tissue analysis of inositol-treated hens showed reductions in concentration of 9% and 9% of cholesterol total and esters in the aorta in 30 days and 14% and 12% in 52 to 68 days. The heart muscle levels dropped 37.5% and 43% in 30 days and 40% and 41% in 55 to 68 days, and those in the liver were 17.6% and 18.3% in 30 days and 25% and 20% in 55 to 68 days respectively below the levels in the controls established in previous studies.[8]

The results seem to indicate that inositol is a decholesterizing agent and effects some mobilization of cholesterol and cholesterol esters from the tissues.

15628

Prolongation of Penicillin Activity with Penicillinase-Inhibiting Compounds.

ROGER D. REID, LLOYD C. FELTON, AND MIRIAM A. PITTROFF.

From the Research Laboratories, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

Methods for prolonging the activity of penicillin in the body by use of various compounds to delay absorption or excretion have been proposed and reviewed fully in the literature. We have made extensive studies on an oral penicillin preparation composed of alum-precipitated penicillin or aluminum penicillin mixed with sodium benzoate. It has been determined from laboratory and clinical experience that sodium benzoate prolongs the blood serum levels when given with penicillin, either orally or parenterally. Other investigators studying this or similar compounds have explained the prolonged blood serum levels on the basis of renal blockage due to absorption of hippuric acid, formed from such compounds, by the renal tubules. Evidence to support this explanation is unconvincing with the dosage we have used. The literature is not clear as to how much sodium benzoate or benzoic acid is needed to produce sufficient hippuric acid to cause an effective renal blockage. In order to find another explanation we have attempted to demonstrate a mechanism that prevents or delays the destruction of penicillin by enzymes.

Normally the intestine contains a large number of "coliform" organisms that are known to produce penicillinase. If this is true then destruction of penicillin in the intestine might be prevented by (1) inhibition of the penicillinase-producing bacteria, or (2) allowing the bacteria to grow while preventing production of the enzyme, or (3) the destruction or inactivation of penicillinase produced.

The following preliminary experiments were made to clarify these points. To begin with, compounds such as sodium sulfathalidine and bromsaligenin, effective in inhibiting or killing the Gram-negative intestinal bacteria, were used. Substances having no inhibitory effect on the Gram-negative bacilli, such as sodium benzoate, sodium sulfanilate and *p*-amino benzoic acid, were selected for study as representing this group of compounds.

In the *in vitro* experiments flasks containing 100 ml of penicillin assay broth, F.D.A. modification, with 10 units of penicillin sodium (Lilly) per ml were inoculated with 0.2 ml of an 18-hour broth culture of *Escherichia coli*. Drugs were added to individual flasks in concentrations dependent upon their solubilities and estimated strength necessary to produce satisfactory results. Control flasks containing the drugs without penicillin were inoculated, and control flasks of penicillin with and without *Esch. coli* were incubated. Another control series in which the broth contained the penicillin and drug was inoculated with coli and incubated.

All flasks were incubated at 37°C. After

TABLE I.
Protection of Penicillin Against *Esch. coli* Factors *in Vitro*.

		Zone of inhibition of *Staph. aureus* 209-P after 18 hours incubation		
		Drug with 10 u/ml penicillin. Incubated with *Esch. coli* mm	Drug with 10 u/ml penicillin. No bacteria mm	Drug alone mm
Sodium benzoate	1.0%	27	34	0
Bromsaligenin	0.1	34	34	0
Sodium sulfathalidine	1.0	19	33	0
Phenylethyl alcohol	5.0	33	33	—
Sodium lauryl sulfate	0.5	28	32	12
" oleate	0.5	25	33	—
" stearate	0.5	0	32	0
" sulfanilate	1.0	0	32	0
Penicillin control, 10 u/ml			32	
Penicillin, 10 u/ml, incubated with *Esch. coli*			0	

TABLE II.
Protection of Penicillin Against Clarase *in vitro*.

Test	Zone of inhibition of *Staph. aureus* 209-P after 3 hr exposure to Clarase mm	Control	Zone of inhibition of *Staph. aureus* 209-P after 3 hr incubation mm
Sodium benzoate 0.5% Penicillin 10 u/ml Clarase 0.25%	15	Sodium benzoate 1.0% Penicillin 10 u/ml	38
Sodium benzoate 1.0% Penicillin 10 u/ml Clarase 0.25%	18	Sodium benzoate 1.0%	0
Sodium sulfanilate 0.1% Penicillin 10 u/ml Clarase 0.25%	16	Sodium sulfanilate 1.0% Penicillin 10 u/ml	40
Sodium sulfanilate 0.5% Penicillin 10 u/ml Clarase 0.25%	20	Sodium sulfanilate 1.0%	0
Sodium sulfanilate 1.0% Penicillin 10 u/ml Clarase 0.25%	24		
Methionine 0.1% Penicillin 10 u/ml Clarase 0.25%	12	Methionine 0.1 % Penicillin 10 u/ml	38
		Methionine 0.1%	0
Penicillin 10 u/ml Clarase 0.25%	0	Penicillin 10 u/ml	40

18 hours some of the material in each flask was passed through a Swinney filter to remove the bacteria and the filtrate added to the cups on a penicillin assay plate, using the standard F.D.A. technic with *Staphylococcus aureus* 209-P as the test organism. In the inoculated flasks there was no growth of *Esch. coli* if the medium contained bromsaligenin or phenylethyl alcohol, but there was good growth in the other inoculated flasks. Plate assays were made from all flasks regardless of growth of *Esch. coli*.

Table I shows the results of such assays.

The results in Table I indicate that there is some mechanism which interferes with penicillinase activity of *Esch. coli* without necessarily inhibiting the growth of that organism. The results indicate further that the inhibition of *Staph. aureus* 209-P on the test plates is not due to the inhibiting drug alone or to a concerted effect of the drug and penicillin but is due solely to the penicillin.

It appeared from the experiments just described that sodium benzoate may exert an antipenicillinase activity. If this were the case, it might be that factor which accounts for prolonged blood serum levels after administration of penicillin with sodium benzoate. To explore this possibility further, penicillin, penicillin plus sodium benzoate, and penicillin plus other compounds were exposed to the action of preformed penicillinase. If penicillin were protected under this set of conditions, it would indicate that protection is not due entirely to the prevention of penicillinase production. Penicillinase was first obtained by filtering broth cultures of a penicillinase-producing strain of *Esch. coli*. Later Clarase*[1] was used as the source of penicillinase.

Penicillin assay broth (F.D.A. modification)[2] was distributed into flasks in 100 ml amounts. To these 0.25% final concentration of Clarase was added. The various drugs were placed in individual flasks to test the antipenicillinase activity. Controls of the same nature as before were run. Mixtures were incubated at 37°C for 3-4 hours and penicillin assay plates were made as before, using *Staph. aureus* 209-P as the test organism. Results are shown in Table II.

These experiments seemed to indicate that certain compounds had the ability to interfere with the activity of penicillinase (in the form of Clarase) *in vitro*. It was decided to examine a rather large group of chemicals for these effects. The methods used in each instance were as described above. The compounds examined and results are shown in Table III. It will be observed that a number of the tested compounds inhibited *Esch. coli* partially or completely. As a result, there was no destruction of penicillin. These same compounds, however, failed to protect the penicillin from the Clarase activity. On the other hand, there were a few compounds which allowed the bacterium to grow, yet protected the penicillin from the enzyme. These compounds were found to confer protection on the penicillin when exposed to penicillinase enzyme. Selection of certain of these compounds for *in vivo* trial was made on the relative efficiency of the drug in protecting the penicillin against the enzyme *in vitro* and, secondly, upon the lack of toxicity of the drug. On this basis, sodium benzoate and sodium sulfanilate were selected as compounds not affecting growth of the bacteria yet protecting against one or the other enzyme, and bromsaligenin was used because of its bacteriostatic or bactericidal activity against Gram-negative bacilli.

Mice weighing 20 g were injected intraperitoneally with 0.5 ml of a 10^{-5} dilution of an 18-hour broth culture of *Diplococcus pneumoniae* Type I. Two hours later treatment of the mice was begun by intraperitoneal injection of penicillin with or without one of the penicillinase-inhibiting drugs. Treatment consisted of one daily dose of 50 units for each mouse. Final readings were made on the 7th day. Results are shown in Table IV.

There was no evidence of toxicity to sodium sulfanilate in the amounts used which was nearly 5.0 g daily per kg of body weight, This is in line with earlier studies on the toxicity of this compound.[3] There were no toxic effects noted in the case of the mice receiving the bromsaligenin (0.25 g per kg of body weight), although the mice were under the effect of general anesthesia for nearly 30 minutes following each treatment. Sodium benzoate given in the same amount as sodium sulfanilate caused death in the

* Clarase—the commercial name for a preparation made by Takamine Laboratories, Inc., which contains an enzyme active against penicillin.

[1] Lawrence, C. A., *Science*, 1943, **98**, 413.

[2] Schmidt, William H., and Moyer, Andrew J., *J. Bact.*, 1944, **47**, 199.

[3] Hebb, Arthur, Sullivan, S. G., and Felton, Lloyd, D., *Public Health Rep.*, 1939, **54**, 1730.

TABLE III.
Effect of Chemical Compounds on Clarase Activity and Activity of Penicillinase Produced by *Escherichia coli in vitro*.

Compound	% conc.	Growth of *Esch. coli*	Zone of inhibition of *Staph. aureus* after exposure to: *Esch. coli* mm	Clarase mm
Sodium caproate	1.0	+	28	0
" stearate	0.5	++	0	0
" oleate	0.5	?	25	14
" succinate	0.5	+	24	0
" adipate	0.1	++++	0	0
" lactate	1.0	++++	0	0
" tartrate	0.5	?	13	0
" citrate	0.5	+	23	0
Calcium gluconate	1.0	+++	0	0
Sodium thioglycollate	0.5	++++	0	0
" lauryl sulfate*	0.5	++	28	32
" stearyl sulfate	0.1	++++	0	0
" formaldehyde sulfoxylate	0.5	++++	0	0
" glutamate	0.5	+++	0	0
Methionine	0.1	+++	0	0
Barbituric acid	0.1	++++	0	0
Hexamethylene tetramine	1.0	0	20	0
Phenylazo-diaminopyridine mono-hydrochloride	0.1	+++	0	0
Ammonium sulfamate	0.5	?	0	12
Bromsaligenin	0.1	0	34	0
Phenylethyl alcohol	5.0	0	35	—
Sodium mandelate	1.0	+	0	12
" benzoate	1.0	++++	27	18
" *o*-chlorbenzoate	0.5	++++	0	0
" salicylate	1.0	0	30	0
" *p*-hydroxybenzoate	0.5	++++	0	0
" dibromsalicylaldehyde†	0.5	?	29	12
" anthranilate	0.5	+++	0	0
" *p*-aminobenzoate	1.0	++++	0	0
" *o*-sulfobenzoate	1.0	++++	11	0
" benzene sulfonate	1.0	++++	0	16
" *p*-chlorphenol sulfonate	0.5	0	28	20
" sulfanilate	1.0	++++	0	24
Sulfanilic acid	0.2	++++	0	16
Sodium acetyl sulfanilate	1.0	++++	0	20
Acetyl sulfanilic acid	1.0	++	0	20
Sodium dibrom sulfanilate	1.0	+++	13	17
" sulfanilamide	0.1	++++	0	0
" sulfathiazole	0.1	++++	0	0
" sulfathalidine	1.0	+++	19	—
p-Carboxybenzene sulfonamide	0.5	+++	0	0
Sodium β-resorcylate	1.0	++++	13	0
" di-iodo-β-resorcylate	1.0	?	26	12
" cholate	0.1	++++	0	0

* Sodium lauryl sulfate alone produced a zone of 12 mm.
† Sodium dibromsalicylaldehyde alone produced a zone of 11 mm.

mice within 30 minutes. Reducing the amount of sodium benzoate to .009 g per dose was not toxic for mice but failed to give added protection. It will be observed that a combination of penicillin and sodium sulfanilate, neither of which alone afforded adequate protection against pneumococci, gave complete protection to these mice. A combination approaching this in effectiveness was penicillin and bromsaligenin. So far as could be determined from the *in vitro* experiments, this effect of the latter drug was solely due to the inhibition of *Esch. coli*.

Rabbits were given penicillin alone and in combination with sodium sulfanilate, sodium benzoate or bromsaligenin. The rabbits

TABLE IV.
Effect of Penicillinase-inhibitors *in vivo*.

Treatment	Total dose	Deaths	Survivors
Penicillin, 50 u/day	200 units		
Sodium sulfanilate, .095 g/day	.37 g	0	20
Penicillin, 50 u/day	200 units		
Sodium benzoate, .0095 g/day*	.04 g	9	1
Penicillin, 50 u/day	200 units		
Bromsaligenin, .0045 g/day	.022 g	4	16
No treatment (controls)	0	20	0
Penicillin, 50 u/day	200 units	16	4
Sodium sulfanilate, .095 g/day	.19 g†	10	0

* There were 20 mice in this group to begin with, all of which died within an hour of the first dose of penicillin (50 units) containing .095 g of sodium benzoate. A second group of only 10 mice was then treated with 0.1 the amount of sodium benzoate, or .0095 g daily, as indicated in the table.

† These mice died before receiving a third dose of the drug.

weighed approximately 1.5 kg and the dose of penicillin in each case was 2000 units. Animals were bled at intervals of 1 to 1½ hours and blood serum levels were determined by the technic of Randall, Price and Welch[4] with modifications suggested by Chandler, Price and Randall.[5]

Sodium sulfanilate given with penicillin, intravenously, gave rise to somewhat prolonged blood serum levels of penicillin, whether the sodium sulfanilate was given intravenously or by mouth. Penicillin given intravenously with bromsaligenin by mouth also gave slightly prolonged penicillin levels in blood.

Discussion. In prolonging the activity of penicillin in the body, one or more of a number of factors may play an important part. *Delayed absorption* may be accomplished by injections of mixtures of penicillin with oil,[6] peanut oil and beeswax,[7] gelatin,[8] or alum[14] or by oral administration with such compounds as aluminum hydroxide gel.[9] Delayed absorption has also been attempted by the use of ice packs at the site of injection.[10]

Methods for *delaying excretion* that have been proposed include the use of penicillin with para-aminohippuric acid,[11] diodrast,[12] benzoic acid[13] and sodium benzoate.[14,15]

Acid-neutralizing agents for combination with penicillin for oral use have been incorporated into many preparations, usually in the form of buffers.

McDermott *et al.*[16] have pointed out that the advantage in protecting penicillin against stomach acidity, "is limited to the difference between the amount of absorption which occurs in the absence of such protection and the maximal absorption when acid destruction is not a factor." Since this amount is very small, they reason that no significant superiority will be noted when an acid-resistant salt of—or acid-protected penicillin is used, over one not so protected.

It therefore appears that any superiority

4 Randall, William A., Price, Clifford W., and Welch, Henry, *Science*, 1945, **101**, 365.

5 Chandler, Velma L., Price, Clifford W., and Randall, William A., *Science*, 1945, **102**, 355.

6 Raiziss, G. W., *Science*, 1944, **100**, 412.

7 Romansky, M. J., and Rittman, G. E., *Science*, 1944, **100**, 196.

8 Parkins, W. M., Wiley, M., Chandy, J., and Zintel, H. A., *Science*, 1945, **101**, 203.

9 Welch, Henry, Price, Clifford W., and Chandler, Velma L., *J. A. M. A.*, 1945, **128**, 845.

10 Trumper, M., and Hutter, A. M., *Science*, 1944, **100**, 432.

11 Beyer, Karl H., Woodward, Roland, Peters, Lawrence, Verway, W. F., and Mattis, P. A., *Science*, 1944, **100**, 107.

12 Rammelkamp, C. H., and Bradley, S. E., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 30.

13 Bronfenbrenner, Jack, and Favour, Cutting B., *Science*, 1945, **101**, 673.

14 Bohls, S. W., and Cook, E. B. M., *Texas State J. Med.*, 1945, **41**, 342.

15 Bohls, S. W., Cook, E. B. M., and Potter, R. T., *J. Ven. Dis. Inform.*, 1946, **27**, 69.

16 McDermott, Walsh, Bunn, Paul A., Benoit, Maria, DuBois, Rebeckah, and Reynolds, Mildred E., *Science*, 1946, **103**, 359.

of an oral penicillin preparation, and indeed a penicillin preparation for parenteral use, will be dependent upon some characteristic of the penicillin itself, or upon its combination with some substance which will protect it against agents in the intestinal tract or body fluids and tissues that destroy or inactivate it.

In the case of oral penicillin preparations, the amount that may be absorbed under the most favorable conditions is dependent upon the protection of penicillin, while in the intestine, from destruction by bacteria or their enzymes. We have demonstrated in the foregoing experiments that protection against the most common of these agents, penicillinase, can be had by a combination with sodium sulfanilate, sodium benzoate or certain other compounds. The mechanism of this protection cannot be explained at this time, since related compounds (chemically) fail to confer this protection.

It has further been observed that certain compounds, such as bromsaligenin, that do not interfere with enzymatic activity may produce a similar result by inhibiting the penicillinase-producing bacteria.

It can be shown that sodium sulfanilate or sodium benzoate protect penicillin to some extent against penicillinase *in vitro*. If breakdown of the penicillin after absorption is accomplished by enzymes in body tissues and fluids (and this seems likely since there is a considerable loss of penicillin even after parenteral administration), it seems possible that this, too, may be reduced by the protective effect of these drugs.

Conclusions. 1. Penicillin blood serum levels can be prolonged by the administration of penicillin with sodium sulfanilate, sodium benzoate, bromsaligenin or certain other compounds. 2. Effect of bromsaligenin on penicillin has been demonstrated *in vitro* to be due to inhibition of growth of *Esch. coli* and other Gram-negative intestinal bacteria. 3. The effect of sodium benzoate or sodium sulfanilate on penicillin has been shown *in vitro* to be due to inhibition, at least temporarily, of the enzyme penicillinase. 4. That such drugs are useful and effective in prolonging penicillin levels in the blood and so increasing the efficiency of penicillin has been shown by *in vivo* tests in mice and rabbits. 5. A list of compounds that do not possess this quality has been given, as well as some chemicals that give questionable results.

15629

Action of Penicillin on Staphylococcus. Effect of Size of Inoculum on the Test for Sensitivity.*

R. F. PARKER.

From the E. H. Cushing Laboratories of Experimental Medicine, Lakeside Hospital, and the Department of Medicine, Western Reserve University Medical School, Cleveland, Ohio.

In some of the early reports[1,2] on the antibacterial effect of penicillin, it is stated that the action of this agent appears to be unrelated to the number of bacteria present. Perhaps as a result of this, the commonly used tests[3,4] for penicillin susceptibility of organisms vary widely from laboratory to laboratory in respect to the size of the inoculum which is used. In the course of certain other work

* This work was supported in part by The Upjohn Company.

1 Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **241**, 177.

2 Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 178.

3 Rammelkamp, C. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

4 Spink, W. W., and Ferris, V., *Science*, 1945, **102**, 221.