

Title	EXPERIMENTAL STUDIES ON THE PATHOGENESIS OF POLYMYOSITIS AND POLYOSTEOMYELITIS : ESPECIALLY ON THE INFLUENCES OF THE EXTRACT OF SKELETAL MUSCLES AND BONE-MARROW ON THE GROWTH OF STAPHYLOCOCCI AND THEIR LECITHINASE ACTIVITY
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EXPERIMENTAL STUDIES ON THE PATHOGENESIS OF POLYMYOSITIS AND POLYOSTEOMYELITIS,*

ESPECIALLY ON THE INFLUENCES OF THE EXTRACT OF
SKELETAL MUSCLES AND BONE-MARROW ON THE GROWTH
OF STAPHYLOCOCCI AND THEIR LECITHINASE ACTIVITY

by

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CONTENTS

Part I. Influences of the extract of skeletal muscles, heart muscles and uterus muscles on the growth of each strain of staphylococci, and heat-proof and undialyzability of the factors influencing the growth of the staphylococci.

Part II. Lecithinase activity of each strain of staphylococci, especially of the muscle-adapted and bone-marrow-adapted strains, myositis strain and osteomyelitis strain.

Part III. Comparison of P³²-uptake into the nucleic acid fractions in the bacterial body of each strain of staphylococci and the influences of the extract of skeletal muscles and bone-marrow on it.

INTRODUCTION

TOSHIKUNI MASAKI of our department studied on the following characters of F. D. A. 209-P strain of staphylococci aurei which was obtained by successive culture in the medium containing the extract of skeletal muscles and bone-marrow of the rabbit, and pointed out the definite effects of their extract, the influences of the muscle extract on the growth of the strain, the bacterial growth in the medium containing the extract of muscle or bone-marrow, peptonase activity and the effects of the extract of muscles or bone-marrow, alkaline phosphatase activity and the activation of Mg ion on it. On the other hand, comparisons were made between these characters and those of the myositis or osteomyelitis strain isolated from clinical materials. Furthermore, typical myositis of rabbit was able to be produced by intravascular or intramuscular injection of the strain which was adapted to the extract of skeletal muscles. Also typical osteomyelitis of rabbit was caused by combinatin of intravascular injection of the strain which was adapted to the extract of bone-marrow and injection of Mg ion of which amount was not sufficient to harm the tissue into bone-marrow. It was found that the minimum dose of staphylococci to cause inflammation varied depended on the strain.

Recently lecithinase was found in various bacteria besides *Cl. Welchii* and it was noted that there was some relation between the lecithinase-forming activity and the pathogenicity of these bacteria. As well known clinically, acute suppurative

* The gist of this paper was reported at the 84th Surgical Congress of Kinki on October 25, 1958.

myositis occurs only in skeletal muscles and not in smooth or heart muscles. TOSHIKUNI MASAKI and ICHIRO DOGURA, the author's co-workers, demonstrated that the extract of skeletal muscles and bone-marrow had specific effects on the growth of the skeletal muscle-adapted and bone-marrow-adapted strains respectively, and such effects seemed to be based on the correlation between the chemical circumstances of these tissues and the specific change of the enzyme system in these bacterial bodies. However, this theory had to be supported from other viewpoints too.

For such reasons the author made experimental studies on the following three items.

PART I. INFLUENCES OF THE EXTRACT OF SKELETAL MUSCLES, HEART MUSCLES AND UTERUS MUSCLES ON THE GROWTH OF EACH STRAIN OF STAPHYLOCOCCI, AND HEAT-PROOF AND UNDIALYZABILITY OF THE FACTORS INFLUENCING THE GROWTH OF THE STAPHYLOCOCCI

CHAPTER 1. COMPARISON IN THE EFFECTS OF THE EXTRACT OF SKELETAL, HEART AND UTERUS MUSCLES ON THE GROWTH OF EACH STRAIN OF STAPHYLOCOCCI

TOSHIKUNI MASAKI of our department demonstrated that the extract of skeletal muscles activated the growth of staphylococci which were adapted to the extract of skeletal muscles of the rabbit and those which were separated from clinical materials of myositis. It is clinically known that acute suppurative myositis occurs only in skeletal muscles and not in smooth muscles. The author tried to clarify the mechanism of these phenomena by comparative investigation on the effects of each extract of skeletal muscles, heart muscles, and smooth muscles on the growth of each strain of staphylococci.

I. Material and Methods

1) Extract of muscle

Fresh materials of skeletal muscles (thigh-muscle), heart muscles and uterus muscles were obtained from adult rabbits of 2kg body weight by surgical operation. They were ground down in a mortar together with the same weight of sea-sand and four-time weight of physiological saline solution, and centrifuged for 30 minutes in 3,000 r. p. m. The transparent extract thus obtained was neutralized to pH 7.0 and aseptitized through Seitz E. K. bacterial filter.

2) Bacterial media

a) Broth medium. The broth medium used in the present studies consisted of polypeptone (TAKEDA) 5.0g, meat infusion (KYOKUTO) 5.0g, NaCl 1.5g and distilled water 500cc. It was neutralized to pH 7.0, repeatedly filtered to complete transparency, distributed in test tubes, 4.5cc in each, and sterilized in an autoclave under pressure of 2kg/cm².

b) Synthetic medium. The following improved synthetic medium which was devised by SHOGO KUWABARA, Department of Bacteriology, Toho University, School of Medicine was used: Na₂HPO₄·12H₂O 5.5g, KH₂PO₄ 1.3g, NaCl 2.5g, MgSO₄·7H₂O

0.1g, glucose 2.0g, niacin 5 γ /cc, thiamine 5 γ /cc, asparagine 0.5g, L-glutamic acid 0.5g, glycine 0.1g, valine 0.1g, L-leucine 0.1g, L-cystine 0.02g, arginine·HCl 0.1g, histidine·HCl 0.05g, L-lysine·HCl 0.05g, L-proline 0.05g, Aq. dest. 1,000cc, pH 7.0 \pm 0.2, and L-tryptophan 0.01g and DL-phenylalanine 0.041g were added. It must be considered that some strain might lose synthesizing activity of nucleic acid factors (uracil, for instance) under the absolute anaerobic condition. The medium was sterilized by Seitz E. K. filter before use.

3) Experimented staphylococci

a) Control strain

b) Muscle-adapted strain. This term was used for F. D. A. 209-P strain of staphylococci aurei which was cultured 70 times successively, each for 24 hours in 37°C, in the 3% broth-agar medium or in the above-mentioned synthetic medium containing the extract of skeletal muscles in 10%. The strain which was cultured in the same way in the same medium not containing the extract was used as control.

c) Myositis strain. The strain was isolated from pus of the patients of myositis in our clinic.

4) Method. Each of the aseptic, fresh extract of skeletal muscles, uterus muscles, and heart muscles in the amount of 1.0cc, 0.5cc and 0.25cc each, was added to 4.5cc of the broth (or the synthetic) medium. The cocci-suspension containing 0.0028g of the examined strain in 10cc of physiological saline solution was prepared by means of Prof. TORIKATA's precipitometer. The homogenized suspension of each strain was added to each broth medium containing the extract and also to the straight broth medium, and incubated in 37°C for 24 hours. The amount of living bacterial cells in each medium was measured by electrocolorimetric turbidity. Therefore, the medium had to be well shaken so that the living cells would be homogenized well in the measurement. The increase of turbidity which resulted from addition of the extract of various muscles was deducted from the total turbidity.

II. Results (Table 1-6, Fig.1-6)

In the straight broth medium, the growth of the muscle-adapted strain was the

Fig. 1. The effect of the extract of skeletal muscles on the growth of staphylococci in the broth media.

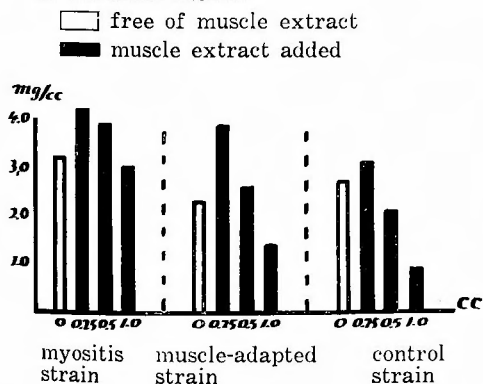


Fig. 2. The effect of the extract of heart muscles on the growth of staphylococci in the broth media.

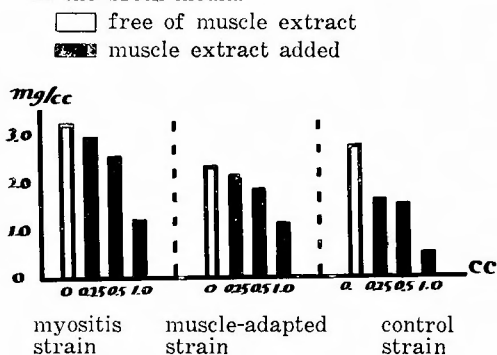


Fig. 3. The effect of the extract of uterus muscles on the growth of staphylococci in the broth media.

□ free of muscle extract
 ■ muscle extract added

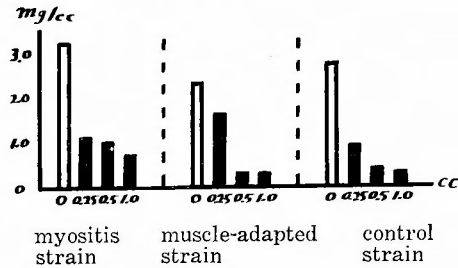


Fig. 4. The effect of the extract of skeletal muscles on the growth of staphylococci in the synthetic media.

□ free of muscle extract
 ■ muscle extract added

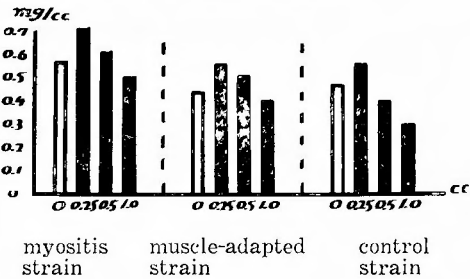


Fig. 5. The effect of the extract of heart muscles on the growth of staphylococci in the synthetic media.

□ free of muscle extract
 ■ muscle extract added

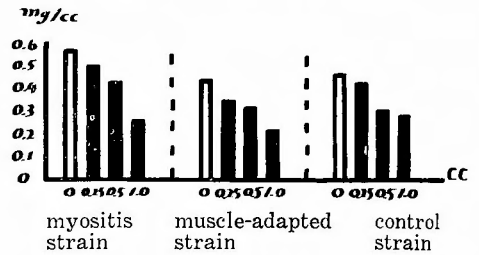


Fig. 6. The effect of the extract of uterus muscles on the growth of staphylococci in the synthetic media.

□ free of extract
 ■ extract added

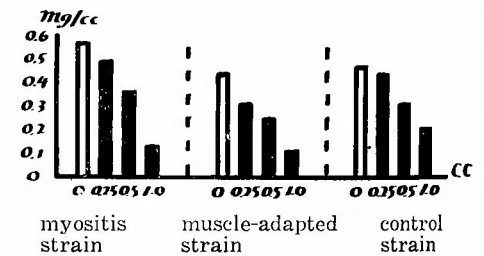


Table 1. The effect of the extract of skeletal muscles on the growth of staphylococci in the broth media.

Quantity of the extract of skeletal muscles added to 4.5cc of broth media (cc)	Strain	Myositis strain	Muscle-adapted strain	Control strain
1.0		3.0	1.4	0.9
0.5		3.9	2.6	2.1
0.25		4.2	3.9	3.1
0.0		3.2	2.3	2.7

(mg/cc)

Table 2. The effect of the extract of heart muscles on the growth of staphylococci in the broth media.

Quantity of the extract of heart muscles added to 4.5cc of broth media (cc)	Strain	Myositis strain	Muscle-adapted strain	Control strain
1.0		1.2	1.1	0.5
0.5		2.5	1.8	1.5
0.25		2.0	2.1	1.6
0.0		3.2	2.3	2.7

(mg/cc)

Table 3. The effect of the extract of uterus muscles on the growth of staphylococci in the broth media.

Quantity of the extract of uterus muscles added to 4.5cc of broth media (cc)	Strain		
	Myositis strain	Muscle-adapted strain	Control strain
1.0	0.7	0.3	0.3
0.5	1.0	0.3	0.4
0.25	1.1	1.6	0.9
0.0	3.2	2.3	2.7

(mg/cc)

Table 4. The effect of the extract of skeletal muscles on the growth of staphylococci in the synthetic media.

Quantity of the extract of skeletal muscles added to 4.5cc of synthetic media (cc)	Strain		
	Myositis strain	Muscle-adapted strain	Control strain
1.0	0.50	0.40	0.31
0.5	0.61	0.51	0.40
0.25	0.71	0.56	0.56
0.0	0.57	0.44	0.47

(mg/cc)

Table 5. The effect of the extract of heart muscles on the growth of staphylococci in the synthetic media.

Quantity of the extract of heart muscles added to 4.5cc of synthetic media (cc)	Strain		
	Myositis strain	Muscle-adapted strain	Control strain
1.0	0.26	0.22	0.29
0.5	0.43	0.32	0.31
0.25	0.50	0.35	0.43
0.0	0.57	0.44	0.47

(mg/cc)

Table 6. The effect of the extract of uterus muscles on the growth of staphylococci in the synthetic media.

Quantity of the extract of uterus muscles added to 4.5cc of synthetic media (cc)	Strain		
	Myositis strain	Muscle-adapted strain	Control strain
1.0	0.13	0.11	0.21
0.5	0.36	0.25	0.31
0.25	0.49	0.31	0.44
0.0	0.57	0.44	0.47

(mg/cc)

worst, that of the control strain was the next, and the myositis strain proliferated moderately. When 1.0cc of the extract of skeletal muscles was added to the broth medium, the growth of the muscle-adapted and control strains was remarkably inhibited and slight inhibition was observed on the growth of the myositis strain. On the contrary, when 0.5cc of the extract of skeletal muscles was added, the growth of the myositis and muscle-adapted strains was markedly accelerated, while the growth of the control strain was still inhibited. In addition of 0.25cc of the extract of skeletal muscles, the growth of all strains especially of the muscle-adapted strain was notably accelerated. Slight inhibition of the growth of all strains particularly of the control was seen in addition of the extract of heart muscles, whereas the extract of uterus muscles suppressed the growth of all strains in almost the same degree. The similar tendencies were observed in the experiments in the synthetic medium.

III. Discussion

The stated results consisted with the clinical fact that only skeletal muscles were affected by myositis and smooth muscles were free from it. Where does this difference between the extract of skeletal and smooth muscles come from? More advanced studies are needed to clarify this problem. YUJI TONOMURA pointed out that the number of ATP-bonds per unit weight of protein in myosin of smooth muscles was significantly less than that in myosin of skeletal muscles. SZENT-GYÖRGYI, A. (1951) considered that actin contained in myosin of smooth muscles was less in quantity than that in skeletal muscles, and the activity of ATP-ase in the former was weaker, too. These observations attracted much attention in connection with the experimental results of ICHIRO DOGURA, one of the author's co-workers, concerning the specific change in ATP-ase activity of each strain of staphylococci, especially of the muscle-adapted and myositis strains.

CHAPTER 2. HEAT-PROOF AND UNDIALYZABILITY OF THE FACTORS CONTAINED IN THE EXTRACT OF SKELETAL AND SMOOTH MUSCLES, INFLUENCING THE GROWTH OF THE STAPHYLOCOCCI

As stated in Chapter 1, a special component which accelerated the growth of the muscle-adapted and myositis strains was contained in the extract of skeletal muscles. To clarify the chemical nature of this component, the author studied its heat-proof and dialyzability.

I. Materials and Methods

1) Treatment of the muscle-extract for the heat-proof test. The muscle-extract prepared in the same method as in Chapter 1 was treated in 50°C for 2 hours, and 60 C for 2 hours.

2) Treatment of the muscle-extract for the dialyzability test. The extract of skeletal muscles or smooth muscles put in fish skin was dialyzed for 48 hours in tap water (in room temperature, 22°C). The water for dialysis was limited to a constant quantity, and concentrated under reduced pressure as a dialyzable component.

3) Method. The effects on the growth of each strain of staphylococci of the extract of skeletal and smooth muscles treated by heating as stated above, and of

the dialyzable and undialyzable components of each extract separated by dialysis were studied by the same method as described in Chapter 1.

II. Results (Table 7-10, Fig 7-10)

The extract of skeletal and uterus muscles treated by heating of 50°C for 2 hours or of 60°C for 2 hours had exactly the same effects on the growth of the control, muscle-adapted and myositis strains as the extract which was not treated by heating. The effects on the growth of each strain of the colloidal component of the extract of skeletal and uterus muscles from which the crystal component was removed by dialysis were almost the same as those of the extract without dialysis.

III. Discussion

From these results it was demonstrated that the factor which was contained in

Fig. 7. Heat-proof of the factors comprised in the extract of skeletal muscles, influencing the growth of the staphylococci.

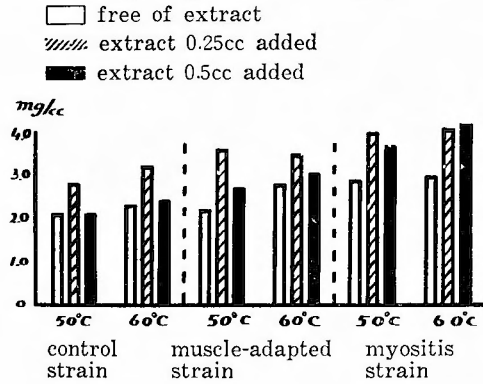


Fig. 8. Undialyzability of the factors comprised in the extract of skeletal muscles, influencing the growth of the staphylococci.

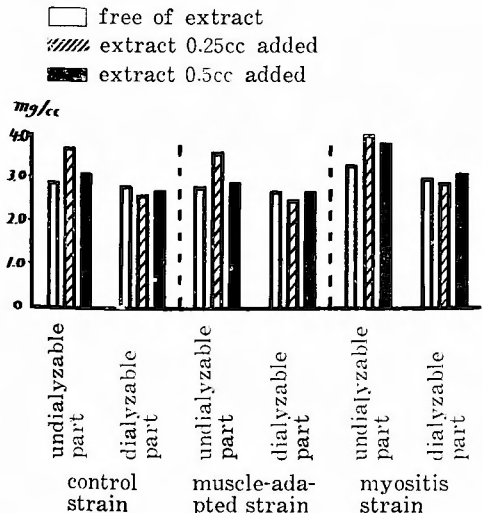


Fig. 9. Heat-proof of the factors comprised in the extract of uterus muscles, influencing the growth of the staphylococci.

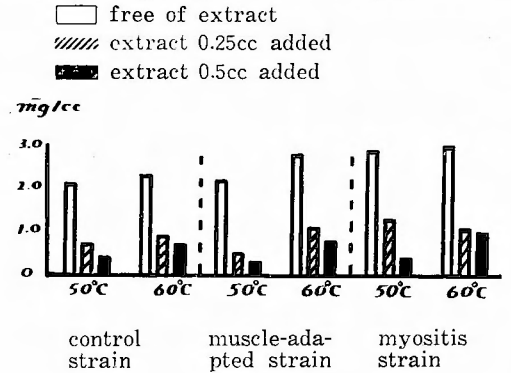


Fig. 10. Undialyzability of the factors comprised in the extract of uterus muscles, influencing the growth of the staphylococci.

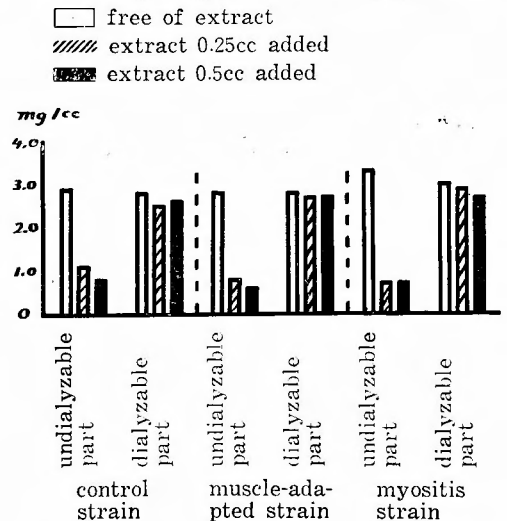


Table 7. Heat-proof of the factors comprised in the extract of skeletal muscles, influencing the growth of the staphylococci.

Quantity of the extract of skeletal muscles added to 4.5cc of broth media (cc)	Strain		Myositis strain		Muscle-adapted strain		Control strain	
	Heat of the extract		50°C	60°C	50°C	60°C	50°C	60°C
			1.0	2.5	3.7	1.5	2.2	1.1
0.50	3.7	4.2	2.7	3.1	2.1	2.4		
0.25	4.0	4.1	3.6	3.5	2.8	3.2		
0.0	2.9	3.0	2.2	2.8	2.1	2.3		

(mg/cc)

Table 8. Undialyzability of the factors comprised in the extract of skeletal muscles, influencing the growth of the staphylococci.

Quantity of the extract of skeletal muscles added to 4.5cc of broth media (cc)	Strain		Myositis strain		Muscle-adapted strain		Control strain	
	Dialysis of the extract		Undial- yzable part	Dialy- zable part	Undial- yzable part	Dialy- zable part	Undial- yzable part	Dialy- zable part
			1.0	2.8	3.0	1.9	2.7	2.0
0.5	3.8	3.1	2.9	2.7	3.1	2.7		
0.25	4.0	2.9	3.6	2.5	3.7	2.6		
0.0	3.3	3.0	2.8	2.7	2.9	2.8		

(mg/cc)

Table 9. Heat-proof of the factors comprised in the extract of uterus muscles, influencing the growth of the staphylococci.

Quantity of the extract of uterus muscles added to 4.5cc of broth media (cc)	Strain		Myositis strain		Muscle-adapted strain		Control strain	
	Heat of the extract		50°C	60°C	50°C	60°C	50°C	60°C
			1.0	0.4	0.6	0.3	0.4	0.3
0.5	0.4	1.0	0.3	0.8	0.4	0.7		
0.25	1.3	1.1	0.5	1.1	0.7	0.9		
0.0	2.9	3.0	2.2	2.8	2.1	2.3		

(mg/cc)

Table 10. Undialyzability of the factors comprised in the extract of uterus muscles, influencing the growth of the staphylococci.

Quantity of the extract of uterus muscles added to 4.5cc of broth media (cc)	Strain		Myositis strain		Muscle-adapted strain		Control strain	
	Dialysis of the extract		Undial- yzable part	Dialy- zable part	Undial- yzable part	Dialy- zable part	Undial- yzable part	Dialy- zable part
			1.0	0.4	2.9	0.3	2.8	0.4
0.5	0.7	2.7	0.6	2.7	0.8	2.6		
0.25	0.7	2.9	0.8	2.7	1.1	2.5		
0.0	3.3	3.0	2.8	2.7	2.9	2.8		

(mg/cc)

skeletal muscles and had specific activation on the growth of the muscle-adapted and myositis strains was the colloidal component which was resistant to the heating of 50-60°C for 2 hours and undialyzable. Further experiments were needed to decide what substance this factor was.

PART II. LECITHINASE ACTIVITY IN EACH STRAIN OF STAPHYLOCOCCI, ESPECIALLY IN THE MUSCLE-ADAPTED AND BONE-MARROW-ADAPTED STRAINS, MYOSITIS STRAIN AND OSTEOMYELITIS STRAIN

CONTARDI, A. and ERCOLI, A. (1933) presumed the existence of four fundamental enzymes, lecithinase A, B, C and D from theoretical consideration of the structural formula of lecithin. ZELLER, E. A. (1951) named the enzymes phospholipase A, B, C and D depending on their split portion of lecithin. Lecithinase D, namely phospholipase C was an enzyme which yielded phosphorylcholine and diglyceride by splitting lecithin. Although there was still something questionable on the direct relation between split of lecithin and invasion of bacteria in vivo, it was noted recently that the infection of bacteria, especially their pathogenesis or virulence was connected with their lecithinase-forming activity.

NAGLER, F. P. O. (1939) noticed turbidity and a floating lipid layer on cultivation of *Cl. Welchii*, type A in human serum. MACFARLANE, M. G., OAKLEY, C. L. and ANDERSON C. G. (1941) considered that this phenomenon was due to α -toxin which was a lethal, haemolytic and necrotic factor contained in the culture filtrate of *Cl. Welchii*, type A. Furthermore, MACFARLANE, M. G. and KNIGHT, B. C. J. G. (1941) insisted that the toxin of *Cl. Welchii* contained lecithinase and that was the α -toxin.

Since the studies on lecithinase of *Cl. Welchii* by GLENNY, A. T. et al. (1932), (without to mentioning the *Clostridium* genus) many studies have been made on lecithinase of various non pathogenic bacteria, including the study on the *Bacillus* genus by COLMER, A. R., the *Vibrio* genus by FELSENFELD, O. (1944), and the *Serratia* genus by MONSOUR et al. According to FUMINORI YANAGIZAWA and TATUJI KIRIBUCHI (1956), lecithinase activity was not proved in the *Escherichia* genus, *Aerobacter* genus, *Proteus* genus and *Enterococcus* group, while the *Serratia* genus, *Bacillus* genus and *Clostridium* genus had fairly remarkable lecithinase activity.

The author made the fundamental studies on the lecithinase activity of F. D. A. 209-P mother strain of staphylococci aurei, and also further investigations on the enzyme activity of the skeletal muscle-adapted and bone-marrow-adapted strains.

CHAPTER 1. FUNDAMENTAL STUDIES ON THE LECITHINASE ACTIVITY OF STAPHYLOCOCCI AUREI

I. Materials and Methods

- 1) Experimented bacteria. F. D. A. 209-P strain of staphylococci aurei preserved at the Department of Microbiology of our University was used.
- 2) Enzymatic material. The colonies of F. D. A. 209-P strain of staphylococci

aurei which were concentrated through the straight broth medium of pH 7.0 and 3% broth agar medium were gathered in physiological saline solution and centrifuged. The sediment suspended equally in the following buffer solution was used as enzymatic material after measuring the quantity of the bacteria by Prof. TORIKATA's precipitometer.

3) Substrate solution. 2.5% lecithin solution. In the early stage, lecithin of TOKYO KASEI Co. which had been purified from egg yolk was used, but later it was switched to lecithin, gliddex-P (GL-5) of DAINIHON SEIYAKU Co., because peroxide was suspected in the former. Lecithin solution was made fresh each time of the experiments, as it easily changed into lysolecithin when it got old.

4) Buffer Solution. MICHAELIS' m/10-veronal-N/10 HCL buffer solution. Boric acid buffer solution had about 20% inhibition of the enzymes.

5) Measurement of the enzyme activity. The measurement was done generally by MACFARLANE'S method. Namely, the mixture of enzyme solution 1.0cc, substrate solution 1.0cc and buffer solution 4.0cc was prepared to obtain the main reaction, and the mixture of substrate solution 1.0cc and buffer solution 5.0cc and the mixture of enzyme solution 1.0cc and buffer solution 5.0cc were prepared for the control. One cc solution was taken from each mixed solution immediately after the mixture was made and also after incubation in 37°C for a constant time, and 9.0cc of 5% trichloroacetic acid was added to inhibit the enzyme reaction. The total acid-soluble phosphor was measured by FISKE-SUBBAROW'S aminonaphthol-sulfonic acid method, and the balance of the increased phosphor amount and the control amount were recorded as the decomposed amount by the enzymes. BECKMAN'S photoelectric spectrophotometer, QB 50 (SHIMAZU Co.), wave length 660m μ was used for the measurement of phosphor amount.

II. Results

1) Optimal pH: It was about pH 7.0 (Fig. 11).

2) Stage of bacterial growth and their enzyme activity: The bacteria which were cultured for about 18 hours had the maximum enzyme activity (Fig. 12).

3) Bacterial amount: In our experiments, free phosphor amount increased in direct proportion to the increase of the bacterial amount, namely the amount of the

Fig. 11. Optimal pH of staphylococcal lecithinase activity.

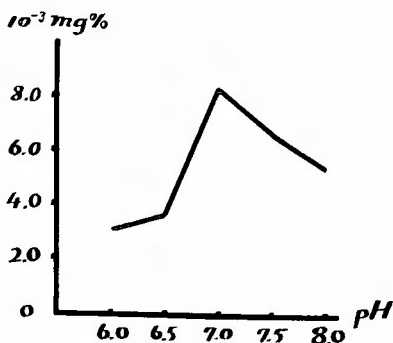


Fig. 12. Stage of staphylococcal growth and their lecithinase activity.

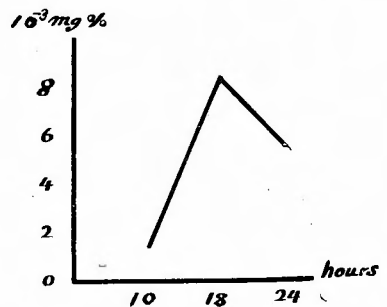


Fig. 13. Bacterial amount and staphylococcal lecithinase activity.

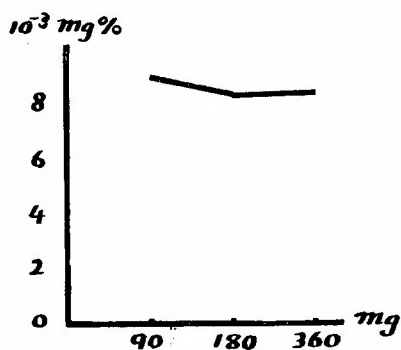


Fig. 15. Temperature of incubation and staphylococcal lecithinase activity.

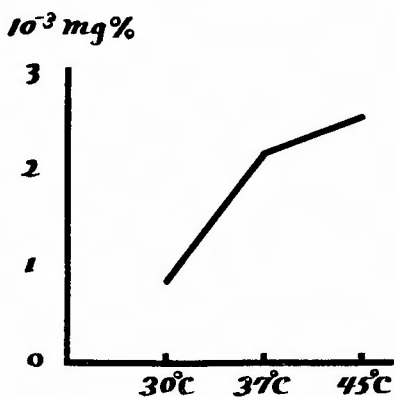


Fig. 14. Substrate concentration and staphylococcal lecithinase activity.

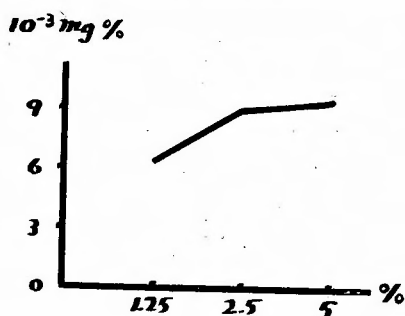
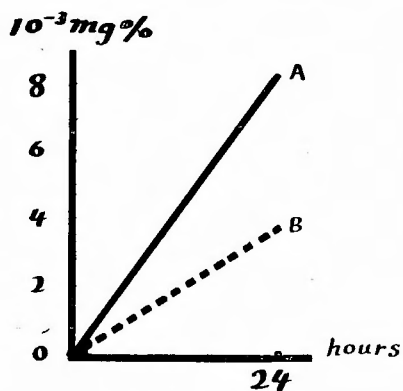


Fig. 16. Activating effect of Ca^{++} on the enzymatic activity.

A 0.01 mol CaCl_2 0.2ml added
B free of Ca^{++}



enzymes (Fig. 13).

In other words, it was demonstrated that the amount of the enzymes could be shown by the amount of free phosphor under our experimental conditions.

4) Substrate concentration: The higher the concentration was, the more the free phosphor amount was (Fig. 14).

5) Temperature of incubation: In incubation of four hours in 30°C, 37°C and 45°C, the maximum enzyme activity was shown in 45°C (Fig. 15).

6) Enzyme activation by Ca^{++} : It was proved definitely (Fig. 16).

III. Discussion

These various experiments proved that F. D. A. 209-P strain of staphylococci aurei had phospholipase C activity the same as *Cl. Welchii*. Lecithinase activity (phospholipase C) seemed to be a factor which could not be neglected in infection by staphylococci, especially in their pathogenicity and virulence. Further studies were needed to decide whether lecithinase of staphylococci aurei was the same as staphylolysin and leukozidin or not.

CHAPTER 2. LECITHINASE ACTIVITY IN THE MUSCLE-ADAPTED, BONE-MARROW-ADAPTED STRAINS AND THE STAPHYLOCOCCI ISOLATED FROM VARIOUS CLINICAL MATERIALS, AND THE EFFECTS OF THE EXTRACT OF SKELETAL MUSCLES AND BONE-MARROW ON IT

I. Materials and Methods

1) Extract of skeletal muscles. It was prepared in the same way as stated in Chapter 1, Part I.

2) Extract of bone-marrow. The bone-marrow of the long tubular bones of adult rabbits of about 2kg body weight or juvenile rabbits was extracted, ground down in a mortar with the same weight of sea-sand and four times weight of physiological saline solution, centrifuged for 30 minutes in 3,000 r. p. m., neutralized to pH 7.0, and aseptitized through SEITZ E. K. bacterial filter.

3) Experimented bacteria. The skeletal muscle-adapted and bone-marrow-adapted strains which were made from F. D. A. 209-P strain of staphylococci aurei in the same way as stated in Chapter 1, the control strain, and the myositis, osteomyelitis and carbuncle strains which were isolated from clinical materials were used.

The other conditions were just the same as in Chapter 1. The tissue extract was added to the reaction mixture in the rate of 1:6.

II. Results

1) Enzyme activity in the skeletal muscle-adapted strain. The enzyme activity shown in 24 hours was about four times greater than that of the control strain (Fig. 17, Table 11).

Table 11. Lecithinase activities of the skeletal muscle-adapted strain and bone-marrow-adapted strain.

Hours incubated	Strain	Isolated P per mg of living cells		
		Control strain	Muscle-adapted strain	Bone-marrow-adapted strain
24		0.008	0.033	0.016
48		0.015	0.038	0.026
72		0.011	0.023	0.017

2) Enzyme activity in the bone-marrow-adapted strain. The enzyme activity shown in 24 hours was about two times greater than that of the control strain (Fig. 17, Table 11).

3) Myositis strain of staphylococci. The enzyme activity shown in 24 hours was about four times greater than that of F. D. A. 209-P mother strain of staphylococci aurei (Fig. 18).

4) Osteomyelitis strain of staphylococci. A slight acceleration of the enzyme activity was observed in comparison with that of F. D. A. 209-P mother strain, but it was not so remarkable as in the myositis strain (Fig. 18).

5) Carbuncle strain of staphylococci. Its enzyme activity was about the same

Fig. 17. Lecithinase activities of the skeletal muscle-adapted strain and bone-marrow-adapted strain.

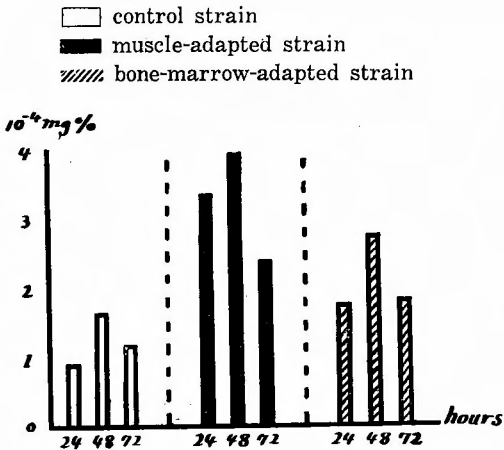
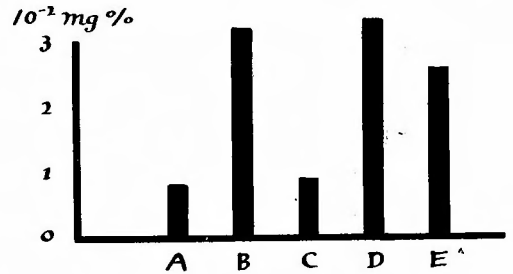


Fig. 18. Lecithinase activities of the myositis, osteomyelitis, carbuncle, and experimental myositis strains.

- A. F. D. A. 209-P mother strain
- B. myositis strain
- C. osteomyelitis strain
- D. carbuncle strain
- E. experimental myositis strain

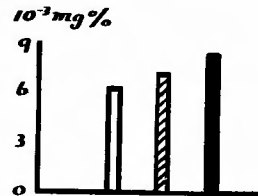


as that of the myositis strain (Fig. 18).

6) Change in the enzyme activity resulted from the development of myositis. F. D. A. 209-P mother strain of staphylococci aurei, 2.5mg was injected into the thigh extensor of the adult rabbit. The strain which was isolated from the myositic focus two weeks after the injection, had three times greater enzyme activity in 24 hours as compared with that of the mother strain (Fig. 18).

Fig. 19. Effect of the extracts of skeletal muscles and bone-marrow on staphylococcal lecithinase activity.

- free of extract
- ▨ bone-marrow extract added
- muscle extract added



7) Effects of the extract of skeletal muscles on lecithinase activity of the bacteria. It activated the enzyme activity in about 1.3 times in 24 hours (Fig. 19).

8) Effects of the extract of bone-marrow on lecithinase activity of the bacteria. It activated the enzyme activity slightly (Fig. 19).

III. Discussion

Lecithinase activity of staphylococci was remarkably accelerated in the muscle-adapted strain, and the enzyme activity of the bone-marrow-adapted strain was also considerably activated in comparison with that of the control strain. It was significant that lecithinase activity of these strains generally corresponded with their inflaming activity in the experimental myositis and osteomyelitis as reported by TOSHIKUNI MASAKI. Besides, the enzyme activity was accelerated after the development of the inflammation even in the same strain. In their experiments on separation and rectification of muscle-ATP-ase which was activated by Mg⁺⁺, KIELLEY, W. W. and MEYERHOF, O. (1949) observed that the enzyme activity existed in the fraction which had the nature of lipoprotein, and clarified that the activity was lost when lecithin

of phospholipid was decomposed by α -toxin of *Cl. Welchii*, namely lecithinase. When the important role played by Mg-ATP-ase in muscle contraction was considered, it could be presumed that lecithinase of staphylococci had a significant meaning in the twitching phenomenon of the affected muscle in acute suppurative myositis.

CHAPTER 3. EXPERIMENTS ON THE DEMONSTRATION OF LECITHINASE ACTIVITY IN THE EXUDATE FROM THE WOUND IN THE EXPERIMENTAL MYOSITIC FOCUS OR THE LYMPH FROM THE AFFECTED REGION

In their studies on the experimental appendicitis of the dog, TANTURI, C. A. and ANDERSON, R. E. (1949) proved lecithinase activity (phospholipase C) in the exudate of the sack in which the appendix was enclosed and in the exudate in the abdominal cavity, and stated that α -toxin of *Cl. Welchii* was the greatest cause of death by appendiceal peritonitis. They also pointed out that streptokinase which broke the barrier localizing the disease and hyaluronidase which helped the intraperitoneal dissemination of the bacteria played important parts too. On the other hand, LAUFMAN, H., TANTURI, C. A. and FURR, W. E. (1951) stated in their experiments, that lecithinase activity was proved in the lymph in the thoracic duct of the dog of strangulated ileus, but no enzyme activity was proved in the blood of peripheral and portal veins.

Injecting 2.5mg of F. D. A. 209-P mother strain of staphylococci into the thigh-extensor of the adult rabbit, the author tried to prove lecithinase activity in the exudate from the wound in the experimental myositic focus and in the lymph from the subinguinal lymphatic vessel two weeks after the injection, but no enzyme activity was proved in either of them. According to BALCH, H. H. and GANLEY, O. H. (1957), when toxin of *Cl. Welchii* (α -toxin, θ -toxin and collagenase) was intramuscularly injected into a goat, no toxin was identified in the exudate which was taken from the wound 24 hours after the injection or in the lymph which drained from the injected area, when the animal was alive. They explained this phenomenon by saying that the toxin was rapidly absorbed into the living tissues and unactivated, therefore impossible to be identified. VAN HEYNINGEN also considered that it was due to the specific, rapid absorption of the toxin into the stroma, or erosion by circulating blood and lymph.

When these opinions were taken in consideration, it would be hasty to deny from the stated experimental result, the actual production of lecithinase of staphylococci at the myositic focus.

CHAPTER 4. THE BIOLOGICAL CHARACTERISTICS OF LECITHINASE PRODUCED BY STAPHYLOCOCCI AUREI

As mentioned in Chapter 1, lecithinase activity exists in F. D. A. 209-P strain of staphylococci aurci. When it works as toxin, as in *Cl. Welchii*, it is important, from the viewpoint of investigation on the role of lecithinase of this strain, to decide

whether it works as exotoxin or endotoxin. Of course, as Prof. TORIKATA has insisted, toxin should be classified by its biological activity and not from the viewpoint of exo- or endotoxin.

Generally, in case of exotoxin, when the amount of toxin in the filtrate and the proliferation of bacteria are compared at each stage of culture, the ogives of both show nearly the same tendency in parallel with time. It is because the amount of toxin increases accordingly as the bacteria decreases by self-destruction after the maximum proliferation.

In order to investigate the relation between the amount of toxin in the filtrate of the liquid medium and the proliferation of bacteria, the author tried to measure the discharged amount of lecithinase into the straight broth medium. It was found, however, that the amount of the enzymes discharged in the straight broth medium was very little, and its measurement was impossible without extracting the enzymes from the medium. Therefore, DEUTSCH et al.'s medium which was supposed to produce much lecithinase in *Cl. Welchii* was used, but no sufficient toxin was produced from it.

SHOGO HOSOYA considered that the toxin which was neutralized immunologically was so-called "exotoxin", and the one which was not neutralized was "endotoxin", and antigen activity should be discussed in their distinction. It was clarified by numerous experiments that α -toxin of *Cl. Welchii*, namely lecithinase was inhibited by antitoxin.

Therefore, the author tried to examine by the following purely immunological method, whether lecithinase of staphylococci was exotoxin or endotoxin.

I. Materials and Methods

Immunization of bone-marrow. 0.5cc of coctigen (boiled in 100°C for 30 min.) of F. D. A. 209-P strain of staphylococci aurei in the third graduation of Prof. TORIKATA's precipitometer was injected in the bone-marrow, after perforating the thigh-bone of the rabbit. The degree of the local immunity of the bone-marrow was tested by agglutination test. The main purpose of this experiment was to measure the temporary antibodies. It was reported, however, that in the stated experimental condition, the value of opsonin of bone-marrow reached the maximum 24 hours after the injection and returned to normal after 168 hours.

The extract of bone-marrow of the rabbit, which showed 100 and 800 times agglutinationtiter, and that of the normal rabbit, were diluted in 4-time solution, 1.0cc of each was added to the reaction mixture. Lecithinase activity of F. D. A. 209-P mother strain of staphylococci aurei in these mixtures were measured in comparison to one another by the same method as described in Chapter 1.

II. Results (Fig. 20)

As described in Chapter 2, the extract of bone-marrow of the normal rabbit showed a slightly activating effect on lecithinase activity of staphylococci, but the extract of bone-marrow of the rabbit with local immunity showed a remarkably inhibiting effect on lecithinase activity. This result corresponded with the fact that lecithinase activity of *Cl. Welchii* was remarkably inhibited by antiscrum (antitoxin).

III. Discussion

From these results, lecithinase produced by F. D. A. 209-P strain of staphylococci was considered to be the toxin which was neutralized immunologically, and belongs to the so-called "exotoxin" in biological classification.

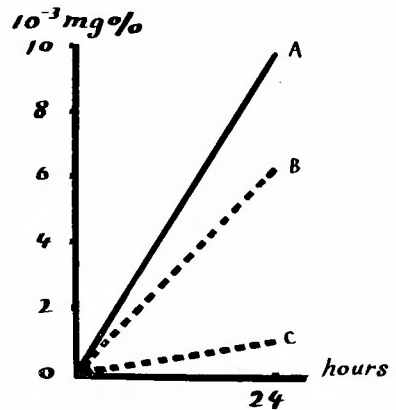
PART III. COMPARISON OF P^{32} -UPTAKE INTO THE NUCLEIC ACID FRACTIONS IN THE BACTERIAL BODY OF EACH STRAIN OF STAPHYLOCOCCI AND THE INFLUENCE OF THE EXTRACT OF SKELETAL MUSCLES AND BONE-MARROW

Nucleic acids which consisted of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) were found in the bodies of bacteria. RNA was related with general metabolism, which DNA was considered to be exclusively connected with cell-division (SACKS, J., 1948). According to SPIEGELMAN, S. and MASAMI SUDA (1950), the giant molecules of RNA were considered to play a part in the formation of the characteristics of each strain, especially of constitutive and adaptive enzymes, in cooperation with genes in the nuclei. Their experimental results also suggested that hereditary qualities such as formation of constitutive enzymes were controlled by DNA in the nuclei, while acquired qualities such as formation of adaptive enzymes were mainly under the control of RNA in the cytoplasm. On the other hand, it had been gradually clarified by the studies of BRACHET, J. (1947), SPIEGELMAN, S. and KAMEN, M. D. (1947) that there was a close relation between the nucleolus containing RNA, the existence of abundant RNA in the cytoplasm and the protein synthesizing function of the cell.

Each strain of staphylococci was cultured in the synthetic medium of which phosphate was partly substituted by a salt of radioactive isotope P^{32} , and RNA and DNA fractions were separated from a certain amount of the cultured bacterial mass. Measuring their radioactivity and the amount of phosphorus, the author tried to investigate the P^{32} -uptake into the nucleic acid fractions of the bacterial body comparatively, the effects of the extract of skeletal muscles and bone-marrow on their P^{32} -uptake, and observe how these kinds of tissue extract worked on the growth of each strain, especially of each adapted strain.

Fig. 20. Enzyme inhibition by the extract of immunized bone-marrow.

- A. normal extract of bone-marrow added
- B. the extract of bone-marrow, which showed 100 times agglutination-titer added
- C. the extract of bone-marrow, which showed 800 times agglutination-titer added



I. Materials and Methods

1) Synthetic medium for the bacteria. The improved synthetic medium devised by SHOGO KUWABARA which was described in Chapter 1, Part I was used.

2) P^{32} salt

3) Experimented staphylococci

a) Control strain

b) Muscle-adapted strain

c) Bone-marrow-adapted strain

4) Extract of skeletal muscles and bone-marrow

3) and 4) were made in the same method as described in Parts I and II.

5) The phosphate of the aforementioned synthetic medium was partly substituted by the amount of P^{32} salt which had been confirmed to give no remarkable effect on the growth of each strain. A constant amount of each strain of experimented staphylococci was cultured in this medium for a constant time in 37°C , centrifuged, and the bacterial mass was collected. Adding cold physiological saline solution, it was again centrifuged, and the staphylococci were sufficiently washed by repeating centrifugation for three times. The RNA and DNA fractions were separated from the bacterial bodies by the following SCHMIDT-THANNHAUSER'S method.

a) The bacterial mass was ground down in a mortar with 10 times distilled water.

b) One cc of the ground solution was taken in a graduated centrifuge tube (the one with graduation of 10-15cc was recommended), and was centrifuged for ten minutes in 3,000 r. p. m. with 12% cold perchloric acid HClO_4 . Repeating the centrifugation twice, the supernatant was collected in a graduated test tube and measured.

c) Mixing 3.0cc of 95% ethyl alcohol well, the sediment was centrifuged. This was repeated three times, and the supernatant was taken in a graduated test tube.

d) After alcohol-extraction and addition of 1.0cc of 1N potassic hydroxide, the sediment was kept in 37°C for 20 hours until the bacterial mass was melted.

e) Adding 0.2cc of 6N hydrochloric acid and 1.0cc of perchloric acid, it was cooled.

f) Sediment made in this way contained DNA and protein.

i) The sediment was filtrated and washed with 1.0cc of 6% perchloric acid. The washed solution was kept with the filtrate.

ii) The sediment was collected and 6% perchloric acid was added to make the total quantity 1.0cc. Adding another 1.0cc of 6% perchloric acid, it was heated at 90°C for 15 minutes.

iii) It was cooled and centrifuged.

iv) The sediment was washed in 1.0cc of 6% perchloric acid, and was put together with the aforementioned supernatant.

The filtrate (f, i) had RNA fraction, and the supernatant (f, iii and f, iv) had DNA fraction. Both fractions were dried in capsules and their radioactivity was

measured by GEIGER-MÜLLER counter. The P^{32} -uptake into the nucleic acid fractions of the bacterial body of each strain was comparatively investigated by these measured values. On the other hand, the extract of skeletal muscles or bone-marrow was added to the synthetic medium at the rate of 1:9 in volume, and performing the same kind of experiment, the effects of the extract on P^{32} -uptake into DNA and RNA fractions were examined.

Because YOSHIKI MIURA (1956) had reported that contamination by absorption of P^{32} seldom occurred in the nucleic acid fractions of F. D. A. 209-P strain of staphylococci, performance of this experiment was facilitated.

II. Results

1) Preliminary experiment

a) State of growth of each strain of staphylococci in the synthetic medium. Each bacterial mass (0.028mg) of the control, muscle-adapted strain, and myositis strain was added in 1cc of the synthetic medium, and was incubated in 37°C . All the strains grew well and each showed the concentration of 0.46mg, 0.42mg and 0.58mg after 24 hours, and were in the stationary phase (Fig. 21).

b) Effects of P^{32} on the growth of staphylococci. P^{32} salt was added to the synthetic medium in the rate of 0.1-20 $\mu\text{c}/\text{cc}$. F. D. A. 209-P strain was inoculated in this medium in the rate of 0.028mg/cc and its growth was observed after incubating for a constant time in 37°C . It was found that P^{32} less than 20 $\mu\text{c}/\text{cc}$ gave no remarkable effect on the bacterial growth (Table 12).

MORSE, M. L. and CARTER, C. E. (1949) stated that in *Escherichia coli*, most of RNA was synthesized in the lag phase of the bacterial growth. According to the experiments of MALMGREN, B. et al. (1945-1947), the total amount of the nucleotides in each cell of the cultured bacteria changed during the bacterial growth, and its maximum was seen in the bacterial cell in the last stage of the lag phase of the growth circle. BELOZERSKY, A. N. (1947) and BOIVIN, A. (1948) observed that the bacterial cell which was obtained from the juvenile medium contained more nucleic acids than that from the old medium. Therefore it was preferred that the comparison of the differences in the nucleic acid fractions of more than two kinds of strains, the strains

Fig. 21. Growth curves of each strain of staphylococci in the synthetic media.

- A. myositis strain
- B. control strain
- C. muscle-adapted strain

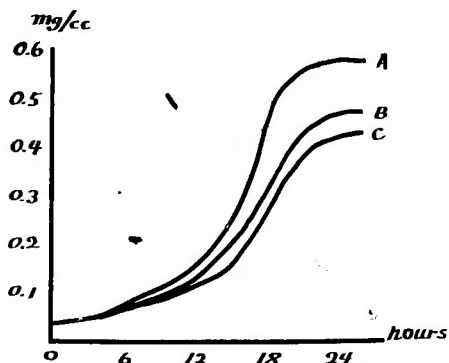


Table 12. Effects of P^{32} on the growth of staphylococci aurei.

Added P^{32} microcurie per cc of media	0.0	0.1	0.5	10	20
Staphylococcal growth (mg/cc)	0.46	0.47	0.50	0.50	0.38

in the same growth stage should be used. Based on the results of the preliminary experiment (a), the bacteria in the 24th hour after the inoculation into the synthetic medium, which were supposed to have reached their stationary phase, were used for the main experiment.

Using *E. coli communior*, *paratyphusbacillus B aneurinaze* (KIMURA, AOYAMA) and *staphylococcus aureus*, SEIICHI MIYOSHI et al. (1956) investigated the effects of P^{32} on the form and growth of the bacteria cultured in the yeast infusion-broth medium and modified EVANS S medium. Addition of P^{32} definitely decreased the number of the bacteria in the media in comparison with the controls, and caused changes on the form and nucleoids of the bacteria. In the modified EVANS S medium containing very little non-radiophosphorus, P^{32} with concentration of $500\mu\text{C}/\text{ml}$ inhibited the growth of the bacteria, but in the yeast infusion-broth medium containing much non-radiophosphorus, P^{32} with concentration of $500\mu\text{C}/\text{ml}$ gave hardly any effects on the growth of bacteria. MORSE, M. L. and CARTER, C. E. (1949) substituted $0.3\mu\text{C}/\text{ml}$ of P^{32} for ordinary phosphate in the medium as an indicator for synthesis of nucleic acids of *Escherichia coli*. Basing on these points and the results of the preliminary experiment (b), phosphate in the synthetic medium was substituted by P^{32} salt in the ratio of 300-500 $\mu\text{C}/1$.

2) Results of the main experiment (Table 13-14, Fig. 22-23)

In the experiments on the muscle-adapted strain and its control, P^{32} salt of $500\mu\text{C}/1$ was added to the synthetic medium, and RNA and DNA fractions were separated from the bacterial mass of 100mg. In the experiments on the bone-marrow-adapted strain and its control, P^{32} salt of $300\mu\text{C}/1$ was added and both fractions were separated from the bacterial mass of 80mg. The natural count was around 33 in each of these experiments.

Fig. 22. Influences of the extract of skeletal muscles on P^{32} -uptake into the nucleic acid fractions in the bacterial body of muscle-adapted strain and control strain.

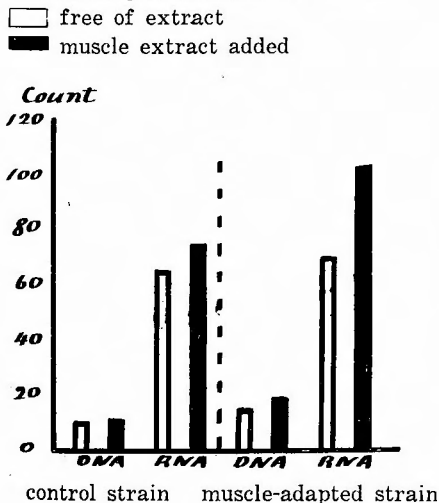


Fig. 23. Influences of the extract of skeletal muscles and bone-marrow on P^{32} -uptake into the nucleic acid fractions in the bacterial body of bone-marrow-adapted strain and control strain.

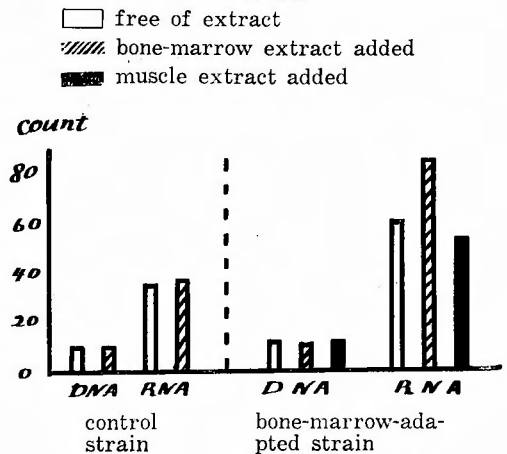


Table 13. Influences of the extract of skeletal muscles on P^{32} -uptake into the nucleic acid fractions in the bacterial body of muscle-adapted strain and control strain.

Strain	Control strain				Muscle-adapted strain			
	DNA		RNA		DNA		RNA	
Added extract	0	0.5cc/5cc	0	0.5cc/5cc	0	0.5cc/5cc	0	0.5cc/5cc
Count	10	11	65	75	15	19	70	113

Table 14. Influences of the extract of skeletal muscles and bone-marrow on P^{32} -uptake into the nucleic acid fractions in the bacterial body of bone-marrow-adapted strain and control strain.

Strain	Control strain				Bone-marrow-adapted strain					
	DNA		RNA		DNA			RNA		
Added extract of bone-marrow	0	0.5cc/5cc	0	0.5cc/5cc	0	0.5cc/5cc	0	0	0.5cc/5cc	0
Added extract of skeletal muscles	0	0	0	0	0	0	0.5cc/5cc	0	0	0.5cc/5cc
Count	10	10	35	37	12	11	12	60	85	53

Rapid uptake of P^{32} or its heavy labeling into the RNA fraction was proved in every strain. In both muscle-adapted and bone-marrow-adapted strains, P^{32} -uptake into RNA fractions was more remarkable than that in the controls. Especially, in the muscle-adapted strain with the effects of the extract of skeletal muscles and the bone-marrow-adapted strain with the effects of the extract of bone-marrow, P^{32} -uptake into both fractions, particularly to RNA fraction was unusually remarkable in comparison with that in the controls. There was a theory that a substance which had relation with gram-positiveness existed on the surface of or around a gram-positive cell, and it consisted of magnesium-ribonucleic acid which was bonded with specific protein in a certain formula by SH radical. Based on this theory, comparison was made on gram-stainability of the stated strains of staphylococci, but no evidence to prove the difference was found.

III. Discussion

These results clarified that the extract of skeletal muscles or bone-marrow remarkably accelerated RNA metabolism of the muscle-adapted strain or the bone-marrow-adapted strain. That is, the extract of these tissues were considered to give remarkable effects on the enzymes which were related to the bacterial metabolism, and induced the formation of the adaptive enzymes. In other words, these experimental results supported the experiments on chemical analysis of the enzymes concerning tissue affinity of each adapted strain which had been demonstrated by TOSHIKUNI MASAKI and ICHIRO DOGURA, the author's co-workers.

CONCLUSION

Experiments were made on the muscle-adapted and bone-marrow-adapted strains

which were made by adapting the same strain of staphylococci to the extract of skeletal muscles and bone-marrow in vitro, and the following results were obtained.

1) In the observation of the effects of the extract of uterus muscles, heart muscles and skeletal muscles on the bacterial growth, only the extract of skeletal muscles accelerated the growth of the myositis strain and the muscle-adapted strain, and the extract of uterus muscles inhibited the growth of any strain.

2) In comparative measurements, the lecithinase activity which was closely connected with the virulence of the bacteria, was remarkably accelerated in the muscle-adapted strain, and in the bone-marrow-adapted strain too considerable enzyme activity was observed in comparison with that in the control.

3) In the observation of P^{32} -uptake into the nucleic acid fractions of the bacterial body of each strain, more uptake into RNA fraction was observed in every adapted-strain in comparison with that in the control strain. Especially, in the muscle-adapted strain with the effects of the extract of skeletal muscles and the bone-marrow-adapted strain with the effects of the extract of bone-marrow more P^{32} -uptake into the both fractions, particularly into RNA fractions was observed.

From these results, it was considered that in the pathogenesis of polymyositis and polyosteomyelitis the formation of adaptive enzymes around skeletal muscles and bone-marrow of the causal staphylococci and the chemical circumstances of these tissues had significant meaning.

The author wishes to thank Dr. KOICHI INHIGAMI, the instructor of our clinic, for his many valuable suggestions and criticisms throughout the present investigation.

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和文抄録

多発性筋炎ならびに多発性骨髄炎の成因に関する実験的研究
とくにブドウ球菌の発育に対する横紋筋ならびに骨髄浸出液
の作用点とブドウ球菌レシチナーゼ能について

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同一菌株に出発したブドウ球菌を横紋筋および骨髄浸出液を含んだ培地に継代培養し、試験管内で横紋筋浸出液および骨髄浸出液に適應せしめて得た筋汁適應菌、骨髄汁適應菌などについて実験を行い次の結果を得た。

(1) 横紋筋、心筋および子宮筋各浸出液のブドウ球菌発育に及ぼす影響を測定したところ、横紋筋浸出液のみが筋炎起炎菌や筋汁適應菌の発育を促進し、子宮筋浸出液はいずれの菌においても発育を抑制した。しかもこれらの菌発育に影響する因子は耐熱性で非透析性であった。

(2) ブドウ球菌について細菌の毒性に密接な関係を有するレシチナーゼの産生を同定し、これを各菌株について測定したところ、筋汁適應菌では著明に亢進し

ており、骨髄汁適應菌もまた対照菌に比べるとかなり著明な酵素能を示すことをしつた。

(3) 各適應菌菌体内核酸磷への P^{32} 導入を測定したところ、いずれの適應菌においても対照菌に比べると、RNA 画分に P^{32} が多く導入された。とくに筋汁適應菌には横紋筋浸出液を、また骨髄汁適應菌には骨髄浸出液を働かせた場合が、他の場合に比較して両核酸画分、とくに RNA 画分に P^{32} が多く導入された。

以上の諸点から、多発性筋炎および多発性骨髄炎の成立には、起炎ブドウ球菌の横紋筋や骨髄に対する適應酵素の形成と、これら組織のもつ化学的環境とが重要な意味をもつものと考えられる。