

# STUDY ON LECITHINASE C ACTIVITY IN THE LIVER NECROSIS AFTER INTERRUPTION OF THE ARTERIAL FLOW TO THE LIVER

by

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## I INTRODUCTION

Two vessels supply blood to the liver—namely the hepatic artery and the portal vein. After the ligation of the hepatic artery, mortality is always expected in dogs, cats, rabbits and human beings since the article of HAEBERER (1905) on the ligation of the hepatic artery performed distally from the junction of the right gastric artery. HUGGINS and POST, reported the death was due to liver necrosis within three days postoperatively as result of the entire ligation of those three arteries——the common hepatic artery, gastroduodenal artery, and right gastric artery——all flowing into the hepatic hilus. This fact has been confirmed by not a few researchers.

In 1949, however, MARKOWITZ, RAPPAPORT, and SCOTT discovered the fact, by chance, that a large dose of penicillin which had been used with the intention to help the recovery after operation, could overcome the fatal influence of the hepatic artery ligation. A large dose of penicillin after the hepatic artery ligation could successfully reduce the high mortality of 100 per cent to 35. Intense follow-up studies have been tried by BOLLMAN and MANN, DAVIS, FITTS, and also FRASER and TANTURI confirming the effectiveness of antibiotics in reducing the mortality after ligation of the hepatic artery.

WOLBACH and SAIKI testified the presence of spore-bearing anaerobic bacillus in the livers in most of adult dogs, and BERG, ZAU, and JOELING proved in their study,

the existence of anaerobic bacillus, similar to welchii bacillus, in the livers of three out of eleven dogs.

ELLIS and DRAGSTEDT investigated histologically the liver necrosis caused by ligation of the hepatic artery confirming that the liver parenchym was entirely destroyed in necrotic areas resulting in the proliferation of spore-bearing anaerobic bacillus caused by anoxia.

TANTURI, SWIGART and CANEPA (1950) recognized the presence of lecithinase in the ascitic fluid of dogs which died after ligation of the hepatic artery. They reported that the lecithinase played the major role in the death of the hepatic artery ligated dogs. To speak in detail, the ligation of the hepatic artery stops the arterial flow to the liver causing deficiency of oxygen in the liver cells, and provides a favorable condition for proliferation of anaerobic bacillus existing in the liver, subsequently leading to the production of toxin which acts as a lethal, hemolytic, and necrotizing factor. Accordingly, lecithinase was looked upon to be the major cause of the death.

In this study, the experiments, following the method of MACFARLANE and KNIGHT, were carried out to investigate enzymatically the activity of lecithinase C in the livers—the necrotic liver after interruption of the hepatic artery, the penicillin administrated liver after interruption of the hepatic artery, and the hepatic artery interrupted livers of ascitic dogs which were produced by constriction of the hepatic vein or constriction of the inferior vena cava, and the interrelations between the activity of lecithinase C and liver necrosis, and accordingly, the cause of death of the hepatic artery interrupted dogs were examined.

## II METHODS AND MATERIALS

### 1 METHODS TO PREPARE LIVER NECROSIS

Healthy normal dogs weighing between five and twelve kg were used. HUGGINS and POST, MARKOWITZ, RAPPAPORT, FRASER, TANTURI, and URABE in our clinic reported that simultaneous ligation of the common hepatic artery, gastroduodenal artery, and right gastric artery always induced liver necrosis and death in 100 per cent. In the following experiments also under intravenous nembutal anesthesia (0.5 cc/kg), the three hepatic arteries mentioned above were separated and exposed from the surrounding tissues through a midline abdominal incision, each being doubly ligated and cut off. In the case of penicillin administration, it was given intraabdominally or intramuscularly after ligation of the hepatic artery. For the ascitic dogs also, the same operation was done. In the case of penicillin non-treated dogs, after cutting off of the hepatic arteries, almost all of the dogs died of liver necrosis within seventeen to twenty four hours postoperatively. On the contrary, penicillin administered dogs and ascitic dogs could survive the cutting off of the hepatic arteries, except a couple of cases which died 24 hours after operation.

The activity of lecithinase C was measured immediately, after the death in the dead cases and same measurement was taken in survival dogs after sacrifice within 24 to 28 hours.

### 2 EXTRACTION OF ENZYME

Irrespective of administration of penicillin, in most cases, massive necrosis was observed in all of the hepatic lobes after cutting off of the hepatic arteries. But, in not a few cases, the writer could hardly recognize liver necrosis in some lobes, particularly in the right lobe, and if any, only a trace of stagnation, although liver necrosis was observed in the predisposing lobes of the liver.

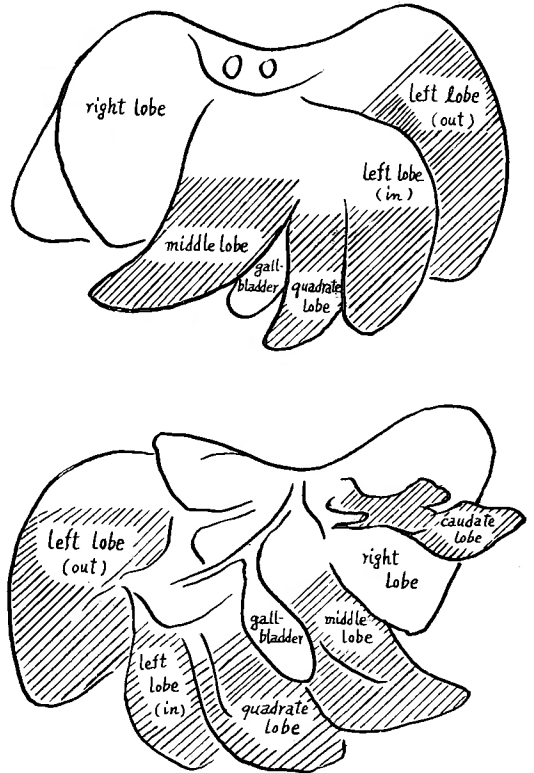
As the activity of lecithinase C could not be demonstrated in such less damaged lobes, the enzyme was extracted exclusively from the lobes which developed necrosis. In the case of penicillin administered dogs and ascitic dogs, the enzyme was extracted from the liver tissue of the left, middle, quadrate and caudate lobes where necrosis was usually found when no penicillin was administered, since there was no sign of liver necrosis (Fig. 1).

The utilization of gross enzyme solution extracted from a certain amount of liver tissue, which was soaked in physiological saline solution or in buffer solution and homogenized, suggested the following possibilities: mixing with a large amount of blood due to the congestion of liver tissue, inhibition of the activity of lecithinase, as TANTURI stated, due to the action of anti-lecithinase which was found in the blood serum; incomplete destruction of the cellmembran and of bacillus; and moreover, intervening of other enzymes besides lecithinase.

If liver tissue was dissolved after freezing for a certain period, and treated with acetone and homogenized, the protoplasmamembranes and bacilli were entirely destroyed with the result that enzyme protein — considered to be non-soluble protein — went out of the cell body, and the intervening proteins degenerated and refinement of extracted enzyme was possible. Although degeneration and inactivation of liver acetone powder thus obtained was conceivable, the toxin of clostridium perfringens was precipitated with acetone, but it could not be extracted with ether, as MURATA reported. Inactivation of this enzyme treated with acetone, as to be mentioned later, was not recognized.

Therefore, homogenized liver powder treated with acetone was prepared and applied to the enzyme reaction. The process was as follows: the removed liver lobe was washed with physiological saline solution, stored in the refrigerator and kept

Fig. 1 Favorite Sites of Liver Necrosis after Interruption of the Hepatic Artery



at the temperature between  $-5^{\circ}\text{C}$  and  $-10^{\circ}\text{C}$  for twenty four hours. A certain amount of this frozen liver lobe (10 g) was measured and ground, put into the homogenizer and dissolved for twenty minutes in lukewarm water of  $30^{\circ}\text{C}$ , and after being refrozen, ground with 30 cc of cold acetone as quickly as possible in cold temperature. The ground substance was to be filtered by suction bottle and sediments thus obtained were washed properly firstly with cold acetone, secondly with cold ether. We could obtain liver acetone powder by drying these washed sediments under the reduced pressure in the desiccator. 1g of liver acetone powder was dissolved into 6 cc of conc. glycerin and kept at  $0^{\circ}\text{C}$  the day before the experiment.

On the day of the experiment, gross enzyme solution was obtained from the soluble part which was separated from the sediments by a centrifugal separator (2,000 rpm. for 20 m.), after stirring up with the same amount of distilled water.

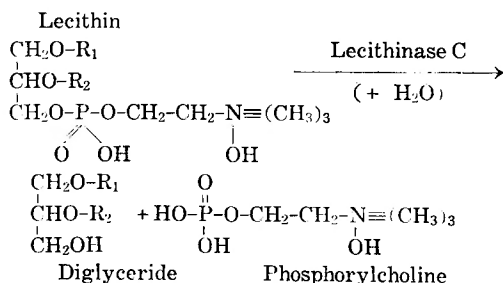
### 3 LECITHINASE C

In 1939, NAGLER recognized the formation of turbidity and floating up of the lipid layer in human serum where clostridium welchii type A was cultivated. MACFARLANE, OAKLEY and ANDERSON presumed this reaction to be due to alpha-toxin composed of lethal hemolytic necrotizing factor in cultivated filtrate. MACFARLANE and KNIGHT proved that lecithinase C was contained in clostridium welchii toxin, and further, they identified lecithinase C with alpha-toxin. The nomination of the lecithinase C was first given by the discoverer, and later it was called as clostridium welchii lecithinase. This enzyme, however, acted not only on lecithin but also on phospholipid like sphingomyelin, and so it was widely called phospholipase C. In the writer's study, the name of lecithinase C was adopted according to the classification by ZELLER.

### 4 METHOD OF MEASUREMENT OF LECITHINASE C ACTIVITY

Lecithinase C, enzymatically, is an enzyme yielding phosphorylcholine and diglyceride by hydrolysis process (Table 1). The activity of lecithinase C is to be

Table 1



measured according to the method developed by MACFARLANE and KNIGHT. The amount of acid-soluble phosphorus, i.e., phosphorylcholine P, which is obtained by the isolation from lecithin solution acted by the gross enzyme solution for a certain period, is measured and indicated as enzyme unit.

The composition of solution in which enzymatic reaction is carried out is as

**Table 2** Composition of Enzyme Reaction Solution

Main Reaction Solution :		Control (1):
0.2cc	0.003M CaCl <sub>2</sub> Solution	0.2cc 0.003M CaCl <sub>2</sub> Solution
1.0cc	Enzyme Solution	1.0cc Enzyme Solution
1.0cc	2.5% Lecithin Solution	2.0cc Palitzsch's buffer
1.0cc	Palitzsch's borate buffer	2.8cc Distilled water
2.8cc	Distilled water	Total Quantity 6.0cc
Total Quantity 6.0cc		Control (2):
		0.2cc 0.003M CaCl <sub>2</sub> Solution
		1.0cc 2.5% Lecithin Solution
		2.0cc Palitzsch's buffer
		2.8cc Distilled water
		Total Quantity 6.0cc

shown in Table 2. Total quantity is 6 cc. The pH of the solution is arranged to be 7.1-7.3 with 1N-Na<sub>2</sub>CO<sub>3</sub> solution. The reaction is processed in the constant hot water (37°C). For the purpose of control, both enzyme solution without lecithin and lecithin solution without enzyme are reacted at the same time. 2 cc of each reacted solutions is taken out with pipette immediately after the reaction and one and a half hours later, further 2 cc is again taken out. The reaction of these prepared solutions was stopped by adding 8 cc of 10 per cent trichloroacetic acid solution. After cooling off for 10 minutes, these mixed solutions are filtered with No. 9 filter (Toyo Roshi Co.). 2 cc of each filtrate is put into the test tubes separately, and then, the acid-soluble phosphor value is measured by BECKMANN'S photo-electric spectrophotometer according to the modification of FISKE and SUBBAROW. The value of lecithinase C is calculated by subtracting the increased values of the two control solutions from that of main solution.

## 5 ENZYMATIC PROPERTY OF LECITHINASE C

### i pH and Lecithinase C Activity

pH test paper, BTB (brom-thymol-blue) of Toyo Roshi Co. and PALITZSCH'S borate buffer solution (pH 7.1) were used in measuring pH of the solution (Fig. 2). Optimal pH was obtained at 7.1-7.6 in this enzymatic reaction. This figure corresponds well with the pH value 7.0-7.6 which was presented by MACFARLANE for clostridium welchii lecithinase in borate buffer solution.

### ii Activator, Especially CaCl<sub>2</sub> Solution and Lecithinase C Activity

Although Ca<sup>++</sup> was best suited for the activation of lecithinase C, in the concentration exceeding more than 0.1 mol, it was impossible to make its measurement, because of the coagulation of lecithin solution. Table 3 tells us that the difference

**Table 3**

Dog No.	Lecithinase C Activity	Concentration of Ca ions (mol)	Isolated total acid-soluble P.mg/dl		Activation Rate ( $\frac{A-B}{A} \times 100\%$ )
			Ca <sup>++</sup> added (A)	free of Ca <sup>++</sup> (B)	
18	Positive	0.003M 0.2cc	0.697	0.357	48.8%
31	Positive	0.003M 0.2cc	0.635	0.302	52.4%
48	Positive	0.003M 0.2cc	0.666	0.285	57.2%
50	Positive	0.003M 0.2cc	0.331	0.050	84.9%

Fig. 2 Effect of pH on Lecithinase C Activity

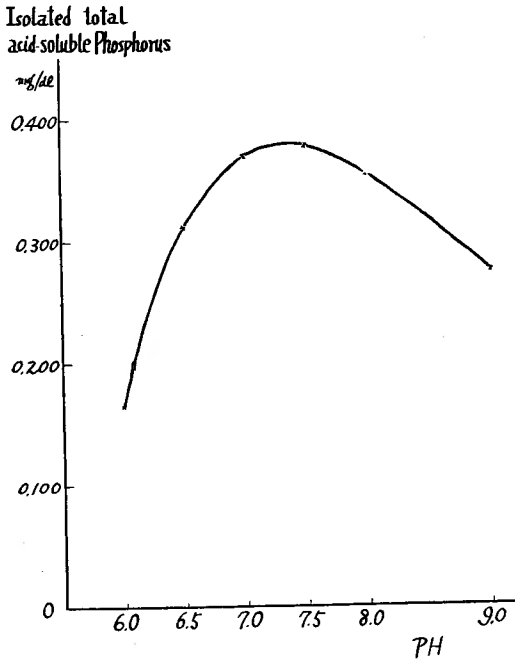
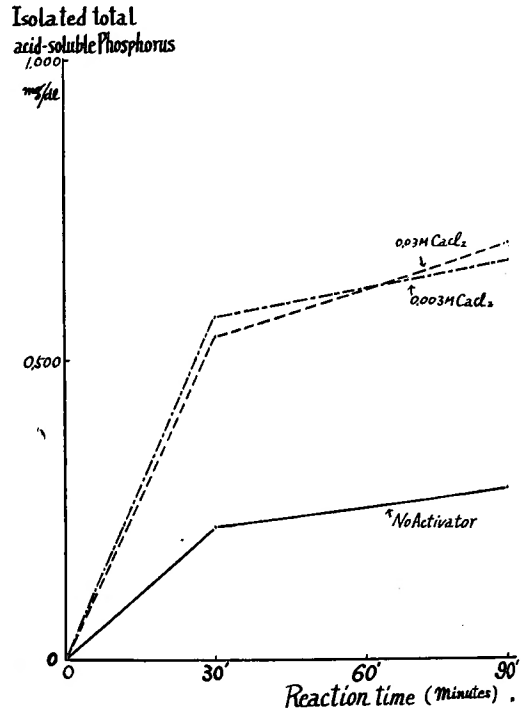


Fig. 3 Effect of Addition of Activator on Lecithinase C Activity



caused by adding or not adding the 2 cc of 0.003 mol CaCl<sub>2</sub> solution, was about 50 per cent of activation rate within one and a half reaction hours. The results obtained by adding 2 cc of 0.03 mol CaCl<sub>2</sub>, and of 0.003 mol CaCl<sub>2</sub> or not adding any, are shown respectively in Fig. 3.

### iii Substrate Concentration and Lecithinase C Activity

The lecithin used in this experiment as substrate is glidex-P (GL-5) (Dainippon Seiyaku Co.), a high pure soybean lecithin, which contains over 95 per cent of phosphatid. Total phosphor amount of 7.145 mg/dl is measured in 1 cc of 2.5 per cent lecithin solution.

The interrelation between substrate concentration and lecithinase C activity is indicated in Fig. 4. Curves vary according to the enzymes used, and the most powerful activity was observed, on average, in the case of lecithin solution of 2.5-2.6 per cent.

Lecithinase C activity was measured manometrically using WARBURG's manometer by ZAMECNIK, BREWSTER, and LIPMANN, who reported the best result was obtained when the lecithin solution was at the concentration of 2.6 per cent.

### iv Concentration of Enzyme Solution and Lecithinase C Activity

Fig. 4 Effect of Substrate Concentration on Lecithinase C Activity

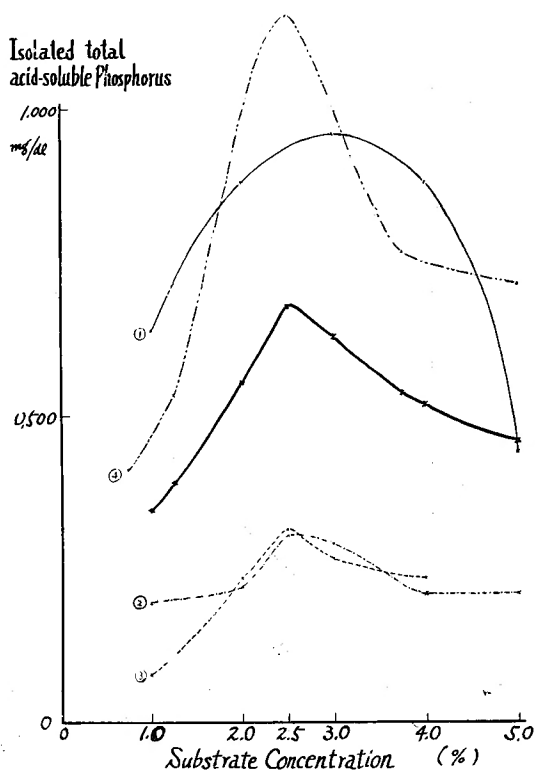
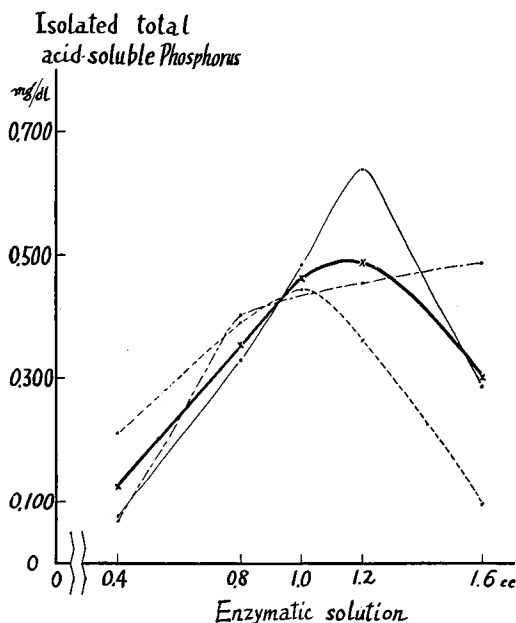


Fig. 5 Effect of Enzyme Concentration on Lecithinase C Activity



Aforementioned gross enzyme solution was used. ZAMECNIK reported, manometrically, the rate of hydrolysis of the substrate, within a certain limit of concentration, has been found to be directly proportional to an increase of enzyme concentration. Although the degree of enzyme reaction differs more or less, as illustrated in Fig. 5, according to the variety of material to be used as enzyme solution, the direct proportional relation to speak on average is confirmed up to 1.0 cc of enzyme solution.

v Reaction Time and Lecithinase C Activity

High-pitched was the velocity of this enzyme reaction until 150 minutes after set-in of the reaction (Fig. 6). MACFARLANE and KNIGHT used clostridium welchii cultivated filtrate directly as gross enzyme solution, and they, on the reason of its great toxicity, converted the value of acid-soluble phosphor obtained in 15 minutes after set-in of the reaction into that of lecithinase C activity. In this experiment, for the prevention of possible error, the value was measured in 90 minutes instead of 15 minutes.

vi Reaction Temperature and Lecithinase C Activity

Direct proportion between reaction velocity and temperature (20°-45°C) has been recognized by ZAMECNIK, BREWSTER, and LIPMANN. In this experiment also, as shown in Fig. 7, it was affirmed that there existed a directly proportional relation between the velocity of reaction and the temperature ranging from 16°C to 45°C.

Fig. 6 Effect of Reaction Time on Lecithinase C Activity

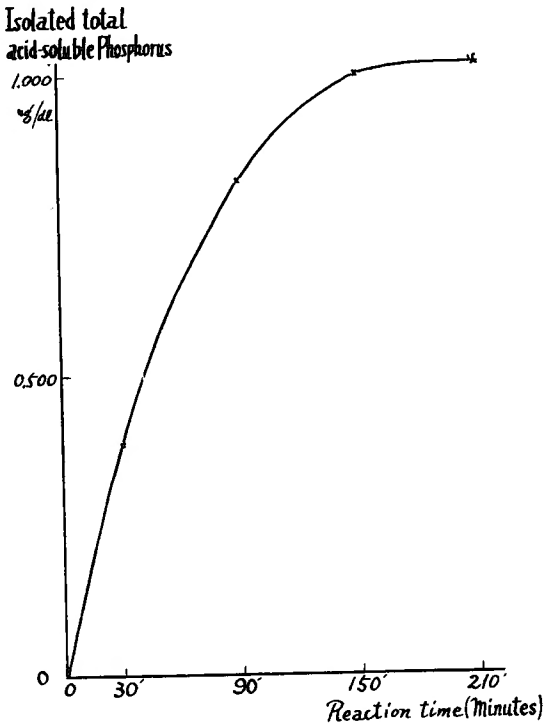
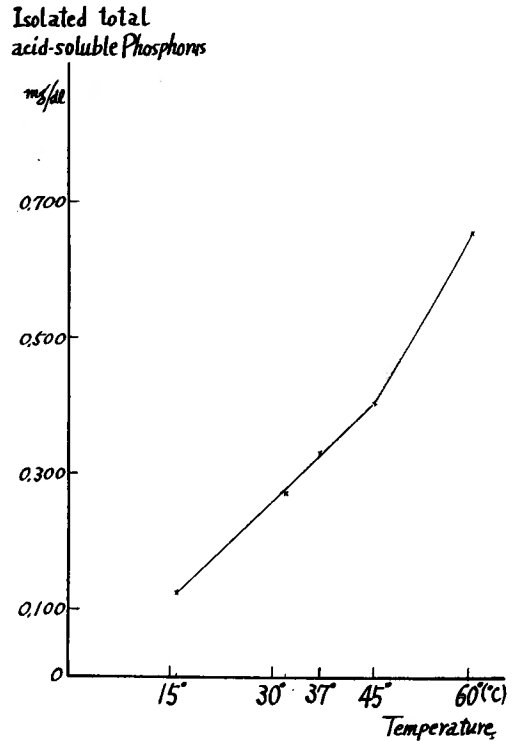


Fig. 7 Effect of Temperature on Lecithinase C Activity



#### vii Gas Gangren Anti-toxin and Lecithinase C Activity

Anti-toxic serum of *clostridium welchii*, as stated by MACFARLANE and KNIGHT, checked phospholipase activity of related toxin. Proliferation of anaerobic bacillus *iike welchii* bacillus was found in most part of necrotic liver after ligation of the hepatic artery. The toxin produced could not be assumed to be the specific one, and accordingly, the anti-toxin should be considered to be polyvalent. As the state of liver necrosis was similar to that of gas gangren, gas gangren anti-toxic serum, a product of Chiba Prefectural Serum Institute, was used for anti-toxin in this experiment. This is a polyvalent anti-gas gangren-toxin which is mixed properly with anti-toxins refined from the horse serum immunized with each antigen of gas gangren bacillus, according to pepsin digestive method and sulphoammonium salt analysis. 1 cc of this anti-toxic serum contains respectively 100 units of *clostridium perfringens* (cl. *welchii*), cl. *oedematiens* (cl. *novyi*), and *vibron septique*.

Should the activity of lecithinase C be inhibited or checked in this enzyme reaction by adding this anti-toxin, it might prove that extracted enzyme solution contained the toxin similar to the toxin which competed with one of the anti-toxins in the gas gangren anti-toxin, and that this enzyme did not lose its activity.

ZAMECNIK and LIPMANN stated, in their article dealing with the antagonistic action between lecithin and anti-toxin for *clostridium welchii*, that if lecithin came into contact with lecithinase firstly, the addition of anti-toxin could not stop the



Table 4

Dog No.	Isolated total acid-soluble Phosphorus mg/dl	Lecithinase C Activity	Quantity of Antitoxic Serum or Normal Serum	Isolated total acid-soluble P. mg/dl		Inhibitory Rate $\left(\frac{B-A}{B} \times 100\%\right)$
				Antitoxic serum added to Main Reaction sol. (A)	Normal serum added to Main Reaction sol. (B)	
29	0.122	Positive	0.2cc	0.188	0.376	50.0%
31	0.805	Positive	1.0cc	-0.017	0.059	128.8%
44	0.091	Positive	1.0cc	-0.035	0.087	140.2%
45	1.649	Positive	1.0cc	-0.071	0.035	302.9%
46	0.369	Positive	0.1cc	0.068	0.237	71.3%
			0.5cc	0.102	0.593	82.8%
			1.0cc	-0.049	0.082	160.0%
50	0.117	Positive	0.5cc	0.017	0.033	48.5%
56	0.133	Positive	0.2cc	0.046	0.141	64.5%
61	0.114	Positive	0.5cc	0.018	0.104	82.7%
62	0.141	Positive	0.5cc	0.156	0.688	77.3%

Fig. 8 Effect of Addition of Antitoxic Serum on Lecithinase C Activity

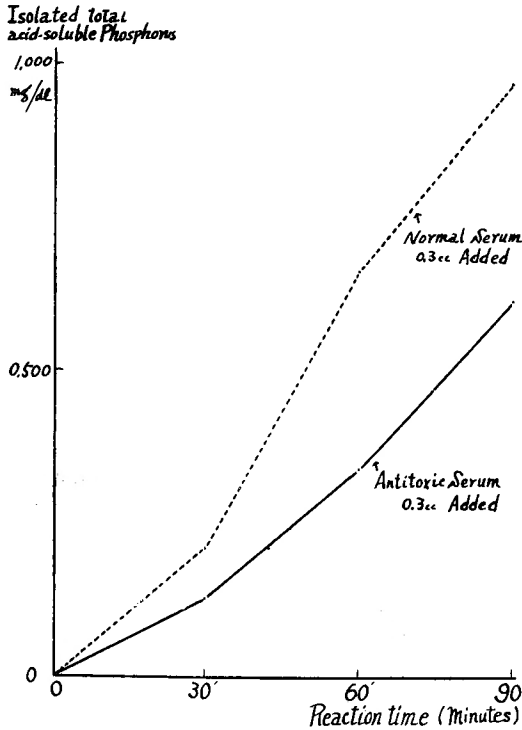
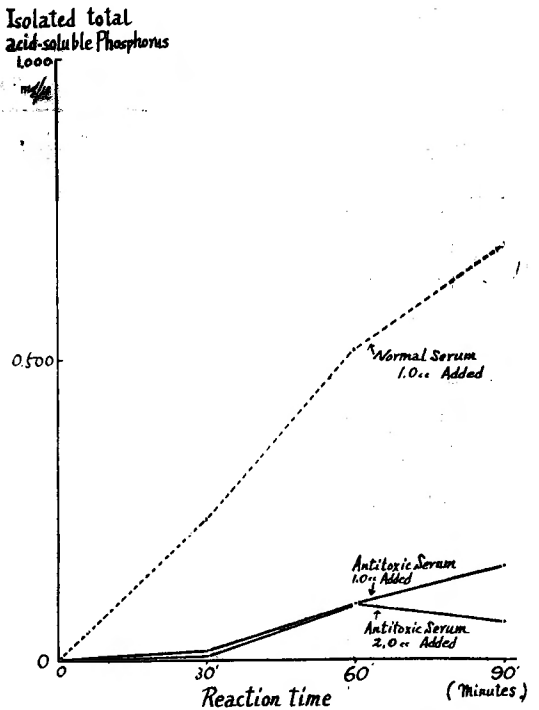


Fig. 9 Effect of Addition of Antitoxic Serum on Lecithinase C Activity



enzyme reaction, but prolonged gradually its velocity.

Table 4 shows the values of reaction obtained one hour and a half after the simultaneous contamination of lecithin solution and anti-toxic serum with the enzyme

solution. Nearly complete inhibition of enzyme activity was shown by adding anti-toxic serum exceeding 1.0 cc (containing respectively 100 units of each anti-toxin). Figs 8, 9 show the protracted enzyme reaction observed with the lapse of time after an addition of lecithin to the enzyme solution, immediately followed by mixing with anti-toxic serum. The difference in the amount of anti-toxin to be added naturally made the difference in the velocity of the reaction. For the control, the serum of healthy normal horse, to which ethyl hydragyryum salicylic acid natium — aseptics for gas gragren anti-toxic serum — was added at the rate of 0.01 per cent, was used.

### III RESULTS OF EXPERIMENTS

#### 1 THE CASE OF THE HEPATIC ARTERY INTERRUPTED DOGS: PENICILLIN NOT ADMINISTERED

In this case, the activity of lecithinase C was demonstrated in 20 dogs out of 23 (87.0 per cent) and not in 3 (13.0 per cent). Necrosis was found with naked eyes all over the livers of dead dogs and positive activity of lecithinase C was also ascertained. Some positive cases of lecithinase C survived the operation for about 24 hours. In these seven cases, liver necrosis was found with naked eyes as shown in Tables 5 and 6.

Three negative cases of lecithinase C were found in the dogs which were living

Table 5

Dog No.	Sex	Weight kgm	Date of operation	Survival	Liver necrosis	Lobe of liver	Isolated total acid-soluble Phosphorus mg/dl
8	♂	6.5	6-3-57	Died 43 hrs.	++	Left lobe	0.680
11	♀	5.6	6-24-57	Died 17 hrs.	++	Left lobe	0.834
12	♀	6.5	7-3-57	Died 20.5hrs.	++	Left lobe	1.060
18	♀	11.0	9-16-57	Died 22 hrs.	++	Left lobe	1.216
22	♀	5.6	9-20-57	Died 24.5hrs.	++	Left lobe	1.188
23	♂	8.5	9-25-57	Sacrificed 28.5hrs.	+	Left lobe	0.129
25	♀	6.0	10-1-57	Died 20 hrs.	++	Left lobe	0.389
26	♀	7.5	10-4-57	Sacrificed 26 hrs.	+	Left lobe	0.424
29	♂	7.0	10-25-57	Died 24 hrs.	++	Left lobe	0.122
31	♂	8.0	11-8-57	Sacrificed 25 hrs.	++	Left lobe	0.805
44	♀	6.5	1-12-58	Sacrificed 24 hrs.	++	Left lobe	0.091
45	♂	10.0	1-30-58	Died 24 hrs.	++	Left lobe	1.649
46	♀	8.2	2-6-58	Sacrificed 21.5hrs.	+	Left lobe	0.369
47	♀	8.0	3-5-58	Died 22 hrs.	++	Middlelobe	0.209
48	♀	7.6	3-26-58	Died 19 hrs.	+	Left lobe	0.032
50	♂	10.8	4-16-58	Sacrificed 24 hrs.	++	Middlelobe	0.117
56	♀	12.5	6-12-58	Died 14.5hrs.	+	Left lobe	0.133
61	♂	6.0	7-30-58	Died 24 hrs.	++	Caudal lobe	0.171
62	♀	7.0	8-22-58	Sacrificed 43 hrs.	++	Left lobe	0.141
65	♀	10.0	10-1-58	Died 15.5hrs.	++	Caudal lobe	0.295

Table 6

Dog No.	Sex	Weight kgm	Date of operation	Survival	Liver necrosis	Congestion of liver	Lobe of liver	Isolated total acid-soluble Phosphorus mg/dl
20	♂	5.0	9-17-57	Sacrificed 28hrs.	-	+	Left lobe	-0.158
28	♂	12.0	10-11-57	Sacrificed 27hrs.	-	±	Left lobe	-0.303
33	♀	5.8	11-14-57	Sacrificed 26hrs.	-	-	Left lobe	-0.674

at the time of 24th postoperative hour, showing no finding of necrosis in all of them at the time of sacrifice, but only a slight degree of congestion in 2 cases out of 3.

## 2 THE CASE OF THE HEPATIC ARTERY INTERRUPTED DOGS: PENICILLIN ADMINISTERED

Reduction of mortality after ligation of the hepatic artery from 100 per cent to 35 per cent by the administration of a large doses of penicillin was successfully achieved by MARKOWITZ and RAPPAPORT. On the other hand, URABE in our clinic proved the mortality to be approximately the same between the cases in which 300,000 units of penicillin per day was administered for nine days extending before and after the operation and the cases in which only a single dose of 100,000 units

Table 7

Dog No.	Sex	Weight kgm	Date of operation	Survival	Liver necrosis	Lobe of liver	Isolated total acid-soluble Phosphorus mg/dl	Amount of Penicillin
Negative cases of Lecithinase C Activity								
10	♀	8.5	6-11-57	Sacrificed 26hrs.	-	Left lobe	-0.100	200,000 Units in Abdom.
27	♀	5.0	10- 9-57	Sacrificed 24hrs.	+	Left lobe	-0.230	100,000 Units in Abdom.
35	♀	7.0	12- 2-57	Sacrificed 16hrs.	-	Left lobe	-0.066	100,000 Units in Abdom.
49	♀	6.5	4- 9-58	Sacrificed 72hrs.	-	Left lobe	-0.889	100,000 Units in Abdom.
55	♀	8.4	5-28-58	Sacrificed 24hrs.	-	Left lobe	-0.201	100,000 Units in Abdom.
58	♀	16.0	7-23-58	Sacrificed 18hrs.	-	Left lobe	-0.152	100,000 Units in Abdom.
59	♀	6.5	7-29-58	Sacrificed 24hrs.	-	Left lobe	-0.638	100,000 Units in Abdom.
Positive cases of Lecithinase C Activity								
16	♀	8.2	8-20-57	Died 20 hrs.	+	Left lobe	0.410	300,000 Units in Muscles
24	♀	6.0	9-24-57	Sacrificed 73.5hrs.	±	Left lobe	1.339	100,000 Units in Portal Vein
37	♂	7.0	12-13-57	Died 26 hrs.	+	Left lobe	0.091	100,000 Units in Abdom.
43	♀	5.2	1- 9-58	Sacrificed 46 hrs.	+	Left lobe Middle	0.762	100,000 Units in Abdom.

of penicillin was injected intraabdominally immediately after the operation. In this experiment, therefore, 100,000 units of penicillin injection was given only once intraabdominally and after that, no antibiotics were administered (Table 7).

11 dogs were administered with penicillin; in 4 of them (36.4 per cent) the activity of lecithinase C was demonstrated, and not in 7 (63.6 per cent).

Liver necrosis was found in all of the positive cases, dead or alive. In these dogs, the findings of liver necrosis were entirely the same macroscopically as in the dogs penicillin not administered. Seven dogs of negative cases were alive after 24 hours postoperative. The dog of Case No. 27 was an exception, in which liver necrosis was found, while the activity of lecithinase C was negative.

### 3 NORMAL DOGS

The presence of spore-bearing anaerobic bacillus in the livers of normal healthy dogs was demonstrated by WOLBACH and SAIKI, and moreover, BERG, ZAU, and JOBLING testified the existence of anaerobic bacillus like welchii bacillus.

The writer measured the activity of lecithinase C of the liver lobes in normal dogs. Although the lobes examined were found to be intact macroscopically, they were naturally predisposed to the development of necrosis after ligation of the hepatic artery, but the activity of lecithinase C was not found in the livers of normal dogs (Table 8).

Table 8

Dog No.	Sex	Weight kgm	Method of operation	Survival	Findings of liver	Liver necrosis	Lobe of liver	Isolated total acid-soluble Phosphorus mg/dl
21	♀	8.3	trial thoracotomy	Sacrificed	Normal	—	Right lobe	-0.663
32	♂	9.0	bilateral vagotomy	Sacrificed	Normal	—	Left lobe	-0.650
36	♂	7.2	death from anaesthesia	Died	Normal	—	Left lobe	-0.198
38	♂	10.0	bilateral vagotomy	Sacrificed	Normal	—	Left lobe	-1.080
40	♂	8.5	bilateral vagotomy	Sacrificed	Normal	—	Left lobe	-0.356

### 4 ASCITIC DOGS

In 1949, RIENHOFF first performed an operation of ligation of the hepatic artery in expectation of obtaining good results in liver cirrhosis with ascites. Since his report, this method of operation has not yet been widely accepted for fear of inducing liver necrosis, CHILD suggested that the cirrhotic liver would tolerate the interruption of the hepatic arterial flow. For the purpose of examining the validity of this operation, the writer produced ascites in dogs by constricting the hepatic vein or constricting the inferior vena cava, and then performed the interruption of the hepatic artery on those dogs investigating whether liver necrosis would occur or not and whether activity of lecithinase C could be demonstrated in the liver or not.

Seven dogs were under investigation (Table 9). The conclusion of the experiment was that neither necrosis nor activity of lecithinase C was demonstrated in the livers of all the ascitic dogs, dead or alive, irrespective of penicillin administration

Table 9

Dog No.	Sex	Weight kgm	Date of operation	Survival	Liver necrosis	Lobe of liver	Isolated total acid-soluble Phosphorus mg/dl	Ascites (l)	Remarks
34	♂	7.0	11-14-57	Died 24 hrs.	—	Left lobe	-0.322	1.5	Constriction of the Hepatic Vein
52	♂	25.0	4-18-58	Sacrificed 26 days	—	Left lobe	-1.654	1.0	Constriction of the Hepatic Vein
66	♂	9.5	11-18-58	Died 15.5 hrs.	—	Left lobe	-0.097	2.0	Constriction of the Inferior Vena Cava
68	♂	7.5	12- 9-58	Sacrificed 24.5 hrs.	—	Left lobe	-0.217	1.5	Constriction of the Inferior Vena Cava
69	♂	16.7	12-13-58	Died 18 hrs.	—	Left lobe	-0.126	6.0	Constriction of the Inferior Vena Cava
72	♂	8.0	12-27-58	Died 18 hrs.	—	Left lobe	-0.729	1.0	Constriction of the Inferior Vena Cava
73	♀	13.8	2-14-59	Sacrificed 27 hrs.	—	Left lobe	-0.266	3.0	Constriction of the Inferior Vena Cava

or not. Although penicillin was administered to three cases (Nos. 34, 52 and 68), the dog No. 34 died. Its liver was, however, found free from necrosis showing no change in colour. 4 died out of 7 ascitic dogs. In all of these four cases, neither necrosis nor activity of lecithinase C was demonstrated in their livers. Considering these data, it is suggested that the cause of death in ascitic dogs might be due to neither liver necrosis nor activity of lecithinase C, but rather to shock which was provoked by the rapid loss of large amount of ascites. The dog No. 73 was trained for the removal of ascites by puncturing ascites prior to the operation. Then the interruption of the hepatic artery was performed on this dog. Relaparotomy was done 27 hours after the previous operation and the left lobe of the liver was resected without using penicillin. The dog survived the last operation for 5 days. The finding of the liver at the time of 2nd operation showed, with naked eyes, no more change than that at the first operation.

#### IV DISCUSSION

Concerning the method of measuring the activity of lecithinase C, ZAMECNIK, BREWSTER, and LIPMANN reported that the value manometrically measured using WARBURG'S manometer nearly corresponded with that calculated by MACFARLANE and KNIGHT'S method. In our experiment, MACFARLANE and KNIGHT'S method was adopted, and the activity was determined by measuring the isolated acid-soluble phosphor value which was increased within one and a half hours after set-in of the reaction.

Enzymatically, the negative activity of lecithinase C in this experiment tells us that in one and a half hours after set-in of the reaction, total value of acid-soluble phosphor which is isolated by autolysis of the enzyme solution and of lecithin solution, is larger than the value of acid-soluble phosphor which was isolated from

the main reaction solution. The rate of autolysis of lecithin solution is almost constant. However, the value of acid-soluble phosphor produced by autolysis of enzyme solution extracted from the normal liver lobes is far larger than that extracted from the necrotic liver lobes.

In the case of negative activity of lecithinase C, the value of acid-soluble phosphor isolated from the main reaction solution is considerably small. This, as stated by ZAMECNIK, can be understood that the autolysis of enzyme solution was inhibited more strongly when lecithin was added to the enzyme solution than when lecithin was not added.

From the viewpoint of bacterial toxin, negative activity of lecithinase C tells us that proliferation of anaerobic bacillus like welchii bacillus doesn't exist in the liver, and that production of toxin, which acts as a lethal, hemolytic, and necrotizing factor, is not carried on. Accordingly, five healthy normal livers, the controls, showed negative lecithinase C activity. This does not mean the disappearance or absence of anaerobic bacillus in the normal liver lobes but it means that anaerobic bacilli still exist but not yet proliferated, and a satisfactory condition is not yet prepared for the production of toxin.

Although in many cases of non-treatment of penicillin after interruption of the hepatic artery, massive necrosis was found to have developed in the liver and no noticeable change was to be identified occasionally in the right liver lobe with the findings similar to those of healthy normal liver. The measurement of lecithinase C activity in this lobe proved to be negative (Table 10). It means that the damage

Table 10

Dog No.	Sex	Weight kgm	Addition of Penicillin	Survival	Lobe of liver	Liver necrosis	Congestion of liver	Haemolytic ascites	Isolated total acid-soluble Phosphorus mg/dl
22	♀	5.6	—	Died 24.5hrs.	Left lobe Right lobe	++ +	++ +	+	1.188 0.388
23	♂	8.5	—	Sacrificed 28.5hrs.	Left lobe Right lobe	+ —	+ +	—	0.129 -0.600
24	♀	6.0	100,000 Units in Portal vein	Sacrificed 73.5hrs.	Left lobe Right lobe	++ —	++ +	+	1.339 -0.149
25	♀	6.0	—	Died 20 hrs.	Left lobe Right lobe	++ ++	++ ++	+	0.389 1.559
27	♀	5.0	100,000 Units in Abdom.	Sacrificed 24 hrs.	Left lobe Right lobe	+ —	+ +	—	-0.230 0
28	♂	12.0	—	Sacrificed 27 hrs.	Left lobe Right lobe	— —	± ±	—	-0.303 -0.116

rendered by the operation is so slight that the proliferation of bacilli and the production of toxin have not taken place. What gives the most fatal influence is the production of toxin from the necrotic liver lobes. The activity of lecithinase C, in this experiment, is classified as to be positive or negative according to the activity value in the necrotic liver lobes.

GALE stated, concerning the clostridium welchii alpha-toxin, that the synthetic finding of experimental reaction in vitro by the enzyme system separated like this enzyme reaction, does not necessarily means the activity of living cells intricated with other various environmental factors. According to this concept, the significance of this experiment must be evaluated in judging whether the activity is positive or negative, but not in determining the absolute value.

The positive activity must be taken as to be the sign of proliferation of bacterial toxin. The living dog in which the activity of lecithinase C is found, is considered to die sooner or later, regardless of value of activity.

Three negative cases in which penicillin was not administered had been alive at the 24th postoperative hour and necrotic livers were not found at the time. Blood congestion was ascertained among two of them. A report has told us that those dogs may die within seven to twelve days after the operation, while others reported that the dogs maintained their lives, though a very few cases, after the perfect ligation of the hepatic artery. The fate of these three dogs might provide us an interest, but no prediction was possible so far, because they were sacrificed.

If we pursued the macroscopical changes in the liver with the lapse of time after interruption of the hepatic artery, the liver turned dark red in general, at first, and then this discolouration began to be localized gradually 3 hours after the operation. About 12 hours after the operation it became entirely localized, not diminishing its size any more, and necrosis might be observed in some of the dogs. Five cases shortly after operation, were under investigation to see the time when the activity of lecithinase C set in (Table 11). Except the dog No. 54 which died

Table 11

Dog No.	Sex Weight kgm	Date of operation	Survival	Liver necrosis	Lobe of liver	Isolated total acid-soluble Phosphorus mg/dl	Remarks
51	♀ 11.2	5-8-58	Sacrificed 12hrs.	Negative	Caudate lobe	-0.285	
53	♂ 5.0	5-21-58	Sacrificed 6hrs.	Negative	Left lobe	-0.100	
54	♀ 11.0	5-22-58	Died 12hrs.	Positive	Caudate lobe	-0.471	Accident death (by Hanging)
60	♂ 5.0	7-30-58	Sacrificed 12hrs.	Negative	Left lobe	-0.116	
64	♀ 10.5	11-11-58	Sacrificed 12hrs.	Positive	Left lobe	-0.062	

of hanging by accident, no dog died within 24 hours after the operation. The activity of lecithinase C was not recognized even in the cases which showed liver necrosis at the time of sacrifice. It was assumed that the activity of lecithinase C, though the number of experimental dogs was very few, was not put into action within 12 hours after the operation.

In four cases out of eleven to which penicillin was administered, the activity of lecithinase C was demonstrated. Necrotic livers were found in all four cases including two dogs which survived the operation. It is widely accepted that the mortality of ligation of the hepatic artery is decreased by the administration of

penicillin. This mortality of 35 per cent corresponds well with positive rate of lecithinase C activity obtained in the cases to which penicillin administered. This proves enzymatically the possible mortality in spite of penicillin administration after operation.

URABE in our clinic demonstrated that administration of penicillin within 15 hours after the operation was decisive in preventing death, and later administration of any large quantity of it was useless. From this fact it was suggested that toxin once produced, was not influenced by penicillin. In order to clarify this point the following experiment was undertaken. 1 cc of 2,000 units of penicillin kalium and that of penicillin natrium was added to the reaction solution. For the control, penicillin, made ineffective by 30 minutes boiling, was used (Table 12). The result

Table 12

Dog No.	Lecithinase C Activity	Isolated total acid-soluble Phosphorus (mg/dl)				
		Main Reaction solution	2,000units of PC kalium added to Main React. sol.	Boiling 2,000units of PC kalium added to Main Reaction sol.	2,000units of PC Natrium added to Main Reaction sol.	Boiling 2,000units of PC Natrium added to Main Reaction sol.
18	Positive	1.357	1.026	1.066		
25	Positive	1.227	1.862	1.513		
26	Positive	0.173	0.311	0.078		
31	Positive	0.661	0.714	0.587		
47	Positive	0.525			0.529	0.661
50	Positive	0.475	0.742	0.453	0.754	0.302
61	Positive	1.624	2.096	1.451	1.613	1.612

was, regardless of penicillin kalium salt or penicillin natrium salt, that there occurred no such remarkable change as would check the enzymatic reaction. Accordingly, it was believed that penicillin would not act on the toxin, that is, lecithinase C.

Inhibitive effects on this enzymatic reaction could be seen, as mentioned above, in gas gangren anti-toxin. It may be possible to conclude that the enzyme solution extracted from the liver is yet effective, and that it contains the same sort of toxin which counteracts with gas gangren anti-toxin. An attempt to save the dogs from death after interruption of the hepatic artery by giving gas gangren anti-toxin after or before the operation, has been tried. The report by TANTURI, dealing on this matter, said that although the length of survival was prolonged from 37 to 98 hours, all of these dogs eventually died. It looks like that the perfect protection

Table 13

Dog No.	Sex Weight kgm	Date of operation	Survival	Liver necrosis	Lobe of liver	Isolated total acid-soluble Phosphorus mg/dl	Remarks
27	♀ 5.0	10-9-57	Sacrificed 24hrs.	+	Left lobe	-0.230	Penicillin 100,000units in Abdomen
54	♀ 11.0	5-22-58	Died 12hrs.	+	Caudate lobe	-0.471	Accident. death (by Hanging)
64	♀ 10.5	11-11-58	Sacrificed 12hrs.	+	Left lobe	-0.062	



against death can not be acquired by giving active immunity.

Much stress must be placed on the fact that there were some cases in which the activity of lecithinase C was not demonstrated, in spite of the existence of liver necrosis observed with naked eyes (Table 13). FRASER reported that in his experiment, 6 out of 7 cases in which liver necrosis developed in spite of penicillin therapy were investigated bacteriologically with the result that the growth of bacilli by cultivation was not observed in 4 out of these 6 cases. He concluded that bacillus had no relation to the development of liver necrosis but the cause of necrosis might be due to ischemia. On the other hand, it is quite reasonable to presume that after interruption of the hepatic artery ischemic necrosis develops at first, and then proliferation of bacilli ensues in that necrotic area leading to the fatal liver necrosis. The necrotic changes observed in a short time of 12 hours postoperatively, such as seen in the dogs Nos. 54, 64, are deemed to be anoxic liver necrosis, while it may be said that the findings of the left liver lobe in the dog No. 27 was identified, enzymatically, to be ischemic necrosis such as claimed by FRASER.

Details were already told about the results of interruption of the hepatic artery on seven ascitic dogs in this writer's experiment. Of course, some survived the operation for a long time under the administration of penicillin, and some, without having any penicillin, maintained their lives for a certain long period showing neither activity of lecithinase C nor liver necrosis, if treated appropriately. It may be said that the liver of ascitic dogs can tolerate the interruption of the hepatic arterial flow.

## V CONCLUSION

1) Measurements of activity of lecithinase C in the liver were performed by the method of MACFARLANE and KNIGHT on normal dogs, on hepatic artery interrupted dogs with or without administration of penicillin, and on ascitic dogs produced by constriction of the hepatic vein or of the vena cava inferior after interruption of their hepatic arteries. According to the results obtained, the interrelationships between activity of lecithinase C, development of liver necrosis and death were examined.

2) Out of 23 dogs which were given no penicillin after interruption of the hepatic artery, 20 showed positive lecithinase C activity (87.0 per cent) and 3 negative activity (13.0 per cent).

Out of 11 dogs which were given penicillin after the operation, four showed positive lecithinase C activity (36.4 per cent), and seven negative (63.6 per cent). In 7 dogs which died after the operation, all of them showed positive activity of lecithinase C and liver necrosis, regardless of penicillin administration.

3) In dogs which survived the interruption of the hepatic artery, some of them showed positive lecithinase C activity, in spite of the existence of liver necrosis. These cases were believed to be doomed to die in near future.

4) Dogs, in which the activity of lecithinase C was negative, had much possibility to survive the interruption of the hepatic artery, and yet occasionally, they

were found to have liver necrosis. This liver necrosis was deemed to be in the stage of anoxic ischemic necrosis.

5) No activity of lecithinase C was recognized in the livers of healthy normal dogs.

6) In seven ascitic dogs after interruption of the hepatic artery, regardless of penicillin administration or not, all of them proved to be free from liver necrosis and showed negative activity of lecithinase C.

7) From these facts stated above, the writer is convinced that although liver necrosis plays an important role in the cause of the death after interruption of the hepatic artery, much more stress must be placed rather on the activity of lecithinase C.

In closing, I wish to thank Prof. Dr. CHISATO ARAKI and Assist. Prof. Dr. ICHIO HONJO for their guidance through out the period of this work.

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## 和文抄録

# 肝動脈血流遮断後の壊死肝に於ける レシチナーゼC活性に就いて

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動脈血と門脈血の供給を受ける肝臓に対して、肝動脈の遮断が如何なる影響を及ぼすかに関しては1905年 Haberer の報告以来、肝門より入る動脈を悉く結紮すると犬、猫、兎、人間に於ては常に肝壊死を來たして死亡すると云う事実が多くの研究者達によつて確認されている。ところが1949年 Markowitz, Rappaport & Scott 等は犬に於て肝動脈遮断後ペニシリンを大量に投与する事によつて、その死亡率を殆ど100%より35%迄に激減せしめる事に成功した。又肝動脈遮断によつて生ずる肝壊死に就いて Ellis & Dragstedt は組織学的に検索し、壊死巣には酸素欠乏に基づく芽胞形成の嫌気性桿菌の増殖を認めており、Tanturi 等は1950年に肝動脈遮断後死亡せる犬の腹水中より、肝内嫌

気性桿菌様細菌の細菌毒素と同一視されるレシチナーゼの存在を認め、之が肝動脈遮断犬の死亡に主要なる役割を演ずるものであると述べている。

従つて私は肝動脈血流遮断後壊死を來たした肝臓に就いて、肝動脈遮断後ペニシリンを投与した肝臓に就いて、更に腹水を伴える肝硬変症の1つの治療法としての肝動脈遮断術を検討すべく、先づ肝静脈狭窄或いは下大静脈狭窄により肝硬変類似の腹水犬を作成し、その腹水犬の肝動脈遮断後の肝臓に就いて MacFarlane & Knight の方法により酵素化学的にレシチナーゼC活性の有無を検討し、レシチナーゼC活性と肝壊死、更には肝動脈遮断犬の死亡との相互関係を究明すべく以下に述べる実験を行つた。

5~12kgの成熟健常犬を用い、ネブタール静脈内注入麻酔により開腹し、総肝動脈、胃十二指腸動脈、右胃動脈を露出し、夫々二重結紮後切断して腹壁を閉じた。ペニシリン投与例には10万単位1回丈腹腔内に注入して閉腹した。ペニシリン無投与の場合は術後17~24時間位で殆ど広範囲の肝壊死を来たして死亡する。ペニシリン投与例及び腹水犬では術後24時間位では大部分生存しており、之等生存例には術後24~28時間で屠殺し、壊死肝葉或いは壊死好発肝葉（壊死の認められない場合）を切除採取した。死亡例には死亡直後の壊死肝葉を切除採取した。酵素の抽出には切除肝葉を凍結し、その一定量を秤量して冷却アセトンと共にホモゲナイザーにて磨砕し、次いで吸引濾過後乾燥して肝アセトンパウダーを作製し、実験に際してこの肝アセトンパウダーをグリセリン水に抽出し、その上澄濾液を酵素液として用いた。

レシチナーゼCに就いて、MacFarlane & Knightが *Clostridium welchii* Type Aの培養濾液中に発見した致死溶血壊死成分たる $\alpha$ -Toxinと同一なる事を証明したが、酵素化学的にはレシチンを加水分解して Phosphorylcholine と Diglyceride を生成する酵素で、酵素活性の測定には MacFarlane & Knightの方法に従い、レシチン溶液に前述の酵素液を作用させて一定時間内に遊離した酸可溶性磷即ち Phosphorylcholine P を測定して酵素単位に換えた。

1) 肝動脈遮断後直ちに閉腹したもの即ちペニシリン無投与例23例中、レシチナーゼC活性陽性例20例、87.0%、陰性例3例、13.0%であつて死亡例に於ては肉眼的に悉く肝壊死像をみとめ、レシチナーゼC活性も総て陽性であつた。術後24時間位では生存していた犬の肝にもレシチナーゼC活性の陽性例があり、之等には悉く肝壊死像を認め、早晚死亡するものと推定され

る。

2) ペニシリン投与例11例中、レシチナーゼC活性陽性例4例、36.4%、陰性例7例、63.6%で陽性例には実験犬の生死と関係なく悉く肝壊死像を認めた。ペニシリン投与により肝動脈遮断犬の死亡率が35%に減少する事は既知の事実であるが、この死亡率とペニシリン投与例のレシチナーゼC活性陽性率とは略一致し、之はペニシリンを投与しても死亡例の存在する事を酵素化学的に証明したものと言える。陰性例7例は共に術後24時間では生存しており、屠殺時肝壊死像は認められなかつた。

3) Wolbach 等により成熟犬の肝臓には芽胞を有する嫌気性桿菌の常在する事が明らかにされたので、本実験の対照として正常なる肝臓5例についてレシチナーゼC活性を測定したが、全例共陰性であつた。

4) 肝静脈狭窄或いは下大静脈狭窄による肝硬変類似の腹水犬7例について肝動脈遮断を行つたが、術後実験犬の生死に拘らず、亦ペニシリン投与の有無に関係なく全例共肝壊死は認められず、レシチナーゼC活性も総て陰性であつた。術前に適当なる処置を行えば、腹水犬はペニシリンを投与しなくても肝動脈遮断に対して抵抗力があり、充分生存し得る可能性は大と考える。

5) 次に肉眼的には肝臓に壊死様変化が認められるにも拘らずレシチナーゼC活性の陰性例が若干ある事で、之等の肝壊死は嫌気性桿菌の繁殖せざる乏酸素性肝壊死、或いは無菌性の ischemic necrosis の状態にあると考えられる。

以上の事実より肝動脈血流遮断犬の死亡に関して遮断後発生する肝壊死も1つの大きな要因であるが、更に肝壊死と共にレシチナーゼC活性の発現がより重要な役割を演ずるものと考えられる。