STUDIES ON THE FORMATION AND ACTIVITY OF THE TRANSFORMATION PRODUCT OF AMPICILLIN

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When ampicillin was injected into a healthy volunteer and experimental animals, an unknown substance with an antimicrobial activity was recovered in the urine together with unchanged ampicillin. This unknown substance was also formed when ampicillin was incubated with urine, serum or homogenate of rat tissues. The causative agent responsible for this new transformation product seemed to be of low molecular weight and stable to heat. This transformation product showed a lower antimicrobial activity than ampicillin against some gram-negative bacteria. The concentrations of this product in the serum and urine of a healthy volunteer receiving ampicillin intramuscularly were far lower than those of ampicillin.

Although a multitude of papers concerning the properties of ampicillin have so far appeared[1~7], few refer to its metabolism in the body. The present paper aims to elucidate the presence of an unknown product, which probably is a transformation product of ampicillin, recovered in the serum and urine of man and animals receiving ampicillin.

Materials and Methods

1. Material: The ampicillin used was Penbritin (potency ; 832 mcg/mg), produced by Beecham Research Laboratories.

2. Subjects and dosages: Ampicillin was injected as a single intramuscular dose of 500 mg to a healthy volunteer (male adult weighing 60 kg), and was also administered to rats of the Webster strain, albino rabbits and dogs of the Beagle strain as a single intramuscular dose of 50 mg/kg.

3. Separation and estimation method: In this experiment ampicillin and related substances were separated and assayed by a chromatographic method already reported by the authors in a previous paper[8].

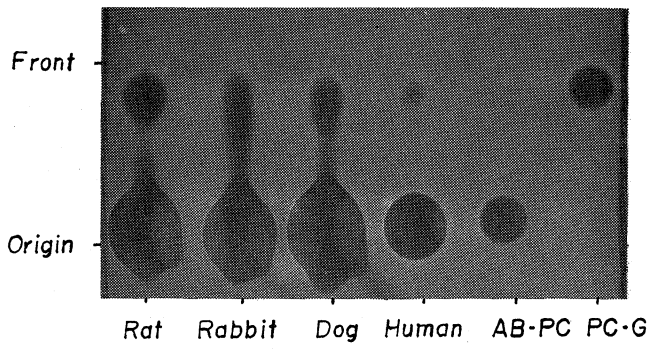
Results

1. An unknown antimicrobial substance recovered in urine samples from a volunteer and animals receiving ampicillin intramuscularly

An unknown antimicrobial substance was recovered in 3-hour urine samples from a volunteer and animals receiving ampicillin by the intramuscular route. Thin-layer chromatograms of the urine are shown in Fig. 1.

All the urine samples produced two spots alike: one remaining near the origin

Fig. 1. Bioautogram of urine after intramuscular administration of ampicillin.
0~3-hour Urine
Dose : 50 mg/kg ; Dog, rabbit and rat.
500 mg ; Human



and being consistent with the spot of ampicillin, and the other, having an Rf value lower than that of benzylpenicillin (shown in the extreme right hand). The spot of benzylpenicillin was detected in traces by the formation of an inhibition zone when a chromatogram of ampicillin at concentrations higher than 2,000 mcg/ml was autobiographed. The unknown spot, with Rf lower than benzylpenicillin when bioautographed, exhibited antimicrobial activity and, to our interest, the same spot was also produced when sera of the test animals were chromatographed, as described below.

These finding suggested to us that the spot of an unknown substance is a metabolite derived from ampicillin, or a transformation product.

2. Concentration of the unknown substance in serum and urine of rats receiving ampicillin intramuscularly

Attempts were made to determine the concentrations of this unknown substance in the urine and serum of rats, prior to its identification.

Ampicillin was administered intramuscularly to five rats at a dose of 100 mg/kg. Their blood was collected at 15 minutes, 30 minutes, 1 hour, and 2 hours, and urine samples were collected over three consecutive periods of 0~3, 3~6, and 6~24 hours. These specimens were chromatographed, and the resultant spots were studied with the aid of bioautography. The sizes of the inhibition zones, produced by ampicillin and the unknown substance, were measured. Concentrations of the unknown substance in the specimens, roughly estimated on the basis of the standard line of ampicillin, turned out to be lower than those of ampicillin, as shown in Table 1. However, since the methods of separating and purifying this unknown substance are not yet established, the absolute values in this table may be uncertain.

Table 1. Serum and urine concentrations of unknown substance after intramuscular administration of ampicillin to healthy rats

Serum (mcg/ml)

Time	Ampicillin	Unknown substance
15 min.	80.0	1.65
30 min.	82.0	1.60
1 hr.	76.0	1.20
2 hr.	12.0	—

Urine (mcg/ml)

Time	Ampicillin	Unknown substance
0~3 hr.	19,000	81.0
3~6 hr.	6,600	54.0
6~24 hr.	550	25.0

Dose : 100 mg/kg
The activity of the unknown substance was determined by using ampicillin as the reference standard.

3. Antimicrobial activity of the transformation product

At this stage of the work we were unable to obtain this unknown substance in a satisfactorily pure form, which is essential for the determination of the antimicrobial activity. With a view to estimating the antimicrobial activity of this substance, the

following experiment was conducted.

One ml of ampicillin solution (500 mcg/ml) was incubated with an equal volume of 30-percent kidney homogenate of rats at 37°C for 1 hour. Without delay, two different volumes of the mixture——5 μl (for use against gram-positive bacteria) and 20 μl (against gram-negative bacteria)——were spotted on thin layers, and developed in the usual manner. Each sheet was then placed on an agar plate previously seeded with a test organism. The results are shown in Table 2. Diameters of inhibition zones produced by ampicillin were compared with those produced by the substance with the use of three test organisms. The ratios of the former to the latter were as follows: 0.66 (28.5 mm/19.0 mm) for *Bacillus subtilis* ATCC-6633, 0.54 for *Staphylococcus aureus* FDA 209P, and 0.40 for *Streptococcus hemolyticus* A-S-8. The unknown substance, though displayed a considerable antimicrobial activity against these gram-positive bacteria, failed to exhibit any inhibition against gram-negative bacteria of *Salmonella enteritidis*, *Proteus vulgaris* IAM-1025 and *Escherichia coli* NIHJ under the same test conditions.

Table 2. Antimicrobial activities of ampicillin (A) and unknown substance (B)

Organism	Inhibition zone (mm)		B/A
	Ampicillin (A)	Unknown substance (B)	
Staph. aureus FDA 209 P	36.0	19.3	0.54
St. hemolyticus A-S-8	22.0	9.3	0.42
B. subtilis ATCC 6633	28.5	19.0	0.66
E. coli NIHJ	11.0	0	—
Sal. enteritidis	26.0	0	—
Pr. vulgaris IAM-1025	20.2	0	—

Rat kidney-homogenate 30% 1 ml
Ampicillin 500 mcg/ml 1 ml
} 37°C, 1 hour

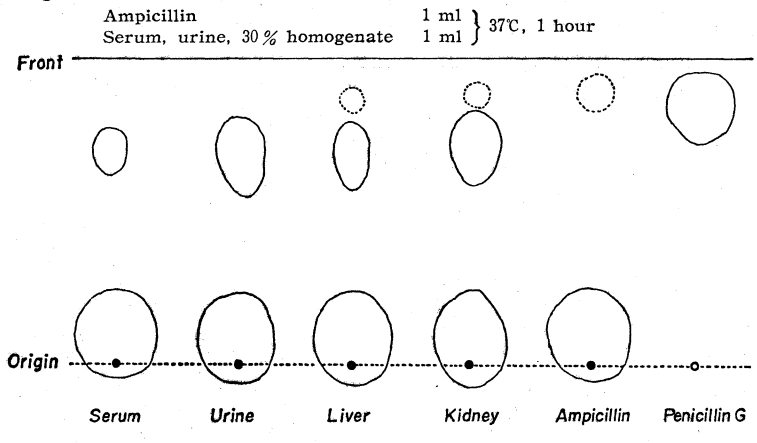
Developed in TLC (gram-positive 5 μl, gram-negative 20 μl), and placed on six agar plates each seeded with different test organisms.

4. Formation of the unknown substance after incubation of the serum, urine and tissue homogenates of rats with ampicillin

An attempt to produce the unknown substance *in vitro* followed the estimation of its antimicrobial activity.

One ml of an ampicillin solution (2 mg/ml) was mixed with an equal volume of the serum, urine or 30-percent liver or kidney homogenate of rats, and incubated at 37°C for 1 hour. The mixture was then subjected to thin-layer chromatography.

Fig. 2. Formation of unknown substance by rat tissue components.



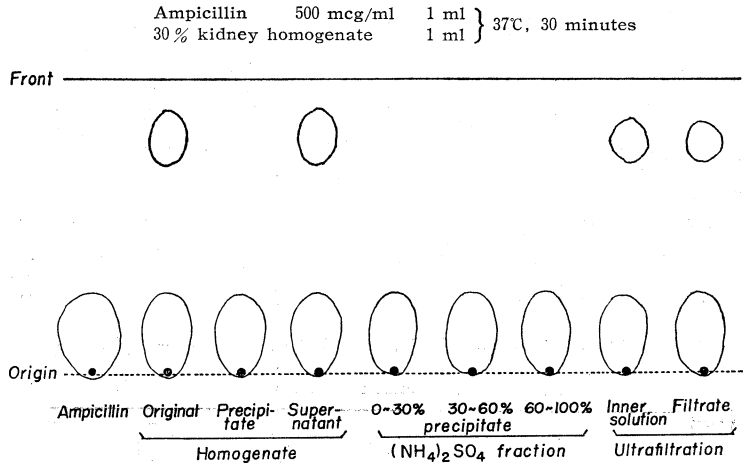
As Fig. 2 shows, these mixture produced a common spot, the Rf value of which was somewhat smaller than that of benzylpenicillin. Furthermore, this spot was consistent with that obtained from the urine samples. These findings indicated that this substance was formed by some mechanism other than enzymatic reactions. Furthermore, the fact that the sizes of the inhibition zones were reduced with the prolongation of incubation period indicated instability of the product.

5. Formation of the substance in fractions of rat kidney homogenate

With the aim of determining whether or not the transformation was of enzymatic origin, the following experiment was conducted.

First of all, in an attempt to determine chromatographically whether the suspected transforming activity depended on the supernatant fluid or on the sediment, rat kidney homogenate was centrifuged at 1,500×*g* for 20 minutes. The sediment thus obtained was suspended in 1/15 M phosphate buffer (pH 7.0) in a volume equal to the supernatant fluid. One ml of this suspension was incubated with 1 ml of ampicillin solution (500 mcg/ml) at 37°C for 30 minutes. As Fig. 3 shows, the spot corresponding to the unknown substance was not obtained with the sediment, but was obtained with supernatant fluid. Subsequently, the supernatant fluid was salted out with ammonium sulfate at various concentrations range to give separate fraction, which were then allowed to react with ampicillin. None of these fraction, however, gave rise to the unknown substance.

Fig. 3. Formation of the substance in fractions of rat kidney homogenate.



An ultrafiltrate of the supernatant fluid, containing neither protein nor other high molecular weight substances, was prepared by the centrifugal ultrafiltration technique using cellulose tubing[9] (Visking Company : size 8/32), and this ultrafiltrate was incubated with ampicillin at 37°C for 1 hour. As Fig. 3 shows, the filtrate obtained under such conditions produced inhibition zones that were similar in size to those produced by the inner solution of ultrafiltration containing both low and high molecular substances. Futhermore, this experiment showed that the interaction of ampicillin with the supernatant fluid was quite rapid and that the product was unstable enough to

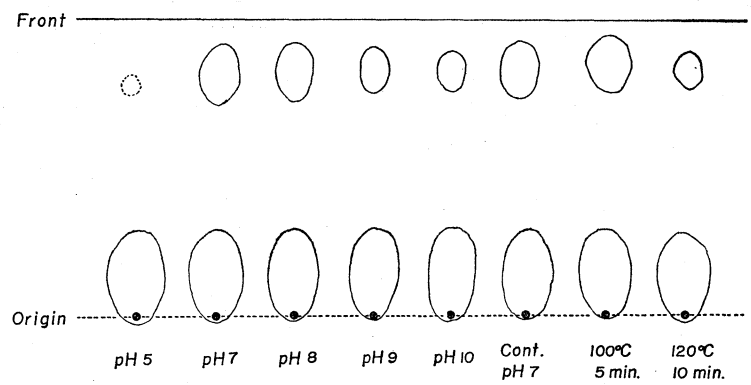
cause the reduction of the size of the inhibition zone when the mixture was incubated at 37°C for 2 hours or more.

These results convinced us that the unknown substance was a transformation product formed by a non enzymatic interaction of ampicillin with low molecular substances existing in the homogenate.

6. Influence of pH and temperature on the transformation in rat kidney homogenate

With a view to examining the influence of pH and temperature on the transformation, the homogenate was incubated with ampicillin under various conditions. The resultant spots were bioautographed for comparison. As Fig. 4 shows, the optimum pH range for the transformation was between 7 and 8. At a lower pH of 5, only traces of the product were formed and, at pH 9, the size of the inhibition zone was reduced owing to the reduced transformation, or owing to instability of the product at this pH range.

Fig. 4. Influence of pH and heat on the transformation in rat kidney homogenate.



The stability of low-molecular substances in the homogenate was examined as follows: the homogenate was treated at 100°C for 5 minutes or at 120°C for 10 minutes and subjected to incubation with ampicillin. As shown in the right hand of Fig. 4, the amount of the transformation product was reduced after heat treatment at 120°C for 10 minutes, but was not altered after heating at 100°C for 5 minutes.

Discussion

An unknown antimicrobial substance, believed to be a transformation product of ampicillin, was recovered in the body of a man and animals receiving ampicillin. This unknown substance was separated and estimated by the authors' method. Studies showed that the transformation product was produced not by an enzymatic reaction but by a low molecular substance in the body. Furthermore, it became clear that only a small proportion of the ampicillin administered was transformed to the product, and that the antimicrobial activity of this product affect significantly the bioassay of ampicillin in the body. We hope to clarify in a later paper the properties of the low molecular substance which causes the formation of the transformation product.

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