effective in cases of chronic bronchitis, particularly those associated with *haemophilus influenzae*. The particular merit, however, of ampicillin is that it is devoid of systemic toxicity, and apart from subjects who are already sensitive to penicillin it can be administered safely in large doses. Most other oral antibiotics suffer from untoward toxic effects which can limit their usefulness.

General Pharmacology of Methicillin and Ampicillin

With regard to toxicity both drugs are remarkably free from acute toxic effects in the same way as penicillin G. In doses up to 3 g/kg both orally and subcutaneously in rats and mice no toxic symptoms have been seen with methicillin, but in the range of 3～5 g, occasional mild convulsions have occurred[10] (ACRED, BROWN, TURNER and WRIGHT). With ampicillin no toxic effects have been observed with doses of up to 5 g/kg[11] (ACRED, BROWN, TURNER, WILSON). In view of the very low order of toxicity we have not tested larger doses. Ampicillin and methicillin have also no long term toxic effects. In prolonged tests in dogs where the drugs were administered at 500 mg/kg daily for 30 days, neither compound has given rise to any sign of toxicity. Neither has the intramuscular injection of a 10% solution of each antibiotic caused a significant degree of irritation.

Absorption and Distribution

Methicillin is not absorbed orally, but by intramuscular injection it gives good blood levels which are comparable with penicillin G. On the other hand, ampicillin is well absorbed orally and intramuscularly in dogs. If administered orally it in fact gives superior levels to penicillin V, though in humans there is no clear advantage (KNUDSEN, personal communication).

Both antibiotics after intramuscular administration are similarly distributed throughout the body. Apart from the liver and kidney neither antibiotic is concentrated particularly in one organ. The high concentrations of antibiotic in these two organs, however, probably reflect the concentration of the antibiotic in the bile and urine and in no way represents an actual concentration in the tissue

Table 1 Activity of ampicillin, chloramphenicol, tetracycline and streptomycin against gram negative and gram positive infections in mice. The results are expressed in terms of the dose of antibiotic, administered either orally or subcutaneously, calculated to protect 50% of a group of infected mice (CD_{50} mg/kg).

Infecting organism	CD_{50} mg/kg							
	Ampicillin		Chloramphenicol		Tetracycline		Streptomycin	
	P. O.	S. C.	P. O.	S. C.	P. O.	S. C.	P. O.	S. C.
Staphylococcus aureus Smith	0.3	0.3	Inactive 100.0	Inactive 100.0	5.2	6.0	—	—
Streptococcus pyogenes Group A	0.1	0.025	3.2	3.2	0.5	0.5	—	—
Salmonella typhimurium	18.0	12.8	310.0	250.0	62.4	59.2	—	—
Klebsiella pneumoniae	11.6	35.4	165.0	280.0	Inactive 400.0	61.0	—	—
Escherichia coli	21.0	8.1	27.0	50.0	36.5	5.6	38.5	1.8

Fig. 6 Serum concentrations in dogs following intramuscular administration of 5 mg/kg. Ampicillin (——), Methicillin (-·-·) and Benzylpenicillin (·····).

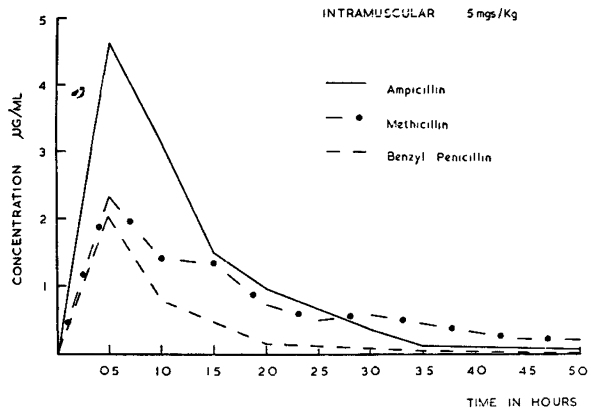


Fig. 7 Serum concentrations in dogs following oral administration of 20 mg/kg. Ampicillin (——) and Phenoxymethyl penicillin (·····).

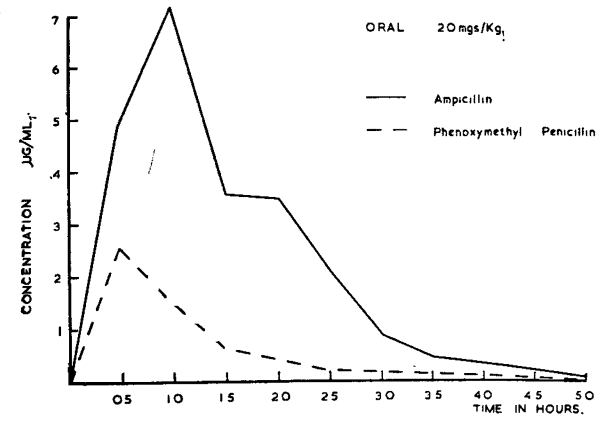


Table 2 Mean concentration µg/g of methicillin and ampicillin appearing in the liver, kidney and serum of groups of ten rats after intramuscular administration of doses of 100 mg/kg of each penicillin.

Ampicillin 100 mg/kg Intramuscularly

Organ \ Time hrs.	Concentration µg/g wet weight			
	0.5 hrs.	1.0 hrs.	2.0 hrs.	4.0 hrs.
Liver	175.0	70.5	5.8	1.0
Kidney	288.0	146.2	6.56	0.75
Serum	64.4	15.3	0.9	0.2

Methicillin 100 mg/kg Intramuscularly

Organ \ Time hrs.	Concentration µg/g wet weight			
	0.5 hrs.	1.0 hrs.	2.0 hrs.	4.0 hrs.
Liver	32.8	23.8	12.9	4.1
Kidney	122.1	88.8	24.1	2.9
Serum	32.5	30.2	5.46	0.18

spaces.

Neither methicillin nor ampicillin readily penetrate into the C. S. F. but both drugs can be given intrathecally provided the administration is controlled. Excess concentrations of both antibiotics can cause convulsions in the same way as penicillin G. Both the new penicillins can penetrate into the aqueous humour, but to obtain the most satisfactory results the drugs have to be given subconjunctivally. In our experiments the penicillins were injected in 0.5 ml volumes subconjunctivally into the right eyes of a group of 6 rabbits. A volume of 0.5 ml saline was injected into the left eye and at 1, 3 and 4 hours after administration two animals from each group were killed and the eyes carefully dissected out. The aqueous humour was drawn asceptically from the anterior chamber. At the same time serum specimens were also collected and the concentration of the antibiotic in the fluids determined.

Elimination

In studying the excretion of an antibiotic, almost invariably only the urine excretion is considered. However, excretion in other secretory fluids are also to be taken into account, particularly in the bile. We have found, for example, that in the conscious rat 15% of methicillin can be eliminated in the bile. Allowing for the errors of assay when the total amount excreted in the urine and in the bile is calculated, nearly all of the administered methicillin can be accounted for. With methicillin the absorption from the gut is negligible and any of the antibiotic which is returned to the gut by the bile or saliva is completely lost to the body. On the other hand, the penicillins which are absorbed orally tend to be re-cycled and this factor alone can acccunt for the more prolonged blood levels seen after the administration of orally absorbed penicillins.

With regard to the mode of elimination, ACRED *et al*[10,11] have shown that both methicillin and ampicillin are removed by the kidneys by renal tubular secretion and glomerular filtration in the same way as penicillin G, although it is much more difficult to block with probenecid the renal tubular secretion of ampicillin than the other two antibiotics. A quantitative study of the clearance rate of ampicillin has not yet been made, but should be of considerable interest to the pharmacologists.

Protein Binding

Finally, in considering the value of an antibiotic it is essential to appreciate the

Table 3 Concentrations of ampicillin and methicillin appearing in the aqueous humour and serum of groups of rabbits injected subconjunctivally in the left eye with 0.5 ml of a 25% solution of ampicillin or 50% solution of methicillin.

Penicillin \ Time	Concentration μg/ml											
	0.5 hrs.			1.0 hrs.			2.0 hrs.			4.0 hrs.		
	L	R	S	L	R	S	L	R	S	L	R	S
Methicillin	14.0	2.4	58.0	8.7	25.7	30.9	6.5	2.7	5.8	0.97	0.48	1.7
Ampicillin	60.0	0.7	12.3	48.0	0.8	5.6	19.0	0.3	0.4	1.1	0.4	0.1

L = Aqueous humour concentration μg/ml in injected eye.
R = Aqueous humour concentration μg/ml in control eye.
S = Serum concentration μg/ml.

significance of protein binding. It is generally assumed that protein binding leads to a decrease in antibacterial activity. Most studies have been carried out by static dialysis and assumptions on *in vivo* activity have been based on these *in vitro* results. However, the conditions in the body are vastly different and we feel that unless some information is available about the nature of the binding it is wrong to extrapolate from *in vitro* results activities *in vivo*. ACRED, BROWN, HARDY and MANSFORD[12] have studied protein binding on several penicillins by gel filtration, static and continuous dialysis techniques and have shown so far as the clinically active penicillins are concerned that the binding is very loose and that the penicillin is readily available. We have also shown that the concentrations of a penicillin which is bound to serum and another penicillin which is unbound to serum are the same or even greater in inflammatory fluids than in the serum. We injected glycogen, a non-chemotactic agent, intraperitoneally into two groups of rats, one which received 100 mg/kg ampicillin which is not protein bound, and the other which received 100 mg/kg of benzyl penicillin which is bound approximately 45% to serum proteins. At 0.5, 1, 2 and 4 hours afterwards 3 rats from each group were killed and the peritoneal fluid removed; at the same time samples of serum were taken for assay. The concentrations of the antibiotics in the peritoneal fluid and the serum are given in Table 4. Apart from the first half an hour after administration when the concentration of the antibiotic in the peritoneal fluid was less than in the serum, the concentrations subsequently were $1\,^1/_2$ to 2 times as great as the serum. This is contrary to what would be expected if the penicillins were firmly bound to the serum proteins, and clear evidence that the penicillin-protein complex breaks down readily and allows the free penicillin to diffuse into the tissue spaces.

In conclusion, the discovery of new penicillins had led to two notable advances in the field of chemotherapy. It is now possible to deal effectively with infections due to resistant staphylococci which have constituted a major problem in hospitals throughout the world. The introduction of oral penicillins effective against these organisms is a still further step towards combating these infections. The development of ampicillin has widened the clinical application of penicillins and much wider range of infections can now be successfully treated.

Table 4 Concentrations of penicillin in peritoneal fluid and serum after intramuscular administration of 100 mg/kg penicillin G and ampicillin to groups of rats.

Penicillin	Fluid	Concentration μg/ml			
		0.5 hrs.	1.0 hrs.	2.0 hrs.	4.0 hrs.
Ampicillin	Peritoneal	50.0	24.1	3.4	0.9
	Serum	62.1	12.7	1.8	0.4
Benzyl Penicillin	Peritoneal	37.0	40.0	18.0	1.0
	Serum	53.0	26.0	12.0	7.8

With these two additions in synthetic penicillins, the primary objectives of our research have been achieved but there is still considerable scope for the development of new penicillins. This applies particularly in the field of allergy where hypersensitivity to penicillins still remains a serious problem.

Bibliography

1) ABRAHAM, E. P., CHAIN, E., FLETCHER, C. M., FLOREY, H. W., CARDINER, A. D., HEATLEY, M. G. and JENNING, M. A. (1941). "Further observations on penicillin" Lancet, Vol. 2, p. 177~188.
2) BATCHELOR, F. R., DOYLE, F. P., NAYLER, J. H. C., and ROLINSON, G. N. (1959). "The synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations" Nature (Lond.) Vol. 183, p. 257.
3) SELBIE, F. R., and O'GRADY, F. (1954). "A measurable tuberculous lesion in the thigh of

the mouse" Brit. J. Exp. Path., Vol. XXXV, p. 556.

4) BROWN, D. M., and ACRED, P. (1960). "Chemotherapeutic studies on a new antibiotic-BRL. 1241" Lancet, September 10, p. 568～569.

5) DOYLE, F. P. D., LONG, A., STOVE, E., and NAYLER, J. H. C. (1961). "New penicillins stable towards both acid and penicillinase" Nature (Lond.), Vol. 192, p. 1183～1184.

6. GOUREVITCH, A., HUNT, G. A., PURSIANO, T. A., CARMACK, C. C., MOSES, A. J., and LEIN, J. (1961). "Microbiological evaluation of 5-methyl-3-phenyl-4-isoxazolyl penicillin" Antibiotics and Chemo., Vol. 11, p. 780～789.

7) ROLINSON, G. N., and STEVENS, S. (1961). "Microbiological studies on a new broad-spectrum penicillin, Penbritin" Brit. Med. J., July, 22, p. 191.

8) BROWN, D. M., and ACRED, P. (1961). "Penbritin——A new broad-spectrum antibiotic. Preliminary pharmacology and chemotherapy" Brit. Med. J., July, 22, p. 197.

9) BRUMFITT, W., PERCIVAL, A. and CARTER, M. J. (1962). "Treatment of urinary-tract infections with Ampicillin. A clinical trial" Lancet, January, 20, p. 130.

10) ACRED, P., BROWN, D. M., TURNER, D. H. and WRIGHT, D. (1961). "Pharmacology of Methicillin" Brit. J. Pharmacol., Vol. 17, p. 70～81.

11) ACRED, P., BROWN, D. M., TURNER, D. H. and WILSON, M. J., (1962). "Pharmacology and chemotherapy of Ampicillin——a new broad-spectrum penicillin" Brit. J. Pharmacol., Vol. 18, p. 356.

12) ACRED, P., BROWN, D. M., HARDY, T. L. and MANSFORD, K. R. L. (1962). "A new approach to studying the protein binding properties of penicillins" To be published.