

**Fluorometric Determination of Amoxicillin<sup>1)</sup>**KATSUMI MIYAZAKI, OSAMU OGINO, HIROYUKI SATO,<sup>2a)</sup>  
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In order to estimate the transfer of amoxicillin in the body fluids, a direct separatory determination method of amoxicillin and its penicilloic acid was investigated. The penicilloic acid of amoxicillin formed an intensive and reproducible fluorescent product in solutions containing mercuric chloride, but the fluorescent product was not formed from amoxicillin directly. From these observations, the measurement can be made of amoxicillin and its penicilloic acid separately in urine and blood following therapeutic doses of the drug. The blood levels and urinary excretion of amoxicillin were compared with those of ampicillin.

**Keywords**—ampicillin derivative; amoxicillin and metabolite; fluorometric analysis; blood level; urinary excretion

Chemical method<sup>3)</sup> based on iodine absorption and microbiological assay method<sup>4)</sup> for the quantitative measurement of amoxicillin (p(-) $\alpha$ -amino-*p*-hydroxy benzylpenicillin) have been described. However, the highly sensitive spectrophotometric determination techniques, which are capable of measuring the low concentrations of unchanged amoxicillin and its penicilloic acid encountered in the biological fluids following therapeutic doses of amoxicillin, have not been reported.

In previous papers,<sup>5)</sup> the authors reported the sensitive fluorometric determination method of ampicillin derivatives in the biological fluids. The present investigation was directed to the development of this technique<sup>5)</sup> for the quantitative measurement of amoxicillin and its metabolite, assumed to be penicilloic acid, in aqueous solution, urine and blood. The intestinal absorption study in the rat and human was investigated and compared with that of ampicillin.

**Experimental**

**Materials and Reagents**—Trihydrate form of amoxicillin (potency: 814  $\mu$ g/mg) was kindly supplied by Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. Amoxicillin stock solutions containing 100  $\mu$ g/ml of amoxicillin (corrected for potency) were prepared fresh with redistilled water. Penicilloic acid solutions containing 100  $\mu$ g/ml of amoxicillin equivalent were prepared with 1 N NaOH solution. All the reagents were of special grade, and were prepared with redistilled water.

Quinine sulfate solution which is a standard fluorescence solution used in this experiment was prepared as described previously.<sup>5a)</sup>

**Apparatus**—Fluorescence intensity was measured by a Hitachi spectrofluorometer, model 203, equipped with a Xenon lamp.

**Urinary Excretion in Man**—Urinary excretion of amoxicillin was determined by the following method. Amoxicillin was given orally at a single dose of 250 mg (one capsule) to a fasting adult human male early in

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- 2) Location: a) *Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo*; b) *Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo*.
- 3) T. Murakawa, Y. Yokota, Y. Kono, M. Nishida, S. Goto, and S. Kuwabara, *Chemotherapy*, **21**, 1399 (1973).
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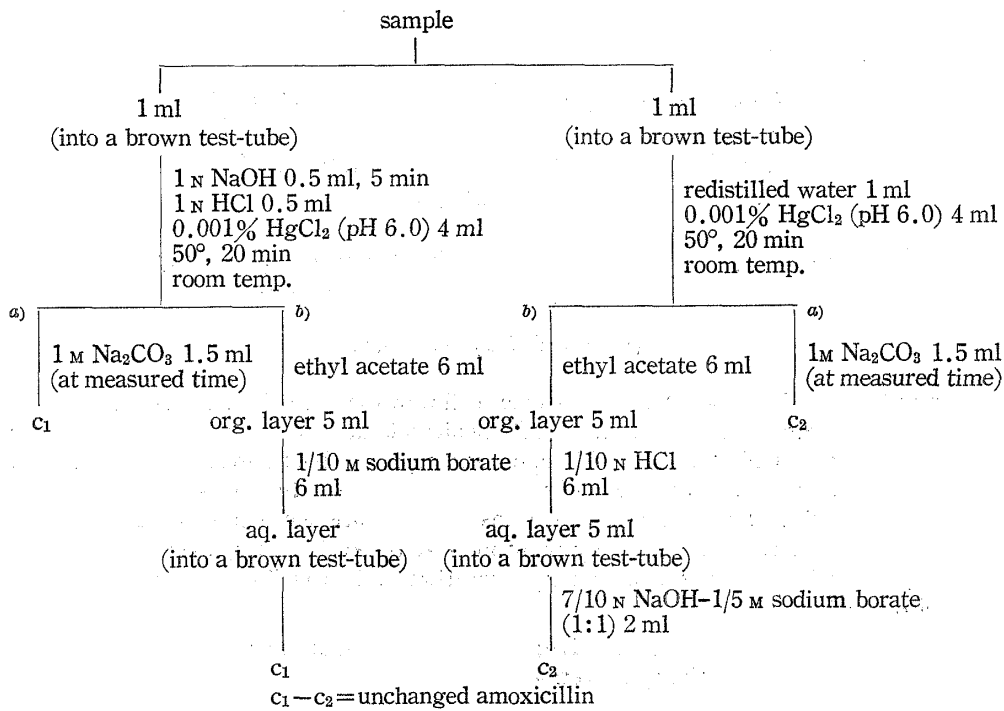


Chart 1. Method of Separatory Determination of Amoxicillin and Its Penicilloic acid in Aqueous Solution and Urine

excitation wavelength 345 nm, emission wavelength 420 nm

a) in aqueous solution, b) in urine sample

c<sub>1</sub> = amoxicillin + penicilloic acid

c<sub>2</sub> = penicilloic acid

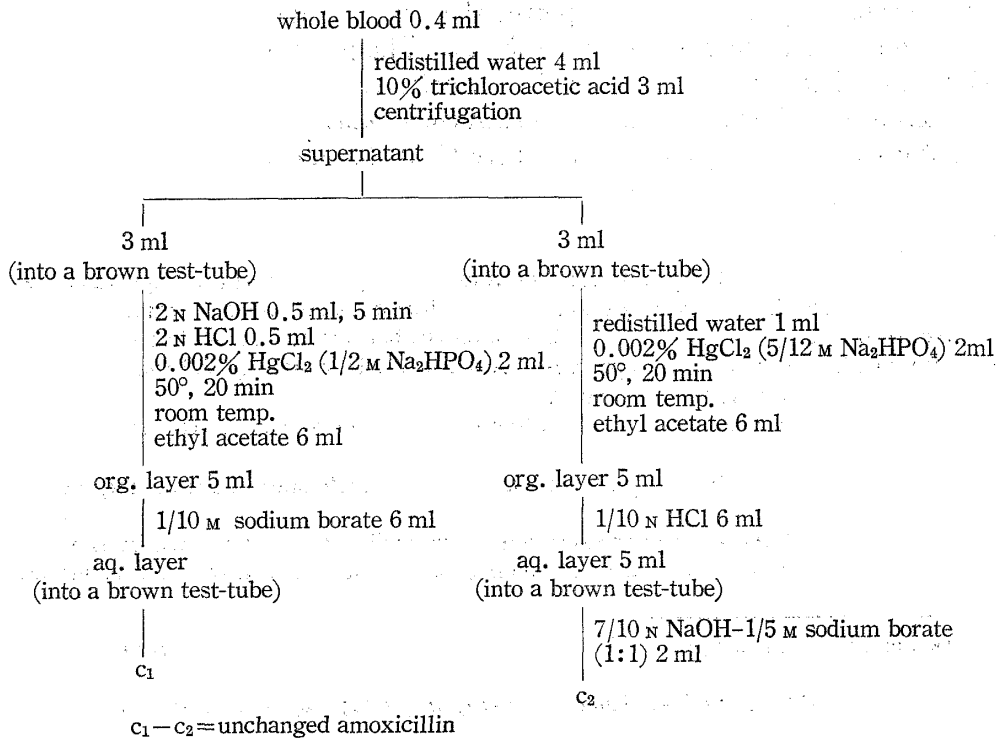


Chart 2. Method of Separatory Determination of Amoxicillin and Its Penicilloic Acid in Blood

excitation wavelength 345 nm, emission wavelength 420 nm

c<sub>1</sub> = amoxicillin + penicilloic acid

c<sub>2</sub> = penicilloic acid

the morning and urine samples were collected with the passage of time. The unchanged antibiotic and metabolite in these urine samples were determined by the separation assay method.

**Blood Levels in Rats**—Blood levels of amoxicillin and its metabolite were determined in the following manners. After the drug solution at a dose of 10 mg/kg, which had been prepared by dissolving the drug in physiological saline, was given to rats (Wistar strain, weighing 300–350 g) intraduodenally, the unchanged antibiotic and its metabolite in the blood taken from the carotid artery with the passage of time were determined by the separation assay method.

**Method of Separatory Determination of Amoxicillin and Its Metabolite**—Procedure 1: Method in Aqueous Solution: According to the procedure shown in Chart 1, a 1 ml aliquot of sample, adequately diluted if necessary, was placed in a brown test-tube. In the measurement of the total amoxicillin ( $c_1$ ), 0.5 ml of 1 N NaOH was added to the sample solution in the brown test-tube and the mixture was allowed to stand for 5 minutes, and 0.5 ml of 1 N HCl was then added. To this mixture, 4 ml of 0.001% (w/v)  $\text{HgCl}_2$  solution prepared with pH 6.0 phosphate buffer solution ( $\text{Na}_2\text{HPO}_4$ : 17.9 g/liter,  $\text{KH}_2\text{PO}_4$ : 35.3 g/liter) was added. A solution of the fluorescent product was obtained by warming this mixture for 20 minutes at  $50^\circ$ , and after cooling, 1.5 ml of 1 M  $\text{Na}_2\text{CO}_3$  solution was added to this test tube at measured time. The fluorescence intensity of the sample solution and reagent blank were measured at an excitation wavelength of 345 nm and emission wavelength of 420 nm, setting the intensity reading for the fluorescence standard solution to 100 units.

In the measurement of penicilloic acid ( $c_2$ ), 1 ml of redistilled water was added to the sample solution in another brown test-tube. And then the mixture was treated following the procedure mentioned in the total amoxicillin determination method. Consequently, the subtraction of  $c_2$  from  $c_1$  gives the concentration of unchanged amoxicillin.

Procedure 2: Method in Urine Sample: According to the procedure shown in Chart 1, 1 ml aliquots of sample solution, adequately diluted if necessary, were placed in brown test-tubes, and these sample solutions were treated according to Procedure 1 modified as follows. In the measurement of total amoxicillin ( $c_1$ ), after warming the reaction mixture for 20 minutes at  $50^\circ$ , 6 ml of ethyl acetate saturated with redistilled water was added, and the mixture was vigorously shaken for 2 minutes and centrifuged. Five ml of the organic layer was then added to 6 ml of 1/10 M sodium borate solution and the mixture was shaken for 5 minutes and centrifuged. The aqueous layer was then placed in a brown test-tube and the fluorescence intensity was measured as mentioned above.

In the measurement of penicilloic acid ( $c_2$ ), 6 ml of 1/10 N HCl instead of 1/10 M sodium borate solution was added to the ethyl acetate layer as shown in Chart 1. This mixture was shaken for 5 minutes, centrifuged and 2 ml of a mixture of 7/10 N NaOH and 1/5 M sodium borate (1:1) was then added to the aqueous layer (hydrochloric acid layer) at measured time. The subtraction of  $c_2$  from  $c_1$  gives the concentration of unchanged amoxicillin.

Procedure 3: Method in Whole Blood Sample: The procedure is as shown in Chart 2. A 0.4 ml sample of whole blood was added to 4 ml of redistilled water in a 10 ml glass-stoppered centrifuge tube. Three ml of 10% trichloroacetic acid (TCA) was added to this hemolyzed blood sample solution and the mixture was centrifuged to obtain a clear supernatant. In the determination of total amoxicillin ( $c_1$ ), 3 ml aliquot of supernatant solution, if necessary diluted adequately with TCA solution (TCA:  $\text{H}_2\text{O}$  = 3:4), was pipetted into a brown test-tube containing 0.5 ml of 2 N NaOH and the mixture was allowed to stand for 5 minutes, and 0.5 ml of 2 N HCl was then added. To this mixture, 2 ml of 0.002% (w/v)  $\text{HgCl}_2$  solution prepared with 1/2 M  $\text{Na}_2\text{HPO}_4$  solution was added to adjust the pH of this mixture to  $6.0 \pm 0.1$ . A solution of the fluorescent product was obtained by warming this mixture for 20 minutes at  $50^\circ$  in a water bath. After cooling, the reaction mixture was treated following Procedure 2 (assay method of total amoxicillin in the urine sample) as described above.

In the measurement of penicilloic acid ( $c_2$ ), 1 ml of redistilled water was added to the 3 ml of centrifuged sample solution in the brown test-tube. To this mixture, 2 ml of 0.002% (w/v)  $\text{HgCl}_2$  solution prepared with 5/12 M  $\text{Na}_2\text{HPO}_4$  solution was added to adjust the pH to  $6.0 \pm 0.1$ . A solution of the fluorescent product was obtained by warming this mixture for 20 minutes at  $50^\circ$  in a water bath. After cooling, the reaction mixture was treated following Procedure 2 (assay method of penicilloic acid in the urine sample) as described above. Consequently, the subtraction of  $c_2$  from  $c_1$  gives the concentration of unchanged amoxicillin.

In these separatory determination methods in aqueous solution, urine and blood samples, the fluorescence intensity of a known amount of each of amoxicillin or penicilloic acid was also measured in order to determine the concentration of unchanged amoxicillin and its penicilloic acid in the unknown sample solutions.

## Result and Discussion

### Formation and Properties of the Fluorescent Product from Amoxicillin

An intensive and reproducible fluorescent product was obtained from penicilloic acid in neutral solution containing  $\text{HgCl}_2$  reagent at  $50^\circ$ . All fluorescence measurements in this study were obtained at the excitation maximum at 345 nm and the emission maximum at 420 nm.

**Effect of the Temperature on the Fluorescence Intensity**—The rate of formation of the fluorescent product from amoxicillin at various temperatures was studied using Procedure 1. As shown in Fig. 1, a maximum and constant fluorescence intensity was obtained at 50° or 60° in Procedure 1 for the determination of total amoxicillin. On the other hand, the fluorescent product was not formed from amoxicillin in these conditions in Procedure 1 for the determination of penicilloic acid.

Consequently, it was found that the measurement can be made of penicilloic acid as well as unchanged amoxicillin in this separatory determination procedure.

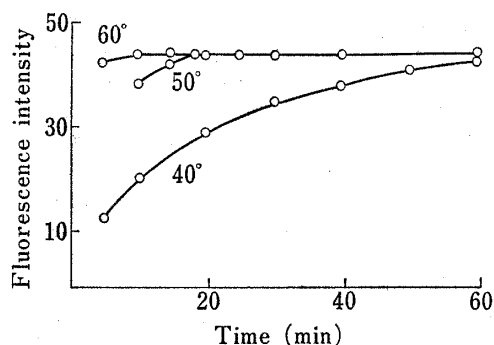


Fig. 1. Relationship between Fluorescence Intensity and Warming Time at 40°, 50° and 60°

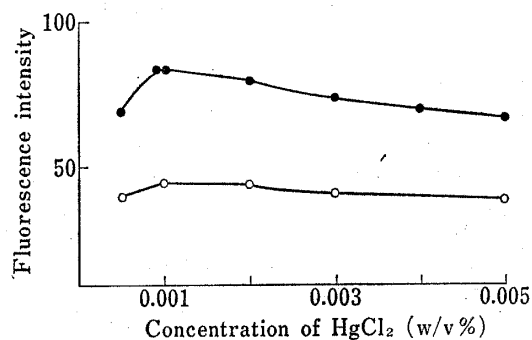


Fig. 2. Effects of Concentration of HgCl<sub>2</sub> Reagent on the Fluorescence Intensity

●—: in aqueous solution and urine sample (procedure 1 and 2)  
○—: in blood sample (procedure 3)

**Effects of the Concentration and pH of HgCl<sub>2</sub> Reagent on the Fluorescence Intensity**—The effects of the concentration and pH of HgCl<sub>2</sub> reagent on the relative amount of fluorescent product formed from amoxicillin in the aqueous solution and blood sample were investigated. As shown in Fig. 2, the maximum and constant fluorescence intensity in aqueous solution and blood sample was obtained at a concentration of 0.001% (w/v) and in the concentration range of 0.001—0.002% (w/v) HgCl<sub>2</sub> reagent, respectively. Furthermore, it was found that the fluorescence intensity was maximum and constant at pH 4.0—6.4.

**Effects of pH on the Formation and Intensity of the Fluorescent Product**—In order to investigate the effect of pH on the relative amount of fluorescent product formed from amoxicillin, the reaction mixtures were warmed for 30 minutes at 50° in media of various pH values, and after cooling, the fluorescence intensities were measured at constant pH 9.4. As shown in Fig. 3, a relative constant fluorescence intensity was obtained over the range of pH 5.5—6.5. From this result, a fluorescent product was obtained at the pH of  $6.0 \pm 0.1$ .

And to increase the sensitivity of the fluorescence intensity, the pH profile of the fluorescence intensity was obtained in media of various pH values (Fig. 4). The fluorescence of the

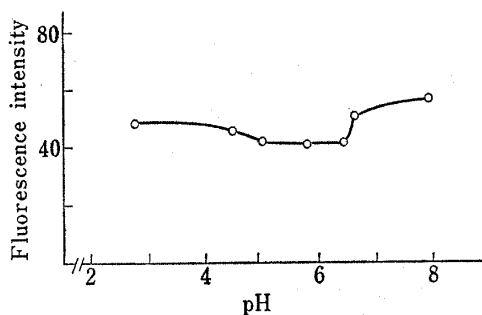


Fig. 3. Effect of pH on the Formation of the Fluorescent Product

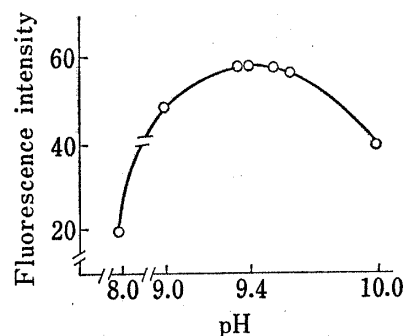


Fig. 4. Fluorescence Intensity in Various pH Sample Solutions Measured

amoxicillin product was greatest at pH 9.4. All measurements of the fluorescence intensity were, therefore, usually made at pH  $9.4 \pm 0.1$  after warming the reaction mixtures for 20 minutes at  $50^\circ$  in pH  $6.0 \pm 0.1$ .

**Solvent Extraction**—In order to separate the fluorescent product from interfering materials in the body fluids, the extractibility of the fluorescent product from various pH media was tested with several organic solvents. The compound was readily extracted into ethyl acetate and isoamyl alcohol from neutral media but was partially insoluble in benzene, chloroform and acetone-chloroform (1:1). The product could be subsequently reextracted into acidic and alkaline solution from organic solvents. On the other hand, amoxicillin itself could not be extracted from aqueous solution into any of the solvents tested, especially into chloroform and ethyl acetate. Consequently, ethyl acetate was suitable for the extraction of the fluorescent product from the body fluids. Furthermore, according to the relationship between the fluorescence intensity and pH at measured time (Fig. 4),  $1/10 N$  sodium borate solution was used as the reextracting medium in the procedure for the determination of total amoxicillin in the urine and blood samples. In the procedure for the determination of penicilloic acid in the urine and blood samples,  $1/10 N$  hydrochloric acid was used as the reextracting medium because of the relative stability of amoxicillin in the acidic solution. And the final pH of aqueous layer was adjusted to pH  $9.4 \pm 0.1$ .

There was a linear relationship between amoxicillin concentration and fluorometric response in the range of  $0.1$ — $0.4 \mu\text{g}/0.4 \text{ ml}$  whole blood.

### Recovery Test of Amoxicillin and Its Penicilloic Acid

A mixture containing a known amount of each standard substance was added to the aqueous solution, human urine or human blood and was separately measured by the procedure in Chart 1 and Chart 2, respectively. The results are given in Table I, in which the agreement between added amount and found value is reasonable. Thus, these methods of separatory determination were found to be applicable to the aqueous, urine and blood samples containing amoxicillin and its penicilloic acid.

TABLE I. Recovery Test of Amoxicillin and Its Penicilloic Acid

Sample	Added ( $\mu\text{g}$ )		Exp. No.	Found ( $\mu\text{g}$ )		Recovery (%)	
	AM-PC	AM-PA <sup>a)</sup>		AM-PC	AM-PA	AM-PC	AM-PA
Aq. soln.	5.00	5.00	(3)	4.80—4.95	4.95—5.17	96.0—99.0	99.0—103
	5.00	2.00	(3)	4.82—5.10	2.06—2.11	96.8—101	103—105
	2.00	5.00	(3)	1.95—2.06	5.00—5.03	97.5—103	100—101
Urine	5.00	5.00	(3)	4.88—4.96	5.03—5.06	97.7—99.3	101—101
	5.00	2.00	(3)	4.86—5.09	2.05—2.10	97.1—102	103—105
	2.00	5.00	(3)	1.92—1.98	4.99—5.20	96.2—98.9	99.9—104
Blood	1.00	1.00	(3)	1.03—1.04	1.03—1.04	103—105	103—105
	1.00	3.00	(3)	0.984—1.02	2.99—3.02	98.4—101	99.7—101
	4.00	2.00	(2)	3.84, 3.84	2.08, 2.08	96.0, 96.0	104, 104

<sup>a)</sup> equiv. of amoxicillin

AM-PC: amoxicillin, AM-PA: penicilloic acid of amoxicillin

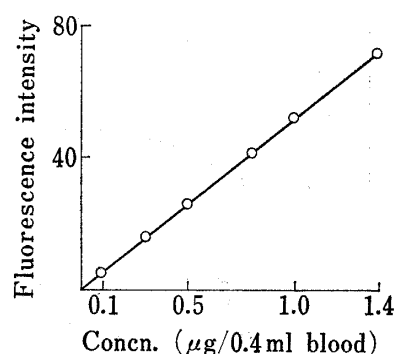


Fig. 5. Fluorometric Standard Curve obtained for Amoxicillin added to Pooled Human Whole Blood

### Urinary Excretion in Man

Urinary excretion of amoxicillin in man was compared with that of ampicillin which had been reported previously.<sup>5b)</sup> The result was shown in Fig. 6. In the urine during 6 hours after oral administration of these antibiotics, unchanged amoxicillin was excreted more rapidly in larger amounts (68% of the dose) than unchanged ampicillin (33% of the dose). And the small amount of 9% and 4% of the dose was excreted as penicilloic acid of amoxicillin and ampicillin, respectively.

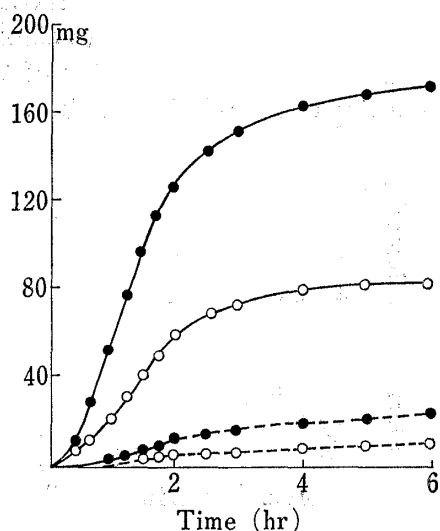


Fig. 6. Cumulative Urinary Excretion of Ampicillin, Amoxicillin and Those Penicilloic Acids after Oral Administration of Ampicillin (O) and Amoxicillin (●) to Man

—: unchanged antibiotics  
 - - -: penicilloic acids

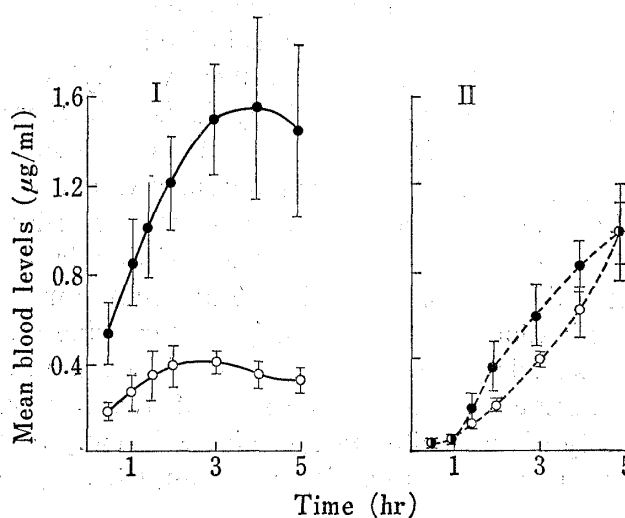


Fig. 7. Mean Whole Blood Levels of Antibiotics and Their Metabolites after Intraduodenal Administration of Ampicillin (O) and Amoxicillin (●) to Rats

Results are expressed as the mean  $\pm$  S.E. of four animals.  
 I : unchanged ampicillin and amoxicillin  
 II : metabolites of ampicillin and amoxicillin  
 dose : 10 mg/kg

### Blood Levels in Rats

Changes in the blood levels of amoxicillin and ampicillin, observed after the drugs were given to rats intraduodenally, were shown in Fig. 7. Unchanged amoxicillin achieved very high blood levels. In addition, as shown in Fig. 7 (II), there was a tendency for the blood levels of the metabolites, assumed to be penicilloic acids of these antibiotics, to increase with time.

Since the blood levels of these metabolites were measured by a method shown to measure penicilloic acids and then there was a tendency for blood levels of aminobenzylpenicilloic acid to increase during the experimental period after its administration, the possibility that the administered antibiotics were decomposed in the digestive tract and the decomposition products were then absorbed cannot be denied. It is necessary to give consideration to the fact that the marked differences in the body fluid concentrations of amoxicillin and ampicillin was observed. In this connection, further study will be undertaken.