NOTE

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

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In the preceding paper[1], we reported that an uncharacterized antimicrobial substance was recovered in samples from animals and healthy volunteers receiving ampicillin. This substance is formed also by the incubation of ampicillin with urine, serum or tissue homogenates. The factors contributing to the transformation of ampicillin were found to be relatively stable to heat, and sufficiently low in molecular weight to permit penetration of a cellophane membrane.

The present paper reports the results of additional experiments to elucidate the mechanism of the transformation and properties of this uncharacterized substance.

Materials and Methods

1. Test antibiotics

Test antibiotics used were ampicillin (Beecham Laboratories) and other penicillins, serving as controls, including benzylpenicillin (Banyu Pharmaceutical Co., Ltd.), carbenicillin (Beecham Laboratories), hetacillin (Bristol Laboratories), acetylaminobenzylpenicillin (Fujisawa Research Laboratories), and O-acetylaminobenzylpenicillin (Fujisawa Research Laboratories).

2. Test animals

Mouse: ICR strain, 20~25 g
Rat: SD strain, 190~220 g
Rabbit: healthy, 2~2.5 kg
Dog: healthy mongrel, 8~12 kg

3. Production of kidney and liver damage in rats

Impaired renal function in rats was induced by an intramuscular dose of 12 mg/kg of $HgCl_2$, and the liver was damaged by an oral dose of 5 ml/kg of a 1:1 mixture of CCl_4 and oilve oil[2].

4. Thin-layer chromatography and bioautography

The same procedures as described in the preceding paper were employed[3].

5. Gas chromatography

Shimadzu Model GC-5 AP_3 was used by the method of BAKER *et al*[4].

6. Determination of aldehyde concentrations in standard solutions

The concentration of aldehydes was assayed by the CLIFT-COOK's iodometric method.

Results

1. Formation of an uncharacterized substance in homogenates of damaged kidney and liver of rats

Thirty percent homogenates of damaged kidneys and livers in phosphate buffer (pH 7.0) were prepared and allowed to react with ampicillin in order to confirm the formation of the uncharacterized substance in these homogenates.

A 1-ml aliquot of the homogenate was incubated with a 1-ml aliquot of a 200 mcg/ml ampicillin solution at 37°C for 30 minutes. A similar incubation was carried out on the normal kidney homogenate (control).

The assay by thin-layer chromatography and subsequent bioautography revealed that the amount of the uncharacterized substance formed in the damaged kidney did not differ significantly from that in the normal kidney (Fig. 1 A). This indicates that kidney damage has litte influence on the transformation of ampicillin.

The experiment was conducted in a manner similar to that involving the kidney. The amount of the uncharacterized substance in the damaged liver did not differ significantly from that in the normal liver (Fig. 1 B).

2. Relationship between Rf values and the structure of penicillins

The uncharacterized substance formed in the homogenates was isolated with a solvent system consisting of ethylacetate, acetic acid and water (8:1:1). The Rf value was about 0.1 for ampicillin and 0.75 for the uncharacterized substance.

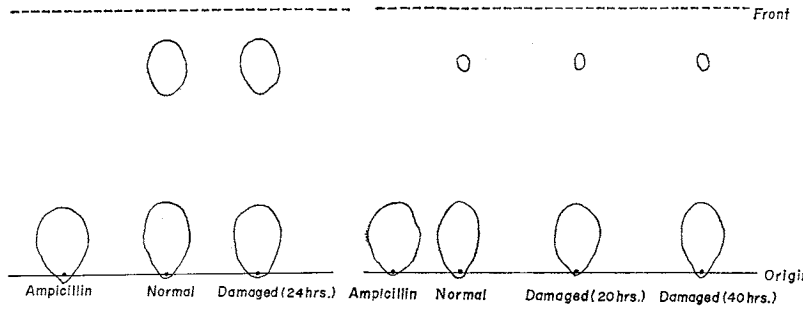
Fig. 1. Production of the uncharacterized substance in homogenates of damaged kidneys and livers of rats

(A) Kidney : $HgCl_2$, 12 mg/kg, I.M.

30% kidney homogenate 1 ml, Ampicillin (500 mcg/ml) 1 ml, incubation 37°C, 30 min.

(B) Liver : CCl_4 and olive oil (1 : 1), 5 ml/kg, P.O.

30% liver homogenate 1 ml, Ampicillin (500 mcg/ml) 1 ml, incubation 37°C, 30 min.

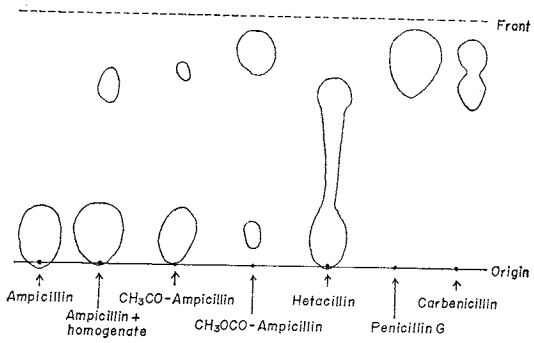


In an effort to presume the structure of the uncharacterized substance by an analogy of Rf values of various ampicillin congeners, the following series of penicillins were chromatographed with the same solvent system: penicillin-G, carbenicillin, in which the amino group of ampicillin is replaced by hydrogen and carboxyl-residue respectively; O-acetylaminobenzylpenicillin, acetylaminobenzylpenicillin and hetacillin, in both of which the amino group of ampicillin is modified by a certain residue, *e.g.*, in hetacillin, by the diacetyl carbonyl group.

As shown in Fig. 2, all these penicillins presented Rf values greater than that of ampicillin. This fact suggests that the amino group of ampicillin is modified by certain materials in the homogenate.

Fig. 2. Chromatogram of penicillins

Solvent : Ethylacetate, acetic acid and water (8 : 1 : 1)
Thin-layer : Silica gel
Concentration : 200 mcg/ml, Amount : 5 μl/spot

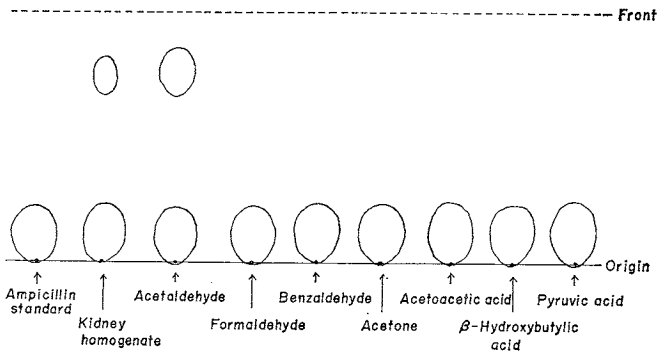


3. Experimental production of the uncharacterized substance by reactions between ampicillin and carbonyl compounds

Since the amino group of ampicillin is known to react with aldehydes and ketones, ampicillin was allowed to react with some carbonyl compounds including those found in serum or urine. A 1-ml aliquot of a 200 mcg/ml ampicillin solution was mixed with a 1-ml aliquot of a 20 mcg/ml solution of each carbonyl compound, and incubated at 37°C for 30 minutes. The mixture was then examined by thin-layer chromatography and bioautography.

Fig. 3. Formation of the uncharacterized substance from ampicillin and carbonyl compounds

Ampicillin (200 mcg/ml) 1 ml, Carbonyl compound (20 mcg/ml) 1 ml, 37°C, 30 min.
→TLC→Bioautography



As shown in Fig. 3, acetaldehyde reacted with ampicillin and produced a substance with an Rf value identical to that of the uncharacterized substance. On the other hand, formaldehyde, at the same concentration, did not produce any spot other than ampicillin

itself.

4. Effect of concentrations of acetaldehyde and formaldehyde on the formation of the uncharacterized substance

The relation between concentrations of the aldehydes and the amount of the uncharacterized substance to be formed was studied by the experiment described below:

A 1-ml aliquot of a 500 mcg/ml ampicillin solution was mixed with a 1-ml aliquot each of various concentrations of acetaldehyde and formaldehyde. The mixtures were incubated at 37°C for 1 hour, then chromatographed and bioautographed by ordinary methods.

The results are shown in Fig. 4-a. The product derived from the reaction between ampicillin and acetaldehyde presented an Rf value of 0.73, which is consistent with that of the product from the homogenate. Of the spots deriving from various concentrations of acetaldehyde, those from 2.5 mcg/ml and 5 mcg/ml solutions appeared to be the closest in size to that from the homogenate. Spots from 10 and 20 mcg/ml solutions were larger than the spot from the homogenate. These findings indicate that very small amounts of acetaldehyde contribute to the formation of the uncharacterized substance.

In order to elucidate the relationship between the acetaldehyde concentration and the resulting spot size, the ampicillin concentration was held constant at 500 mcg/ml, and acetaldehyde at various concentrations was allowed to react with the ampicillin. As shown in Fig. 4-b, the relationship between the diameter of the spot on the ordinate and the logarithm of the acetaldehyde concentration on the abscissa proved linear.

Meanwhile, formaldehyde was allowed to react with ampicillin under similar conditions. No spot was observed when the formaldehyde concentration was as low as 6.25 mcg/ml, and even the spot from a con-

Fig. 4-a. Formation of the uncharacterized substance from acetaldehyde and formaldehyde at various concentrations

Ampicillin (500 mcg/ml) 1 ml, Aldehyde 1 ml } 37°C, 1 hr. Bioautography

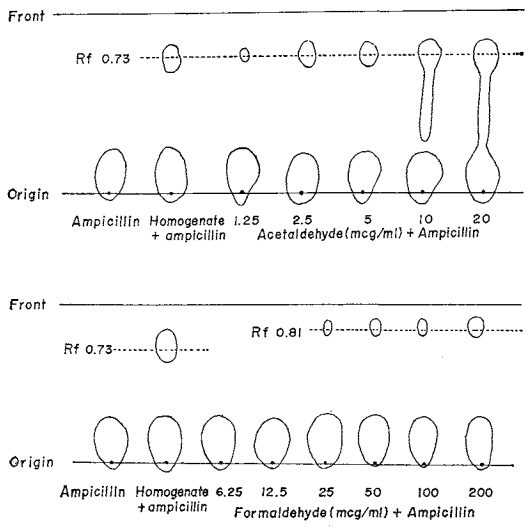


Fig. 4-b. Standard curve of acetaldehyde derived from the inhibition zone produced by the uncharacterized substance

Ampicillin (500 mcg/ml) 1 ml, Acetaldehyde 1 ml } 37°C, 1 hr. Bioautography

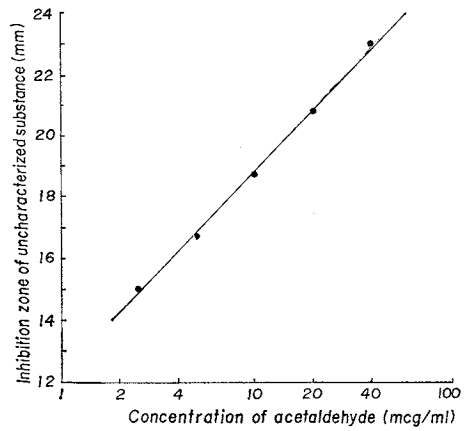


Fig. 5. Formation of the uncharacterized substance in the kidney and liver homogenates of rats receiving ethanol

30% homogenate* 1 ml, Ampicillin (500 mcg/ml) 1 ml } 37°C, 30 min.

* Prepared 4 hours after oral administration of 4 ml/rat of 50% ethanol.

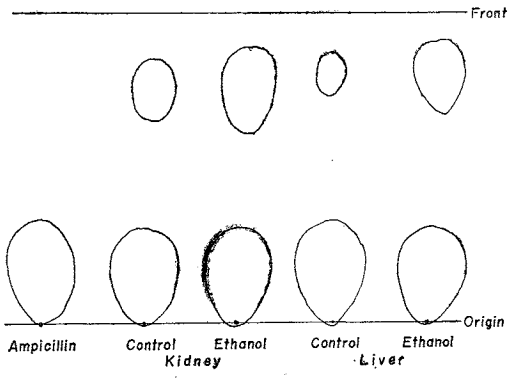
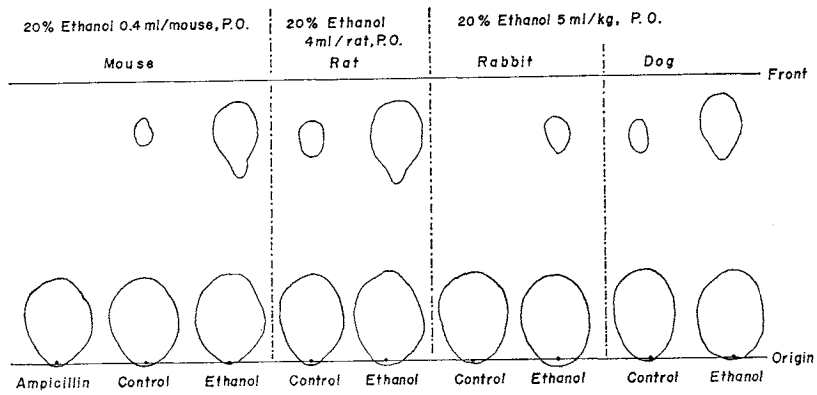


Fig. 6. Formation of the uncharacterized substance in the liver homogenate of rats receiving ethanol

30% liver homogenate 1 ml
(Prepared 1 hour after administration) } 37℃, 30 min.
Ampicillin (500 mcg/ml) 1 ml



centration as high as 200 mcg/ml was smaller than that from the homogenate. Moreover, the Rf values of the spots from formaldehyde and ampicillin were inconsistent with that of the uncharacterized substance.

These findings suggest that the material causing the transformation of ampicillin is acetaldehyde but not formaldehyde.

5. Production of the uncharacterized substance in homogenates of kidneys and livers of rats receiving ethanol

The acetaldehyde concentration in the livers and kidneys has been known to increase by administration of ethanol. On the assumption that the increased acetaldehyde concentration in these organs contributes to elevated production of the uncharacterized substance, the following experiment was conducted.

Three normal rats were orally given 4 ml of 50% ethanol. Four hours afterwards, these animals were killed, and the kidney and liver were homogenized. These homogenates were mixed with ampicillin and incubated at 37°C for 1 hour.

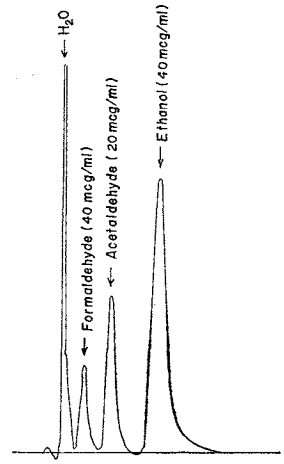
The results are shown in Fig. 5. Production of the uncharacterized substance markedly increased in the liver and slightly increased in the kidney.

6. Formation of the uncharacterized substance in the liver homogenate of various species of animals receiving ethanol

The elevated production of the uncharacterized substance in the liver was confirmed

Fig. 7. Gaschromatogram of acetaldehyde

Column : Porapak Q(1=2 m)
Detector : FID
Carrier gas : N_2 (60 ml/min.)
Temp. Column : 110℃
Inlet : 120℃
Sample applied : 5 μl



in the tested species of animals. Animals were given 20% ethanol orally as follows: 0.4 ml to mice, 4 ml to rabbits, and 5 ml to dogs.

As Fig. 6 shows, the liver homogenates showed an increased production of the substance in the mice, rats and dogs. However, the production was remarkably less in the rabbits.

7. Assay of acetaldehyde residing in the kidney and liver homogenates

Since acetaldehyde was found to be the suspected causative factor in the transforma-

Fig. 8. Gaschromatogram of liver and kidney homogenates

Supernatant fluid of 30 % homogenate used 5 μl applied

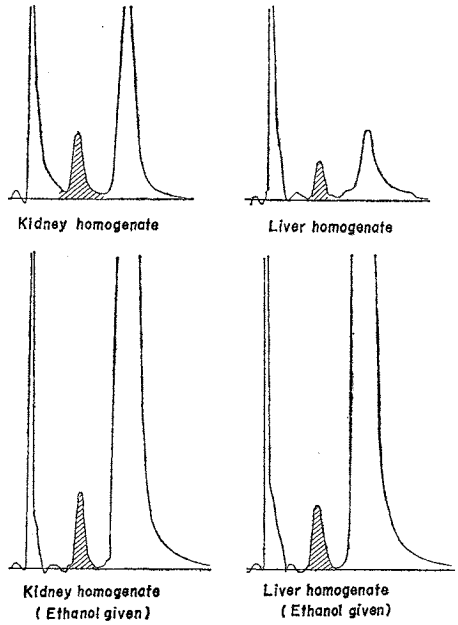
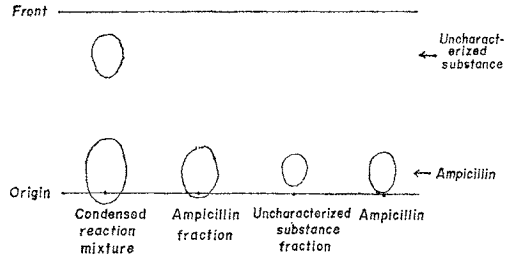


Table 1. Acetaldehyde concentration in the supernatant fluid of the homogenate

	Assay method (mcg/ml)	
	Bioautography	Gas-chromatography
Kidney	4~5	5~8
Kidney (ethanol given)	18~28	20
Liver	2~4	3~5
Liver (ethanol given)	15~23	20

Fig. 9. Chromatograms of fractionated samples

TLC : Chromatogram Sheet (Eastmann) No. 6061 (silica gel)
Solvent : Ethyl acetate-acetic acid-water (8 : 1 : 1)
Organism : *B. subtilis* ATCC-6633



t
tion of ampicillin in the kidney and liver, he homogenat es were assayed for formaldehyde, acetaldehyde and ethanol by gas-chromatography. Acetaldehyde was conveniently partitioned and assayed from accompanying formaldehyde and ethanol under the conditions specified in Fig. 7.

The upper part of Fig. 8 shows gas-chromatograms obtained from homogenates of the normal liver and kidney under the same conditions as in Fig. 7. The presence of acetaldehyde is clearly demonstrated. The lower part of Fig. 8 denotes the apparently increased acetaldehyde content in the homogenates after ethanol administration.

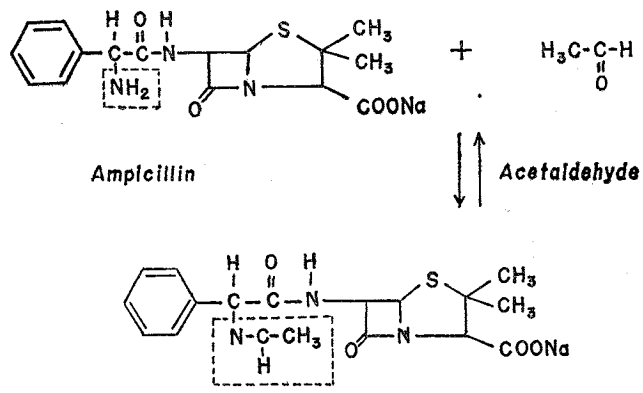
Table 1 shows the acetaldehyde concentrations in the homogenates assayed by both bioautography and gas-chromatography. It is evident that the concentrations assayed by the two procedures are consistent and that the concentrations greatly increase after ethanol administration.

These findings strongly convinced us that acetaldehyde, which normally exists in the living tissue, is the causative substance in the transformation of ampicillin into the uncharacterized substance.

8. Isolation of the uncharacterized substance

A 5-ml aliquot of a 2 mg/ml ampicillin solution and a 20-ml aliquot of supernatant fluid of 30 % rat kidney homogenate (14,000 r.p.m., 30 minutes) were mixed and incubated at 37°C for 1 hour. The reaction mixture was condensed to about 4 ml under reduced pressure. Twice the volume of ethanol was then added, and centrifuged. The supernatant fluid was spotted to a thin-layer

Fig. 10. Presumed structure of the uncharacterized substance



chromatographic plate of 1-mm thickness, and developed with ethyl acetate-acetic acid-water (8 : 1 : 1). After drying in an air current, the plate was divided into 10 equal horizontal parts, and the fraction bearing the uncharacterized substance was scraped off and extracted with 0.1 M phosphate buffer (pH 7.0). When the fraction containing the uncharacterized substance was chromatographed under the same conditions, the spot obtained did not correspond to that of the uncharacterized substance but was consistent with that of ampicillin itself (Fig. 9). This fact suggests that the transformed product is readily reversed to ampicillin.

9. Presumptive structure of the uncharacterized substance

We came to consider that the structure of the transformation product is as shown in Fig. 10, on the basis of various data such as transformation conditions, reversibility of the transformation, *etc.* This structure closely resembles that of hetacillin, and accounts for all the findings concerned.

Discussion

In the present paper, the authors have reported on an antimicrobial transformation product first recovered in urine samples of animals receiving ampicillin. This substance was assayed by the TLC-bioautographic method described in our preceding paper, and proved to be formed from ampicillin and acetaldehyde. Moreover, the amount of this product increased in the living body with the accumulation of the acetaldehyde.

A great number of papers have been presented concerning the metabolism of antibiotics. In general, most antibiotics are enzymatically inactivated into metabolites in the living body, *e.g.*, penicillins into penicilloic acid[5], cephaloglycin into desacetylcephaloglycin and further into certain other degradation products[6], and chloramphenicol, into the glucuronate form[7].

The transformation product is recovered in small quantities, and possesses little or no antimicrobial activity against gram-negative organisms. This transformation has proved to be reversible.

This transformation product is considered to be formed by the addition of acetaldehyde to the amino group of ampicillin. Reactions similar to this have been observed also in some cephalosporins such as cephaloglycin and cephalexin. Various transformation mechanisms of antibiotics are considered to occur in the living body. The present experiment suggests that a similar transformation involving active materials such as acetaldehyde may take place in antibiotics other than ampicillin.

Summary

The *in vivo* production of an uncharacterized substance derived from ampicillin was reported previously. In the present study, it was found that this substance is transformed from ampicillin and is readily reversed to ampicillin. This substance resembles hetacillin in structure, and is formed when the amino group of ampicillin reacts with acetaldehyde which exsists in small amount in kidneys, liver, serum and urine.

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