Metabolism of Penicillins to Penicilloic Acids and 6-Aminopenicillanic Acid in Man and Its Significance in Assessing Penicillin Absorption

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Penicillins can be metabolized to penicilloic acids in man, the extent being dependent on the penicillin structure. In the phenoxy penicillin series, phenoxymethyl penicillin was found to be particularly unstable, but the higher homologues were more stable. In the isoxazolyl series, oxacillin was unstable, and progressive insertion of halogen in the phenyl ring increased stability. Ampicillin and amoxycillin showed some instability, ampicillin possibly being the more stable. After intramuscular administration, carbenicillin was very stable in the body, ampicillin was fairly stable, and benzyl penicillin was unstable. It is important to take into account the penicilloic acid content of urine when estimating total absorption of a penicillin. Increased stability in the body as well as slower renal clearance can lead to high concentrations in the serum. Penicilloic acids seemed to be more slowly cleared from the body than penicillins. The liver is probably the site of inactivation.

The present investigation of the fate of penicillins in healthy humans was carried out as a preliminary to studying what happens in humans with various types of bacterial infection. Penicillins can break down to bacteriologically inactive penicilloic acids by hydrolysis of the β-lactam ring, a reaction which occurs slowly in aqueous solution but rapidly in the presence of β-lactamase, an enzyme produced by many bacteria.

There has been very little work published on the occurrence of this reaction while penicillins are circulating in the body of man. However, Birner (2) reported the chromatographic detection and assay of penicilloic acids in human urine samples after oral administration of penicillin V and phenethicillin. Birner found high concentrations of penicilloic acid in urine, the concentrations often being as high or higher than those of the penicillin. From this it was clear that there was considerable destruction in the body, which is confirmed by our own work, but Birner did not calculate the proportion of the dose excreted as penicillin or discuss the significance of his results. His methods were very involved and depended on solvent extraction followed by thin-layer chromatography, removal from the plate, nitration, and spectrophotometric assay. This method is thus only applicable to solvent-extractable penicillins. Rosenblatt et al. (7) implicated the destruction of penicillin in the liver of man to help explain differences in concentrations in the blood after dosing with oxacillin, cloxacillin, and dicloxacillin. This work was extended by Standiford, Jordon, and Kirby (8) to cover benzylpenicillin, ampicillin, and carbenicillin, and similar conclusions were reached. Kind et al. (5) reported some inactivation of penicillin G and ampicillin in rat liver.

Removal of the side chain of penicillins is another possible route by which penicillins could be inactivated in the body, and English, Huang, and Sobin (4) noted the presence of 6-aminopenicillanic acid (6-APA) in the urine of man after oral administration of penicillin G, but they did not report the extent of the conversion. These workers concluded that gut bacteria were responsible for this reaction in animals as they could not find activity in tissue homogenates.

This present report gives the results of a survey of the extent of breakdown in man of a range of penicillins by the two routes, β-lactam hydrolysis and side-chain removal. The structure-stability relationships and the importance of measuring breakdown when assessing absorption of penicillins are also discussed.
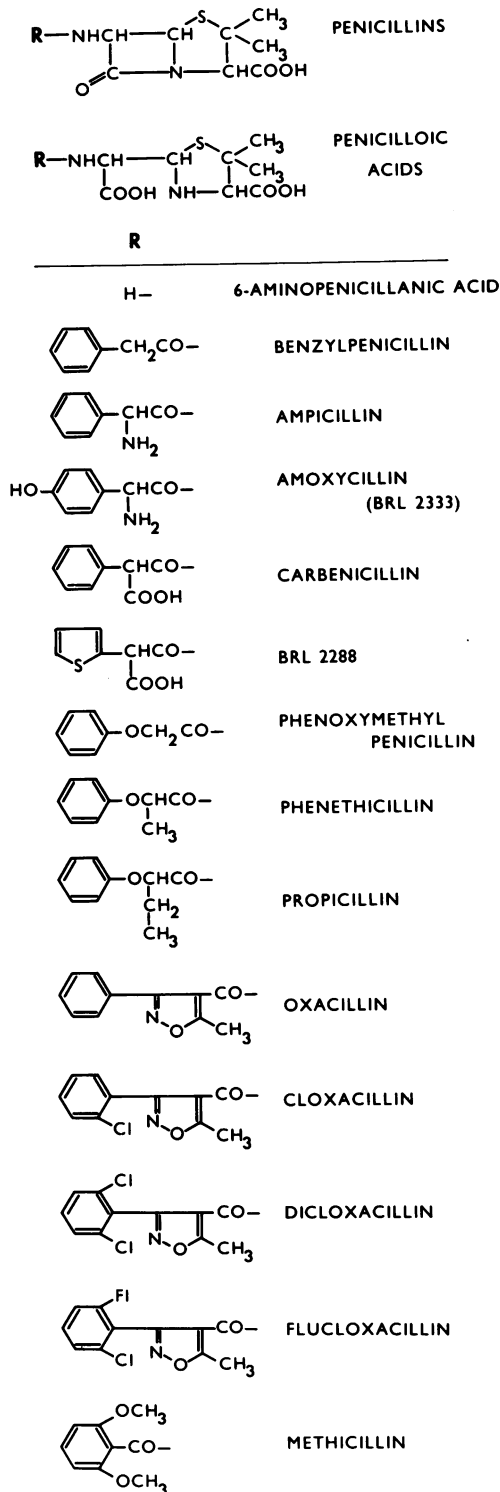
FIG. 1. *Structures of the penicillins and penicilloic acids.*

MATERIALS AND METHODS

Penicillins. All penicillins were made by Beecham Research Laboratories except oxacillin, which was obtained from Bristol Laboratories, Syracuse, N.Y., and penicillins V and G, from Glaxo Laboratories, Greenford, England. Amoxycillin is the *p*-hydroxy derivative of ampicillin, i.e., 6[D(−)α-amino-*p*-hydroxyphenylacetamido] penicillanic acid and BRL2288 is the 3-thienyl analogue of carbenicillin, i.e., α-carboxy-3-thienylmethyl penicillin. The former is now marketed as a well-absorbed broad-spectrum penicillin (10), and the latter is undergoing trial as an improved anti-*Pseudomonas* penicillin (9). The structures of these compounds are shown in Fig. 1. The purity of all penicillins was taken into account when determining the dose, which was expressed in terms of pure free acid penicillin. The penicilloic acid content of penicillins was determined by the iodometric assay as described later and was usually less than 5%. When penicillins were given by the intramuscular route, the penicilloic acid administered in the dose was subtracted from that recovered in the urine.

Preparation of penicilloic acids. To a 5% solution of penicillin in deionized water was added 5 N NaOH dropwise to bring the pH to 12.0. As the β-lactam ring opened, a new carboxyl group was generated, so the pH was kept constant by occasional addition of NaOH. The reaction was complete when the pH ceased to fall, usually within 3 h at room temperature. A measured amount of HCl was then added to bring the pH to 7.0 and the solution was freeze-dried or evaporated to dryness under vacuum.

All preparations were shown by thin-layer chromatography to give a single starch-iodine reacting zone characteristic of penicilloic acid. This zone migrated to the same position as the penicilloic acid made by reacting the penicillin with *Escherichia coli* β-lactamase (1 mg/ml, pH 7.0, 1 h). This test could only be applied to the β-lactamase-labile penicillins. All preparations were also shown to be devoid of residual penicillin by bioautograms. Analysis of sulphur content of the preparations was obtained by conversion to sulfate, precipitation with excess $BaCl_2$, and back titration of residual $BaCl_2$ with ethylenediaminetetraacetate. The sulphur content was in turn used to calculate the purity, which was usually at least 80%.

Experimental design and human volunteers. Wherever possible, the same six volunteers were used in each experiment. All volunteers were healthy and between the ages of 21 and 45 years. Each experiment had equal numbers of males and females, and they were given their penicillin dose after fasting overnight. The dose of each penicillin was expressed in terms of pure free acid penicillin. Approximately 1 h after the dose, they had breakfast and thereafter their usual meals.

Urine samples were usually collected over 0- to 6-h and 6- to 12-h periods after dosing with penicillin. Control urine samples were collected over a 0- to 6-h period from each volunteer 1 day before the experiment or, very occasionally, 2 days after. Treatment and control urine samples were assayed for iodine uptake by the method described below, the difference

being used to estimate the penicilloic acid content of the treatment samples.

Penicilloic acids in urine samples. The method of estimating penicilloic acids was based on the iodometric assay of Alicino (1), modified to reduce and make allowance for the iodine uptake by other substances present in urine samples. Theoretically, the method will estimate any penicillin decomposition product which readily reacts with iodine. However, for all of the penicillins tested, thin-layer chromatographic examination of urine samples from volunteers showed that the only starch-iodine reacting zone other than that for the penicillin was that running in the same position as the penicilloic acid marker.

The penicillins were shown not to contribute significantly to the iodine uptake by performing the penicilloic acid assay immediately after addition of penicillin to urine. The very small amount of iodine uptake was probably the result of penicilloic acid in the penicillin preparations. When the penicillins were incubated in urine for 6 h at pH 6.5 and 37 C, the penicilloic acid assay indicated a breakdown in the region of 5%.

Assay procedure for penicilloic acids in urine. The treatment and control urine samples were frozen at −18 C, thawed, and centrifuged to remove precipitate. A 2-ml sample was adjusted to pH 5.0 with 0.1 N HCl, and excess iodine was added (5 ml of approximately 0.01 N iodine solution in 3.2 M KI). The mixture was continuously stirred for 1 min; then the excess iodine was titrated with 0.005 N sodium thiosulfate with 2% soluble starch solution as indicator. By using water instead of the sample in the above procedure, the volume of 0.005 N thiosulfate equivalent to the 5 ml of iodine solution was determined. The difference between the two thiosulfate titrations was used to calculate the iodine uptake by the urine sample. The starch was obtained from B.D.H., Poole, England.

Subtraction of the iodine uptake value for the urine control from the value for the treatment sample gave the iodine uptake attributable to the penicilloic acid in the sample. This in turn was converted to micrograms of penicilloic acid per milliliter by reference to a standard line. Linear standard lines were obtained for each penicilloic acid by use of the above method to assay the iodine uptake of solutions in the range of 100 to 1,000 μg/ml. These solutions were made up in pH 5 pooled human urine, and a correction was made for the iodine uptake in a urine blank. The standards were corrected for purity and the penicilloic acid contents were expressed as pure free acid.

Factors affecting the penicilloic acid assay. Iodine uptake by urine was low and was little affected by pH in the range of pH 4 to 6, but above pH 6 it rose rapidly. At pH 5.0, the reaction between iodine and penicilloic acids was much more rapid than the reaction between iodine and urine. The pH values of urine samples containing penicilloic acids were thus adjusted to 5.0 and the iodine reaction was carried out over 1 min with continuous stirring.

Thin-layer chromatography of penicilloic acids and penicillins. Three 5-μliter urine samples were applied to Merck Silica Gel plates with markers of the administered penicillin and its penicilloic acid (5 μliters of 1 mg/ml in pooled urine). The solvent system was butanol-ethanol-water (2:1:1, vol/vol), and the starch-iodine-acetic acid spray of Thomas (11) was used to detect penicillin and penicilloic acid zones.

Paper chromatography of 6-APA. Urine samples were examined for 6-APA content by the phenylacetylated bioautographic method of Cole and Sutherland (3) with the use of the butanol-ethanol-water solvent system (3) and detection of zones by contacting with agar seeded with *Bacillus subtilis* ATCC 6633. 6-APA was run as a marker alongside all urine samples and migrated at a slower rate than penicillins and their active metabolites.

Bioassay of penicillins. The penicillin content of urine samples was assayed by the agar diffusion method on large plates. *Sarcina lutea* ATCC 9341 was used as assay organism for all penicillins except carbenicillin and BRL2288, for which *Pseudomonas aeruginosa* T 447 B was used, and benzyl penicillin, for which *B. subtilis* was used. The administered penicillin was used as standard with purity correction, and a doubling dilution series was placed on each assay plate. Each urine sample was assayed at three dilutions, all of which fell within the range of the standards. It is known that certain penicillins, notably ones with the isoxazolyl side chain and propicillin, undergo some transformation in humans to other antibacterial substances (6). However, it has been shown that these substances are present either in small amounts or have similar activity to the parent penicillin (6), from which it is concluded that their effect on the bioassays will be small.

RESULTS AND DISCUSSION

6-APA in urine of man. The results in Table 1 suggest that removal of the side chain of penicillins is a minor transformation route in man, the proportion of the dose appearing as 6-APA never exceeding 1%. However, if 6-APA is excreted by the biliary route, the transformation could be underestimated. The substrate profile for 6-APA liberation is quite unlike that for the penicillin acylase of enterobacteria, which suggests that it is not the gut flora which is responsible for this activity. This conclusion is contrary to that of English, Huang, and Sobin (4), but they gave no quantitative data and did not examine a range of penicillins.

Penicilloic acid levels in the urine of man: effect on estimation of absorption. It was established by thin-layer chromatography of all urine samples that in the quantitative iodometric assay procedure the major iodine-absorbing penicillin-derived material in urine was penicilloic acid (see Materials and Methods: Penicilloic acids in urine samples). Birner (2) recently reported penicilloic acid zones on thin-layer chromatograms after oral administration of phenoxymethyl penicillin and phene-

TABLE 1. *Presence of 6-aminopenicillanic acid (6-APA) in human urine samples after oral administration of penicillins*[a]

Penicillin (250 mg, oral)	Width of 6-APA zone (mm)		
	Subject 1	Subject 2	Subject 3
Phenoxymethyl penicillin .	18	14	17
Phenethicillin	trace	trace	13
Propicillin	15	13	17
Ampicillin	trace	trace	16
Benzyl penicillin	0	0	0
Oxacillin	0	0	0
Cloxacillin	0	0	0

[a] Urine samples (three superimposed spots each of 5 µliters) were applied to 1-cm-wide Whatman no. 1 paper strips. After chromatography in the butan-1-ol-ethanol-water system and phenylacetylation of one of a pair of strips, 6-APA and other zones were revealed by contacting with agar seeded with *B. subtilis*. Markers of 6-APA (5 µliters of 10 µg/ml solution) gave zones of about 18 mm, R_f 0.13. Benzylpenicillin is not usually administered by the oral route, but urine recoveries were 13, 3, and 6% for the three subjects.

thicillin to man, thus confirming our own observations with these compounds.

Results in Table 2 are the penicillin and penicilloic acid recoveries for a range of penicillins administered by the oral route to humans. It is clear that for most penicillins, a significant amount of the dose appears in the urine as penicilloic acid (y)% and that when this is added to the penicillin (x)% recovered in the urine it can have quite a dramatic effect on the estimated absorption of the penicillin $(x + y)$%. The highest penicilloic acid levels did not always occur in the same person.

Absorption and destruction of oral phenoxy penicillins. The most notable increased estimate of absorption obtained when excreted penicilloic acid was taken into account was found with phenoxymethyl penicillin and oxacillin, where the figures increased from 26% (x) to 60% $(x + y)$ and 17% to 33%, respectively (see Table 2). The experiments with these two compounds have been repeated with the mean results being 36% increased to 73% for phenoxymethyl penicillin and 23% to 45% for oxacillin.

The increased recovery of phenethicillin in urine compared with phenoxymethyl penicillin is in part due to higher total absorption $(x + y)$ increasing from 60% to 72%) and in part due to increased stability of phenethicillin in the body (penicilloic acid level, y, down from 34% to 23%). Propicillin is similar to phenethicillin.

The stability in the body is estimated by the percentage breakdown to penicilloic acid, $y/(x + y)$%, being 57% for phenoxymethyl penicillin and 31 to 32% for phenethicillin and propicillin. Birner (2) also reported high percentage breakdown figures which were in the region 60 to 80% and 30 to 55% for the 4- to 6-h period after administration of phenoxymethyl penicillin and phenethicillin, respectively.

Absorption and destruction of oral isoxazolyl penicillins. The pattern of results for the isoxazolyl series (Table 2) again shows that increased recovery of penicillin in the urine, when comparing oxacillin with its halogenated derivatives, is not only associated with increased total absorption $(x + y)$ but also with increased stability of the compounds in the body. Increased halogenation of the phenyl isoxazolyl side chain has a pronounced effect on stability in the body, the percentage breakdown figures, $y/(x + y)$, being down from 49% for oxacillin to 22% for cloxacillin and 10% for dicloxacillin. The penicilloic acid level in urine after oral administration of dicloxacillin and flucloxacillin is quite low, i.e., 4% of dose.

Absorption and destruction of oral α-amino penicillins. In the α-amino series, breakdown to penicilloic acid was again seen (Table 2), being in the region of 20 to 30% [$y/(x + y)$]. A greater proportion of the dose of amoxycillin appears as penicilloic acid (20 to 25%) than is the case for ampicillin (7 to 11%). This may be associated with the fact that amoxycillin is better absorbed and gives higher concentrations in the body. The results for total absorption $(x + y)$ at the 250-mg dose were 84% for amoxycillin and 54% for ampicillin. At the 500-mg dose the total absorption results were lower, namely, 74% for amoxycillin and 33% for ampicillin. However, in this 500-mg experiment, the urine recoveries of amoxycillin (49%) and ampicillin (26%) were lower than in an earlier 500-mg experiment (crossover with 12 volunteers), where respective mean values of 60% and 40% were obtained. With the use of an iodometric method which was a forerunner of the method described in this paper, penicilloic acid recoveries of 14% and 11% were obtained in the earlier experiment, giving total absorption figures $(x + y)$ of 74% for amoxycillin and 51% for ampicillin.

It is clear that amoxycillin is better absorbed than ampicillin, but, unlike the halogenated isoxazolyl penicillins, it does not have improved stability in the body.

Late excretion of penicilloic acids. It was noted that 2 to 9% of the 0- to 12-h penicillin

TABLE 2. *Urinary recovery of penicillins and penicilloic acids after oral administration of phenoxy, isoxazolyl, and amino penicillins to humans[a]*

Penicillin	Dose (mg)	No. of subjects	Percentage of dose recovered in 0–12 h		$(x + y)\%$ (Measure of absorption)	$(y)/(x + y)\%$ (Measure of breakdown)
			As penicillin (x)	As penicilloic acid (y)		
Phenoxy penicillins						
Phenoxymethyl	500	6	25.9 ± 12.3	34.4 ± 19.7^b	60.3 ± 22.7	56.6
Phenethicillin (α-phenoxyeth-yl)	500	6	49.8 ± 12.2	22.2 ± 14.4^b	72.4 ± 17.0	30.9
Propicillin (α-phenoxypropyl)	500	6	44.9 ± 21.2	21.2 ± 14.2	66.1 ± 24.2	32.2
Isoxazolyl penicillins						
Oxacillin	500	6	16.9 ± 13.2	16.1 ± 13.3^c	33.0 ± 23.9	48.7
Cloxacillin	500	6	38.1 ± 17.9	11.1 ± 12.9	49.2 ± 25.5	21.6
Dicloxacillin	500	6	32.9 ± 20.0	3.8 ± 7.6	36.7 ± 22.5	10.2
Flucloxacillin	500	6	40.6 ± 30.1	3.7 ± 5.1^c	44.3 ± 27.8	9.6
Amino penicillins						
Ampicillin	500	6	25.8 ± 17.1	6.7 ± 5.9^d	32.5 ± 19.0	20.9
Amoxycillin (p-hydroxy-α-aminobenzyl penicillin)	500	6	49.1 ± 13.4	24.7 ± 10.7^d	73.9 ± 18.6	33.4
Ampicillin[e]	250	10	43.3 ± 18.2	10.8 ± 6.4	54.0 ± 19.6	20.5
Amoxycillin[e]	250	10	63.4 ± 18.8	20.2 ± 11.4	83.6 ± 22.1	24.0

[a] The values in the table are the means for the numbers of subjects shown, and the limits of error are at the 95% probability level.
[b] $p < 0.05$ by the *t*-test.
[c] $p < 0.01$ by the *t*-test.
[d] $p < 0.001$ by the *t*-test.
[e] Recovery in 0 to 6 h.

TABLE 3. *Urinary recovery of penicillins and penicilloic acids after intramuscular injection of various penicillins to humans[a]*

Penicillin	Dose (mg)	Percentage of dose recovered in 0–12 h		$(x + y)\%$ (measure of absorption)	$(y)/(x + y)\%$ (measure of breakdown)
		As penicillin (x)	As penicilloic acid (y)		
Benzylpenicillin	300	66.4 ± 26.5	15.6 ± 12.7	81.9 ± 28.0	19.0
Methicillin	500	75.0 ± 13.3	6.9 ± 10.5	81.9 ± 19.8	8.1
Ampicillin	500	72.5 ± 21.0	10.0 ± 4.1^b	82.5 ± 21.5	12.2
Carbenicillin	500	81.5 ± 8.6	1.8 ± 4.0^b	83.3 ± 10.6	2.1
BRL2288 (α-carboxy-3-thienyl-methyl penicillin)	1,000	85.5 ± 10.1	14.6 ± 5.2	100.1 ± 8.2	14.6

[a] The values in the table are the means for six subjects, and the limits of error are at the 95% probability level.
[b] $P < 0.001$ by the *t*-test.

recovery was collected in the 6- to 12-h period, whereas the comparable figures for the penicilloic acids were 10 to 16% for the phenoxypenicilloic acids and 20 to 60% for the isoxazolylpenicilloic acids. It might be predicted that the time taken for transformation of the penicillin to penicilloic acid could result in a later peak of penicilloic acid excretion compared with penicillin. However, the proportion of the penicilloic acid which is excreted late, particularly for the isoxazolyl compounds, is such as to suggest a slower rate of excretion of the penicilloic acids. The results of Birner (2) for the penicilloic acid of phenoxymethyl peni-

cillin also show delayed excretion compared with the penicillin, but his results for phenethicillin do not show this trend so clearly.

Stability of penicillins after intramuscular administration. The results for the intramuscular route of administration are given in Table 3. The most surprising result in this set is that for carbenicillin, for which the breakdown to penicilloic acid is so small as to make it difficult to measure $[y/(x + y) = 2\%]$. The percentage breakdown $[y/(x + y)]$ for methicillin, ampicillin, and benzylpenicillin was, respectively, 8, 12, and 19%. When the penicilloic acid is taken into account, it gives total recovery data $(x + y)$ which are very similar for benzyl penicillin, methicillin, ampicillin, and carbenicillin, i.e., 82 to 83%. The remaining 17 to 18% presumably represents degradation beyond penicilloic acid or loss by other routes. For BRL2288, 100% recovery was obtained when allowance was made for penicilloic acid.

Relationship between steady-state serum levels and penicillin breakdown. Rosenblatt et al. (7) determined the mean drug concentrations in human plasma after administering 250 mg per h intravenously for 3 h and reported steady-state levels of 9.4 μg/ml for oxacillin, 14.4 μg/ml for cloxacillin, and 24.4 μg/ml for dicloxacillin. To explain the differences among these levels, Rosenblatt suggested that in addition to differences in rate of renal clearance (oxacillin twice as great as dicloxacillin) there was degradation by nonrenal mechanisms, probably the liver. The results of our present studies are in accord with these suggestions because we found an inverse relationship between percentage breakdown and the serum levels reported by Rosenblatt; thus, the penicillin showing the greatest breakdown, i.e., oxacillin, gives the lowest concentration in serum.

Standiford, Jordan, and Kirby (8) extended the studies on steady-state plasma levels after intravenous infusion to a comparison of benzyl penicillin, ampicillin, and carbenicillin. Again, there was an inverse relationship between these plasma levels and our percentage breakdown figures for these compounds. As in Rosenblatt's publication, it is suggested by Kirby's team that, although the renal mechanism was the most important reason for different serum levels (renal clearance rate for carbenicillin was half that of ampicillin and one-quarter that of benzyl penicillin), there was also inactivation by nonrenal mechanisms. This inactivation, as

shown by the rate of decline of plasma levels in uremic patients, was greatest for benzyl penicillin (half-life, 2.6 h), intermediate for ampicillin (half-life, 7.8 h), and slowest for carbenicillin (half-life, 15 h). These results tie in very well with our findings that carbenicillin is very stable in the body (2.1% breakdown) followed by ampicillin (12.2% breakdown) and benzyl penicillin (19% breakdown). High drug concentrations in serum can thus be associated with high stability in the body as well as slow renal clearance.

Support for the theory that the liver is the site of inactivation is provided by the experiments of Kind et al. (5), in which they infused rat liver. They found a much greater rate of inactivation of benzyl penicillin than ampicillin.

LITERATURE CITED

1. Alicino, J. F. 1946. Iodometric method for the assay of penicillin preparations. Ind. Eng. Chem. (Anal. Ed.) **18**:619–620.
2. Birner, J. 1970. Determination of phenoxymethyl penicilloic acid and phenoxyethyl penicilloic acid in urine in the presence of the parent penicillins. J. Pharm. Sci. **59**:757–760.
3. Cole, M., and R. Sutherland. 1966. The role of penicillin acylase in the resistance of gram-negative bacteria to penicillins. J. Gen. Microbiol. **42**:345–356.
4. English, A. R., H. T. Huang, and B. A. Sobin. 1960. 6-Aminopenicillanic acid in urine after oral administration of penicillins. Proc. Soc. Exp. Biol. Med. **104**:405–406.
5. Kind, A. C., H. N. Beaty, L. F. Fenster, and W. M. M. Kirby. 1968. Inactivation of penicillins by the isolated rat liver. J. Lab. Clin. Med. **71**:728–735.
6. Rolinson, G. N., and F. R. Batchelor. 1963. Penicillin metabolites. Antimicrob. Ag. Chemother. 1962, p. 654–660.
7. Rosenblatt, J. E., A. C. Kind, J. L. Brodie, and W. M. M. Kirby. 1968. Mechanisms responsible for the blood level differences of isoxazolyl penicillins. Arch. Intern. Med. **121**:345–348.
8. Standiford, H. C., M. C. Jordan, and W. M. M. Kirby. 1970. Clinical pharmacology of carbenicillin compared with other penicillins, J. Infec. Dis **122**(Suppl.):S9–S13.
9. Sutherland, R., J. Burnett, and G. N. Rolinson. 1971. α-Carboxy-3-thienylmethylpenicillin (BRL2288), a new semisynthetic penicillin: in vitro evaluation. Antimicrob. Ag. Chemother. 1970, p. 390–395.
10. Sutherland, R., E. A. P. Croydon, and G. N. Rolinson. 1972. Amoxycillin: a new semi-synthetic penicillin. Brit. Med. J. **3**:13–16.
11. Thomas, R. 1961. Colorimetric detection of penicillins and cephalosporins on paper. Nature (London) **191**:1161–1163.