

EXPERIMENTAL STUDY ON THE PATHOGENESIS OF POLYMYOSITIS AND POLYOSTEOMYELITIS (AFFINITY OF THE PATHOGENIC ORGANISM FOR SKELETAL MUSCLE VIEWED FROM AMINO ACID METABOLISM, WITH ESPECIAL REFERENCE TO THE CORRELATION OF MUSCLE ADAPTATION THEORY TO VITAMIN B₁ DEFICIENCY THEORY (OZAWA))

by

MASAHIRO NISHINO

From the 2nd Surgical Division, Kyoto University Medical School
(Director : Prof. Dr. YASUMASA AOYAGI)

Received for publication Jan. 17, 1961

INTRODUCTION

Acute suppurative myositis and acute infectious osteomyelitis often assume the form of multiple involvement. In this case, the pathogenic staphylococcus which has first attacked a skeletal muscle or a bone marrow, continues a selective attack on either of these systems. As multiple involvement is caused by hematogenous infection, it seems quite possible that a patient might be simultaneously affected with myositis and osteomyelitis, but this seldom occurs. How is this strict discrimination to be explained? To solve this problem, many investigations have so far been conducted either from the standpoint of the pathogenic organism, or from that of the infected individual. Those who pay special attention to the pathogenic organism seek the cause of selective multiple involvement in the tissue affinity of the pathogenic organism; C. MARTINOTTI (1898), for example, isolated a staphylococcus with strong muscle-affinity from clinical cases, and noted that its intravascular injection never failed to cause muscle abscesses. He further showed the unchangeability of its pathogenic character for many months, and designated it as *Staphylococcus polymyositicus*. And also ISHIHARA (1955) noted that staphylococci isolated from suppurative foci such as furuncle, carbuncle, osteomyelitic and myositic lesions, had characteristic genetically-fixed tissue-affinities and cell chemical properties; and gave a pluralistic explanation for the tissue-affinities of different pathogenic strains of staphylococci.

According to recent genetic biochemical studies, the metabolic process of the bacterium, or the enzymatic structure which determines the character of metabolism does not permanently remain stable, but undergoes peculiar changes under the influences of nutrition supply and other environmental factors, and it is possible for these changes to be genetically fixed. Accepting this new view, MASAKI, a member of our laboratory, subcultured successively one and the same strain of staphylococci in the media, added either with rabbit skeletal muscle extract or with

rabbit bone marrow extract, and succeeded in producing the strains whose enzymo-chemical properties were respectively similar to those of the so-called myo- and osteo-strains. He further showed that these strains, when used in animal experiments, respectively caused typical myositis and osteomyelitis. DOGURA, enlarging the above view, divided the alkaline phosphatase of the bone-marrow-adapted, and the osteomyelitis strain into apo- and co-enzyme, and discovered that the apo-enzyme was activated with the addition of the co-enzyme of the bone marrow extract, and likewise noted that the activation of the ATPase of the muscle-adapted and the myositis strain occurred through the addition of the muscle extract. MAEDA, noting that adapted and pathogenic strains possessed increased lecithinase activity, and that the addition of tissue extracts to the respective media of these strains caused increased induction of P^{32} into RNA fraction of the staphylococcus, emphasized that the tissue affinity of the staphylococcus depended to a large extent on tissue chemical environment and the formation by adapted and pathogenic strains of adaptive enzymes for skeletal muscle or bone marrow. On the other hand, FUJIWARA noted that pyruvic acid splitting enzyme activity, especially pyruvic dehydrogenase activity was increased in the muscle-adapted and the myositis strain; and that the vitamin B_1 deficiency brought on quantitative increase of intramuscular pyruvic acid; and he used these two experimental results to explain the fact that the skeletal muscle tissue of the vitamin B_1 deficient individual was a more favourable environment for the growth of myostrains. These researchers regarded the above-mentioned increased enzymatic activities as due to enzymatic adaptation; they tried to clarify the tissue-affinity of the staphylococcus monistically.

Many papers have been published on the changes of amino acid metabolism in penicillin-resistant strains (BELLAMY and KLIMEK, 1947; GALE and RODWELL, 1948), and in strains adapted to specific materials added to media. As HIRANO, an associate of the present author, has stated, it is quite reasonable to suppose that the amino acid metabolism, or the protein metabolism of the bacterium stands in close relationship with its organ-affinity. MASAKI, DOGURA, MAEDA and FUJIWARA had produced strains of staphylococci adapted to tissue extracts, but the present author and HIRANO tried to obtain from purified form myosin, a principal protein constituent of muscle, as a first step to the clarification of adaptation material. When myosin was obtained, it was used for gradual replacement of amino acids in the medium. *Staphylococcus aureus*, F. D. A. 209-P strain was then subcultured successively in this myosin-containing medium to change it into the myosin-adapted strain. For the purpose of ascertaining the nature of the staphylococcal tissue-affinity, this myosin-adapted strain was investigated for the behavior in amino acid metabolism together with DOGURA and MAEDA's muscle- and bone-marrow-adapted strains and pathogenic strains isolated from clinical cases.

EXPERIMENT I. PAPER CHROMATOGRAPHICAL INVESTIGATION OF STAPHYLOCOCCAL AMINO ACID METABOLISM

Paper chromatography was used for the screening of staphylococcal amino acid

metabolism. This method may be used for investigation of

- (1) Amino acid composition of bacterial protein,
- (2) Free amino acid composition in the bacterial metabolic pool,
- (3) Free amino acids in various tissues and organs, and
- (4) Increase or decrease of the amount of amino acids in the synthetic medium following bacterial growth.

In the investigation of (1) it is difficult to detect fine differences of experimental results, while in those of (2) and (3) the purification and comparison of test materials are rather troublesome, and so in this experiment (4) was mainly investigated.

CHAPTER 1. FREE AMINO ACIDS IN RABBIT SKELETAL MUSCLE AND BONE MARROW

Q. N. MYRVIK and R. N. WEISSER (1955) reported that fresh muscle and bone marrow extracts contained bactericidin which exerted mild inhibiting effects on staphylococcal growth, but according to FUJIWARA, *Staphylococcus aureus* can grow in fresh tissue extracts without any addition of nutrition from the outside, first, in the degree of suitability for growth the muscle extract, and then second, the bone marrow and the skin extract.

Such a heterotrophic bacterium as the staphylococcus has an exacting demand for amino acids; it needs them for the synthesis of protein and enzymes; and so even if bactericidin is taken out of consideration, proper amounts of amino acids must be contained in tissue extracts in which staphylococcal growth is possible. On the other hand, if staphylococcal tissue-affinity is related with free amino acids of tissue, it may rightly be supposed that free amino acid composition varies with different tissues. In the following experiment the muscle and the bone marrow were comparatively examined for free amino acid composition.

I. Materials and Methods

Rabbits weighing 0.6-1.5 kg were used. The experimental animal, after being bled to death by severing A. carotis, was immediately placed in ice, and 10 g of thigh muscle, and 5 g of thigh bone marrow were surgically taken from it. Because inorganic and organic salts, sugars, soluble protein and other colloidal substances contained in these tissues made it impossible to achieve sufficient separation of amino acids in paper chromatography, their condensed extracts were not readily utilizable as test material. Deproteinization and desalination were necessary before they were put to experimental use.

(a) Deproteinization. 10 g of muscle kept cold in ice was added to 5 times as much absolute alcohol, and then homogenized, being kept cold all the while, in a Waring blender. After being boiled for several minutes it was centrifuged, and the supernatant was heated at 70°C to volatilize alcohol. The condensed material was dissolved by adding 30 cc of distilled water. 0.3 g of activated charcoal powder was then mixed up well with it. The solution obtained by its filtration was desalinated by the procedure which will be described later. Activated charcoal powder was

used to remove colloidal substances; colloids damage the function of ion exchange resin, and impede the paper-chromatographical investigation.

As for bone marrow, 5 g added to the same amount of sea sand, and 5 times as much absolute alcohol, was triturated in a mortar, and after being boiled for several minutes it was centrifuged. Colloidal substances were removed from the supernatant with activated charcoal powder.

(b) Desalination. The deproteinized solution still contains organic and inorganic ions, and sugars which impede the separation of amino acids in paper chromatography. Therefore, ion exchange resin, Amberlite IR-120 (ORGANO) was used to remove them. Glass cotton was put on the bottom of a permutit tube, and the tube was stuffed with 5 g of Amberlite IR-120 wet and swollen with 20cc of distilled water, leaving no room for air bubbles. Following this procedure, 50 cc of a 2 N HCl solution was passed through the tube at the rate of 1.5 cc per minute so as to make resin of H type. The HCl still attached to resin was washed away with about 20 cc of distilled water. As soon as the pH of the outflow became 5, the test solution was passed at the rate of 1.5 cc per minute. Most of amino acids, and Na^+ were thus fixed by resin. Though mostly all other organic acids, inorganic negative ions and sugars passed through, about 20 cc of distilled water was used to wash away some remaining portions. When 50 cc of a 2 N ammonia solution was passed at the rate of 2-3 cc per minute, the fixed amino acids were freed from resin, and flowed out. By the above procedures, such inorganic positive ions as Na^+ mostly remained unfreed, when using such a strong acid ion exchange resin as Amberlite IR-120. Caution should be exercised never to let air bubbles into resin. The presence or not of amino acids in solutions passed through resin was ascertained by using ninhydrin. The solution obtained by passing a 2 N ammonia solution was desiccated by decompression, and used as test material.

(c) Paper Chromatography and Development of Test Material.

(1) Placing of Test Material on Paper. A part of the desiccated test material was dissolved in 0.2 cc of distilled water, and by using a micropipette, a certain fixed amount was dropped on a sheet of Toyo filter paper No. 50 40 cm $\times$ 40 cm in size at the point 6 cm $\times$ 6 cm from a corner. During this procedure the sheet was dried by heated air from below, to keep the spread of the spot within 0.5 cm.

(2) Development was done after the two dimensional ascending method. In the one-dimensional method the test material was developed at 25°C for 30 hours with a phenol solution containing a 0.1 % ammonia solution at the rate of 20 %, and then sufficiently dried.

As a developing solvent for the two dimensional method, a mixed solution of butanol, distilled water, and glacial acetic acid (4:2:1) was used. The test material was developed at 25°C for 24 hours along the direction at a right angle to that of the one-dimensional method. The tip of solvent permeation was always marked, whether the one-dimensional or the two-dimensional method was used.

(3) Readings of Results. The developed filter paper, after being sufficiently dried, was sprayed with a 0.2 % ninhydrin solution of water-saturated butanol, and

again dried in the air. When heated at 90°-100°C for 10 minutes, each amino acid showed its own peculiar color at its place of development. The identification of amino acids was chiefly based on their rates of flow (Rf: migration distance of solute from origin/permeation distance of solvent from origin). The tone of color was also taken into account. Results were checked by the simultaneous development of purified amino acids. The fading of color began 2 days later. Those which had showed large Rf's, exhibited a tendency to fade rapidly.

(4) Quantitative Measurement of Amino Acids. Quantitative measurement by paper chromatography includes the following methods:

- (i) Conversion from the minimum detected quantity,
- (ii) Measurement of the area of the spot,
- (iii) Use of the densitometer, and
- (iv) Colorimetry on the extracted materials from paper chromatogram.

The procedures of (i) and (iv) are very inconvenient and troublesome when many materials are comparatively tested, and the same is true with the method of (iii) when the two dimensional method is used. As K. B. FISCHER (1948) and others reported, the area of the developed spot is proportional to the logarithm of a solute concentration. Though the error of 2-10 % is said to be unavoidable, the method of (ii), namely, spot-area measurement is fairly dependable in comparative tests, and so it was used in this experiment.

The present author found that the above-mentioned proportional relationship existed in the majority of cases, but with regard to glutamic acid, tryptophane, aspartic acid, proline, and phenylalanine, the spot area tended to increase parabolically with the increase of concentration after the latter had reached a certain level (Fig. 1-2). Quantitative measurement, however, was conducted on these amino

Fig. 1 Relations between the logarithm of a solute concentration and the area of its developed spot on paper chromatography.

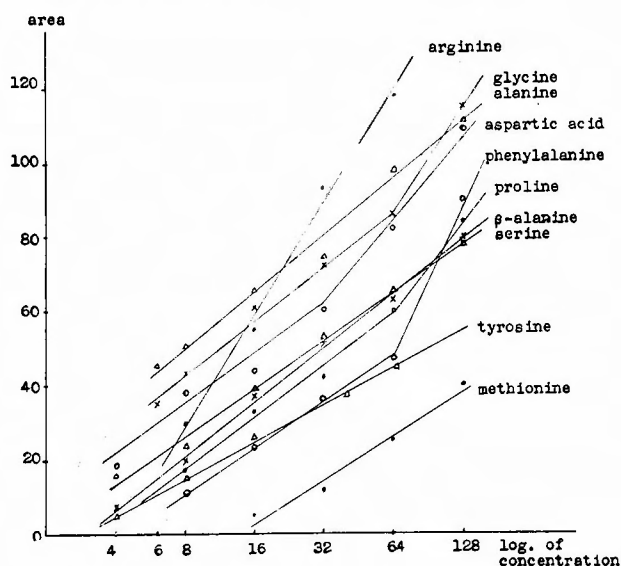
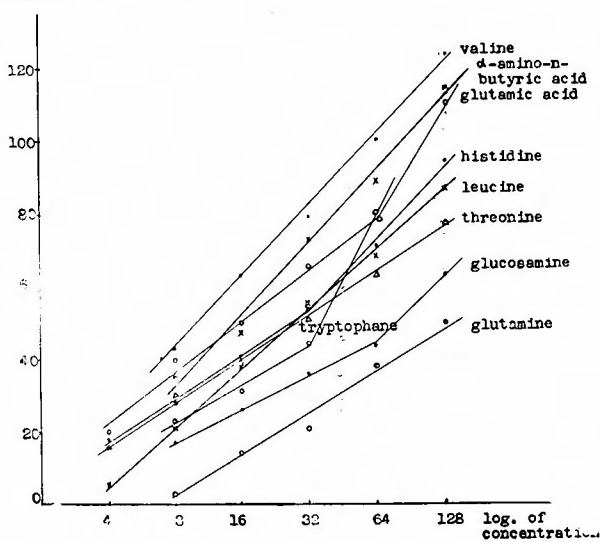


Fig. 2 Relations between the logarithm of a solute concentration and the area of its developed spot on paper chromatography.



acids, too, referring to their parabolic curves. The spot was demarcated from the back of filter paper, and measured by a planimeter.

Table 1 Free amino acids in rabbit skeletal muscle and bone-marrow.

tissue body weight amino acid	skeletal muscle		bone-marrow		SURGALLA'S medium
	0.6 kg	1.5 kg	0.6 kg	1.5 kg	
cystine	—	—	—	—	24
serine	2	5.6	5.1	8.4	0
aspartic acid	—	4.2	5.8	8.4	98
glutamic acid	2.3	2.5	10.6	10.3	98
glycine	8	14	9.3	16.6	378
lysine	—	—	—	—	55
threonine	2	1	—	—	0
alanine	4.5	5	5.1	5	59
hydroxyproline	—	—	—	—	87
β-alanine	4.2	9	—	—	0
proline	—	—	—	—	411
tyrosine	—	—	5	—	45
histidine	2	—	0.8	—	57
arginine	—	—	—	—	1909
valine	—	—	—	—	78
methionine	1.6	2.5	4	6.4	37
leucine	5	4	10.8	4	87
phenylalanine	—	—	—	—	41
tryptophane	—	—	—	—	10
α-amino-n-butyric acid	2	—	—	—	0
total	33.6	47.8	56.5	59.1	3474 _(γ/g)

II. Results and Summary (Table 1) (Fig. 3-4)

The amount of amino acids differed to some degree running parallel with the weight of experimental animals. The bone marrow extract contained aspartic acid, glutamic acid and leucine in larger quantities than the muscle extract did, but the latter had more of threonine, β -alanine and α -amino-n-butyric acid. Thus, the muscle extract did not necessarily contain amino acids in larger quantities than other tissue extracts. As FUJIWARA reported, the muscle extract is most favourable for staphylococcal growth, but this fact, as mentioned above, is unexplainable by mere quantitative differences of amino acids between this extract and other tissue extracts. This problem demands more careful consideration. It is considered that not only quantities of amino acids, but also the problem concerning the correlation-ship between their mutual antagonism and the tissue-affinity of the staphylococcus should be investigated more fully in the future: valine is said to inhibit the utilization of leucine and threonine (GLADSTONE: 1939), glycine, serine, β -alanine, and threonine that of alanine (SNELL and GUIRARD: 1943); and threonine that of serine (MEINKE and HALLAND: 1948).

Compared with the SURGALLA's amino acid synthetic medium, the muscle and the bone marrow extract contain free amino acids in by far smaller quantities, but it is to be noted that the muscle and the bone marrow extract contain such amino acids as are not in the SURGALLA's medium; that is, the former has serine, threonine, β -alanine, and α -amino-n-butyric acid; and the latter serine.

CHAPTER 2. AMINO ACIDS FORMED THROUGH RESOLUTION OF MYOSIN BY MYOSIN-ADAPTED, CONTROL, MYOSITIS AND OSTEOMYELITIS STRAINS

In bacterial growth the protease activity is a prerequisite to the synthesis of bacterial protein, and closely connected with bacterial vitality. The staphylococcus absolutely needs some amino acids, and so it may be assumed that it first utilizes free tissue amino acids when it invades the host, and then by resolving tissue protein, secures essential amino acids. While the variety and quantities of free tissue amino acids vary with each tissue, as mentioned in Chapter 1, we might be able to suppose the higher the tissue-affinity of pathogenic strains are, the more their tissue protein-splitting enzyme increase.

MASAKI and KATAOKA reported that the caseinase and peptonase activities of tissue-adapted strains were activated by adding respective tissue-extracts, but they did not ascertain what component of tissue extracts this activating effect had. As there is a relationship between enzymatic activity and protein structure, it is conceivable that a bacterium finds some proteins more easily resolvable than others. When two kinds of bacteria with different enzymatic activities are respectively cultured on a substrate easily resolvable by one of them, amino acids thereby formed will have a different composition in each case. In the following experiment myosin was used as substrate, and subjected to the protease activities of various strains of staphylococci; and the present author tried to determine the nature of staphylococ-

cal tissue-affinity by investigating the composition of amino acids formed by each strain.

I. Materials

(a) Casa-Amino Acid Semi-Synthetic Medium (Table 3). With reference to the SURGALLA's amino acid synthetic medium (Table 2), lacking amino acids and salts were added to this medium. To prevent denaturation of the ingredients, the medium was sterilized in an autoclave by heating at 120°C for 10 minutes under atmospheric pressure. The aseptic condition of the medium was further confirmed after incubation at 37°C for 24 hours.

Table 2 SURGALLA's synthetic medium (1947).

KH ₂ PO ₄	4.536 g	L-glutamic acid	98 mg
glucose	2.250 g	L-histidine	57 mg
L-arginine-HCl	1.909 g	L-oxypoline	87 mg
L-proline	0.411 g	L-leucine	87 mg
glycine	0.378 g	L-lysine-HCl	55 mg
MgSO ₄ ·7H ₂ O	20 mg	DL-methionine	37 mg
L-cystine	24 mg	L-tryptophane	10 mg
FeSO ₄ (NH ₄) ₂ SO ₄ ·6H ₂ O	14.5 mg	L-tyrosine	45 mg
DL-alanine	59 mg	nicotinic acid	1.23mg
DL-phenylalanine	41 mg	vitamin B ₁	33.4 γ
DL-valine	78 mg	Aq. dest.	1000 cc
L-aspartic acid	98 mg		pH 7.5

(b) Enzymes

(1) Production of Myosin-Adapted, and the Control strain. Myosin was obtained, and purified from rabbit skeletal muscles after SZENT-GYORGI's KI method (1951). Amino acids and salts were removed by dialysis. On the other hand, amino acids were gradually withdrawn from the casa-amino acid semi-synthetic medium which contained, besides amino acids, sugars, vitamins and salts; and instead a solution of purified myosin was added to prepare synthetic media containing myosin in graded quantities. Staphylococcus aureus, F. D. A. 209-P strain, was subcultured successively in these media to change it into the myosin-adapted strain. The strain subcultured in the original casa-amino acid semi-synthetic medium was used as control.

(2) Production of Enzymes. It seemed desirable to use casa-amino acid semi-synthetic media for this purpose, but from the need of obtaining large quantities

Table 3 Casa-amino acid semi-synthetic medium

casa-amino acid (NISSAN)	7 g
tryptophane	10 mg
cystine	24 mg
nicotinic acid	1.23 mg
vitamin B ₁	33.4 γ
KH ₂ PO ₄	4.536g
MgSO ₄ ·7H ₂ O	20 mg
FeSO ₄ (NH ₄) ₂ SO ₄ ·6H ₂ O	14.5 mg
glucose	2.25 g
Aq. dest.	1000 cc
	pH 7.5

of staphylococci, the myosin-adapted, the control, the myositis and the osteomyelitis strain were cultured in broth agar media. It was taken for granted that the acquired enzymatic system was genetically-fixed.

Staphylococci, after being cultured at 37°C for 24 hours, were washed three times with an isotonic saline solution, and collected. One volume was suspended in 5 volumes of an isotonic saline solution, and toluol was placed on this suspension. Staphylococcal suspension autodigested at 37°C for 48 hours were used as enzyme material.

(c) Myosin Substrate Solution. Myosin was dissolved at the rate of 2 % in SPRENSSEN'S phosphate buffer solution consisting of 2 volumes of M/15 KH_2PO_4 and 8 volumes of M/15 Na_2HPO_4 . The pH of the solution was adjusted to 7.5.

II. Methods

2 cc of the enzyme suspension was added to 10 cc of 2 % myosin substrate solution. Immediately after the addition, 5 cc of this mixed solution was withdrawn. The remaining solution, after toluol had been placed on it, was incubated at 37°C for 24 hours, and 5 cc of this solution was again withdrawn as test material. The buffer solution added with the enzyme suspension was used as control. Deproteinization, detection of amino acids and other procedures were carried out, as described in Chapter 1.

III. Results (Table 4) (Fig. 5-8)

Table 4 Amino acids formed through resolution of myosin by myosin-adapted, control, myositis, and osteomyelitis strains.

amino acid	strain	myosin-adapted strain (My_{19})	myositis strain	osteomyelitis strain	control strain
aspartic acid		4.8	11.4	8	6
glutamic acid		5.4	27.5	3	6.8
serine		4	12	3.3	2
glycine		4.4	6	7.1	6
threonine		7	4.2	—	3.1
alanine		2.4	—	0.1	5
β -alanine		2.4	2.4	—	4.4
α -amino-n-butyric acid		4.2	6.4	8.4	—
valine (methionine)		4.8	5.8	7.5	3.8
phenylalanine		74	88	54	20
proline		—	9	—	—
arginine		6	6.2	3.1	—
histidine		—	4	—	—
total		113.4	182.9	94.9	57.1

(γ /0.25g)

Generally-speaking, the myosin-adapted, and the myositis strain showed a vigorous myosinase activity. The production of aspartic acid, glutamic acid, and phenylalanine was more marked with the myositis strain than with other strains. Serine and threonine, of which more mention will be made later, were produced in larger quantities by myostrains, namely, myosin-adapted and myositis strains.

As to the osteomyelitis strain, its production of α -amino-n-butyric acid was proportionately larger, while that of threonine was smaller, as compared with other strains. It was conspicuous that the control strain hardly produced these amino acids. The osteomyelitis and the myositis strain produced alanine and β -alanine in only small quantities.

In nearly all the cases the quantities of amino acids produced from myosin by the experimental strains showed correspondence with the myosinase activities of these strains as measured by HIRANO; only with the myosin-adapted and the myositis strain this correspondence failed. This is due to the fact that the method of formol titration used by HIRANO gives the number of severed peptide-bonds, but does not necessarily measure the quantities of produced amino acids.

CHAPTER 3. UTILIZATION OF AMINO ACIDS IN SYNTHETIC MEDIA BY ADAPTED, CONTROL AND PATHOGENIC STRAINS

It may be assumed from the nature of tissue-affinity that the altered enzymatic system of tissue-adapted strains and pathogenic strains will necessarily cause alterations in the metabolic process of amino acids. In the following experiment certain fixed quantities of staphylococci of each strain were cultured in synthetic media of same kind, and changes in the amino acid composition of the medium were paper-chromatographically investigated.

I. Materials

(a) Casa-Amino Acid Semi-Synthetic Medium. With reference to the SURGALLA's synthetic medium lacking amino acids and salts were added to this medium.

(b) Test Strains. The myosin-adapted strain; DOGURA and MAEDA's muscle- and bone-marrow-adapted strains; and the myositis and the osteomyelitis strain.

II. Methods

Staphylococci cultured at 37°C for 24 hours in casa-amino acid semi-synthetic media were again inoculated into semi-synthetic media in 0.1 mg/cc quantities, and incubated at 37°C. The media were shaken well at certain intervals. 4 cc and 2.5 cc of culture media were respectively aseptically withdrawn. The 4 cc was used as test material for growth measurement by electrocolorimetric nephelometry, while the 2.5 cc was used for paper-chromatographical investigation of amino acids, the procedures of which are described in Chapter 1.

III. Results and Summary

(a) Growth Curve (Fig. 9). The carbuncle, the osteomyelitis and the myositis strain showed a very active growth, in comparison with the mother strain, F. D. A. 209-P, the control and the adapted strains originating from the mother strain. The myosin-adapted strain had a shorter maximum stationary phase than other strain, and staphylococci belonging to this strain rapidly decreased in number during the logarithmic death phase.

With pathogenic strains and the mother strain the maximum stationary phase ranged from the 14th hour to the 18th following the inoculation, but with adapted

Fig. 9 Growth curves of each pathogenic and tissue-adapted strain in the casa-amino acid semi-synthetic medium.

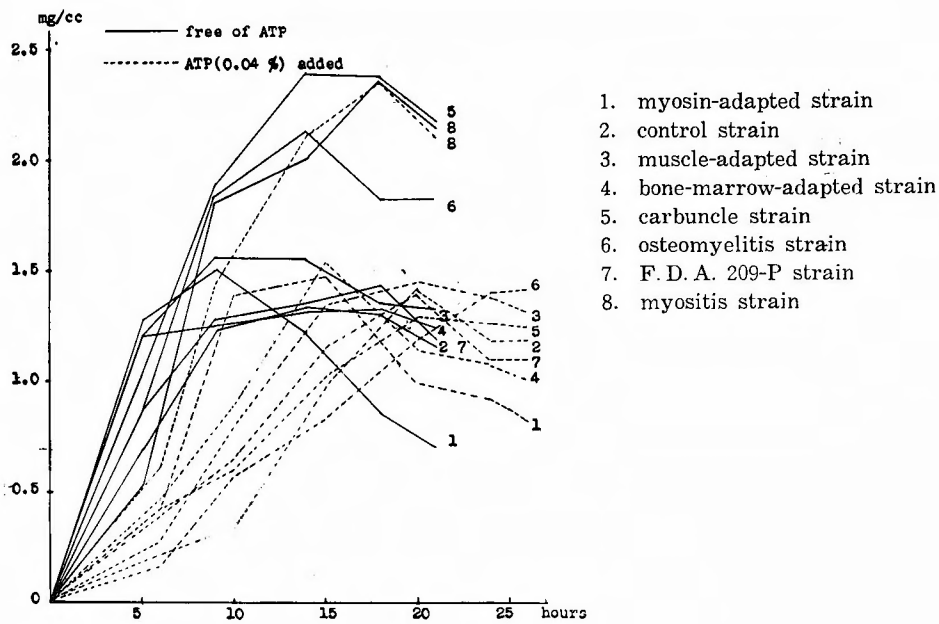


Fig. 23 Utilizations of serine in the casa-amino acid semi-synthetic media by each strain of staphylococci.

- A. myosin-adapted strain
- B. control strain
- C. muscle-adapted strain
- D. bone-marrow-adapted strain
- E. F.D.A. 209-P mother strain
- F. osteomyelitis strain
- G. myositis strain
- 1. 14 hrs. after inoculation
- 2. 24 hrs. after inoculation

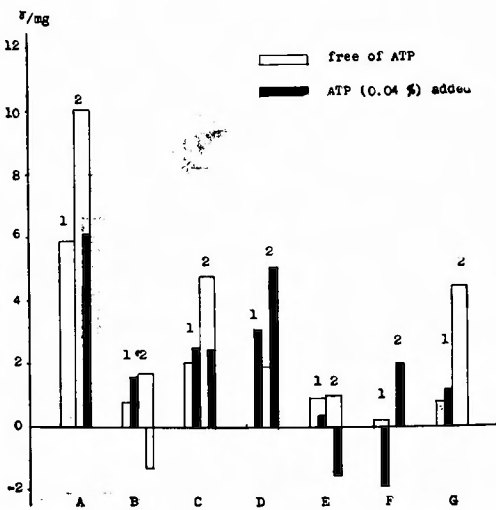
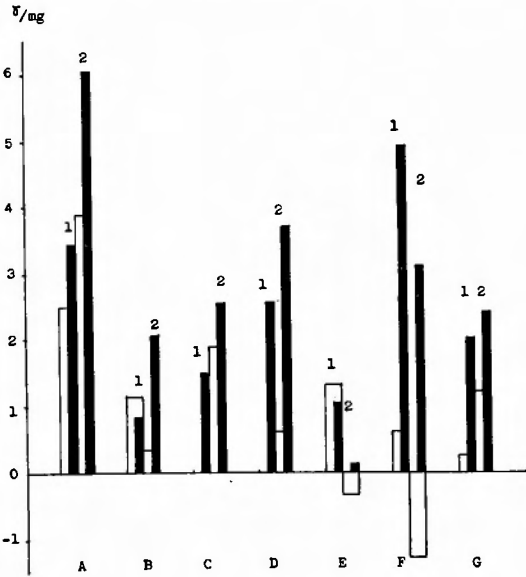


Fig. 24 Utilizations of threonine in the casa-amino acid semi-synthetic media by each strain of staphylococci.



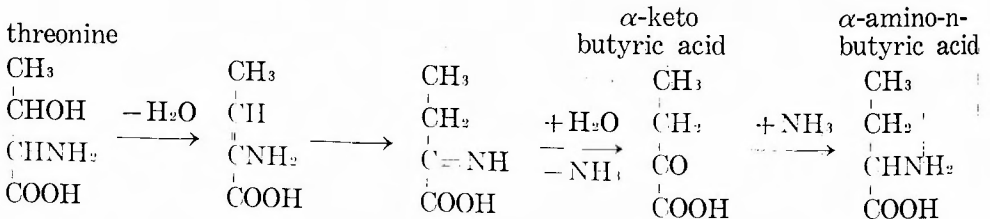
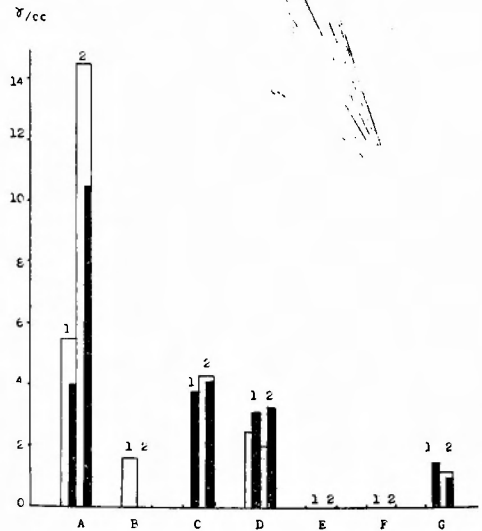
and control strains from the 9th hour to the 14th. This fact partially reveals the varied degree of adaptation which adapted and control strains have attained to the casa-amino acid semi-synthetic medium.

(b) Consumption or decrease of amino acids in the medium was rather similar with each strain. But the myosin-adapted strain showed a higher utilization of serine and threonine than the control strain (Fig. 11, 13, 15). This tendency increased running parallel with the number of subculture (Fig. 16-19). It was also strong in muscle-adapted and myositis strains (Fig. 20), but not noticeable in bone-marrow-adapted, osteomyelitis, control and mother strains (Fig. 10, 12, 14, 21-24).

With myosin-, muscle-, and bone-marrow-adapted, and myositis strains, the spot of α -amino-n-butyric acid enlarged with the elapse of time, but with mother, osteomyelitis and control strains, the appearance of α -amino-n-butyric acid was hardly noted (Fig. 25). It was also interesting to note that on the whole, the decrease of threonine went in parallel with the increase of α -amino-n-butyric acid.

LIEN and GREENBERG (1953) showed the formation of α -keto butyric acid and α -amino-n-butyric acid from threonine by hepatic threonine-dehydrase of rat. As this process seemed to have something to do with the above fact, the serine- and threonine-dehydrase activities of each strain were investigated in Experiment II.

Fig. 25 Productions of α -amino-n-butyric acid in the casa-amino acid semi-synthetic media by each strain of staphylococci.



CHAPTER 4. THE EFFECTS OF SERINE, THREONINE, AND α -AMINO-N-BUTYRIC ACID ON THE GROWTH OF THE MYOSIN-ADAPTED, AND THE CONTROL STRAIN

In the preceding chapter it was shown that the myosin-adapted strain actively utilized serine and threonine, and produced instead α -amino-n-butyric acid. The following experiment was conducted to ascertain the effects of serine, threonine and

α -amino-n-butyric acid upon the growth of the myosin-adapted strain and the control strain.

I. Materials and Methods

By eliminating glycine which seems to have much to do with serine and threonine, the SURGALLA's amino acid synthetic media were remade into new media whose ingredients are shown in the Fig. 26. Staphylococci were inoculated into the media in 0.1 mg/cc quantities, and the growth curves of the myosin-adapted and the control strain were drawn. The degree of growth was indicated by the rate of optical density so that the effects of media on growth might be known in detail.

II. Results and Summary (Fig. 26)

In the case of elimination of glycine from the SURGALLA's medium the myosin-adapted strain, when serine and threonine was added, kept growing, and moreover, showed mild multiplication, but the control strain not only showed no growth, but also gradually decreased in number. The effect of α -amino-n-butyric acid was not appreciable, but strictly speaking, this acid seemed to possess, if any, a very feeble growth-inhibiting action. The addition of glycine had a quick, remarkable promoting effect on staphylococcal growth, especially on the growth of the myosin-adapted strain.

The foregoing results seem to preclude any relationship of transfer between glycine and serine, but at any rate it may be concluded that the myosin-adapted strain has developed the enzymatic system which can readily utilize glycine, serine and threonine.

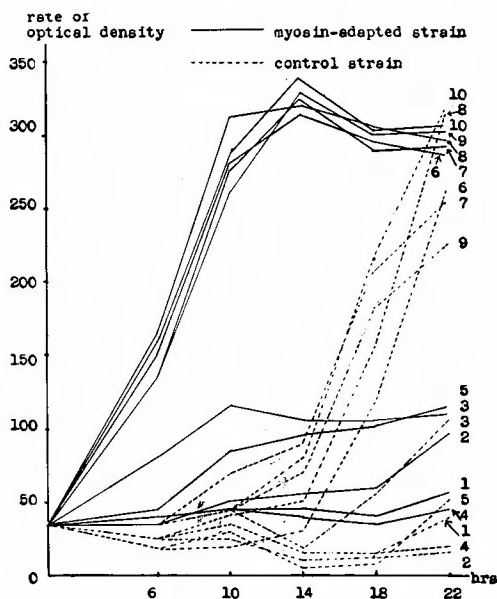
These growth-curves show that MEINKE's so-called serine-threonine antagonism is nearly non-existent in the myosin-adapted and the control strain.

CHAPTER 5. THE EFFECTS OF ATP ON THE METABOLISM OF AMINO ACIDS CONTAINED IN THE MEDIUM OF EACH STRAIN

OCHOA stated that ATP played an important role in the synthesis of protein as a carrier of amino acids. DOGURA, a member of our laboratory,

Fig. 26 Effects of serine, threonine, and α -amino-n-butyric acid on the growth of the myosin-adapted, and the control strain in the amino acid synthetic media.

1. S. (SURGALLA's amino acid synthetic medium)-glycine
2. S. -glycine + serine
3. S. -glycine + threonine
4. S. -glycine + α -amino-n-butyric acid
5. S. -glycine + serine + threonine
6. S. + serine
7. S. + threonine
8. S. + serine + threonine
9. S. + α -amino-n-butyric acid
10. S. + serine + threonine + α -amino-n-butyric acid



noting that the ATPase activity of the muscle-adapted strain increased with the addition of the muscle extract, argued that the tissue-affinity of this strain had a close bearing on the myosin-ATPase system peculiar to the skeletal muscle. MASAKI, on the other hand, reported that staphylococcal growth was accelerated in the medium containing ATP in proper concentration. It is, therefore, natural to suppose that ATP, when added to the medium in proper concentration, will have some kind of effect on the metabolism of amino acids in the staphylococcus. The following experiment was carried out under this supposition.

I. Materials and Methods

ATP-Na was added to 50 cc of casa-amino acid semi-synthetic medium at the rate of 0.05 % (growth-promoting concentration, as shown by MASAKI). This medium was incubated at 37°C for 24 hours, and ascertained to be aseptic. Staphylococci of each strain was then inoculated into the medium in 0.1 mg/cc quantities. Changes in amino acid composition occurring with staphylococcal growth were investigated in the same way as described in Chapter 3.

II. Results

In place of bouillon media in MASAKI's experiment, casa-amino acid semi-synthetic media were used. Contrary to experimental results obtained by MASAKI, the addition of ATP prolonged the time from the growth phase to the maximum stationary phase with each strain, and seemed rather to inhibit staphylococcal growth. Only with the myosin-adapted strain was this prolongation of time a little shorter (Fig. 9).

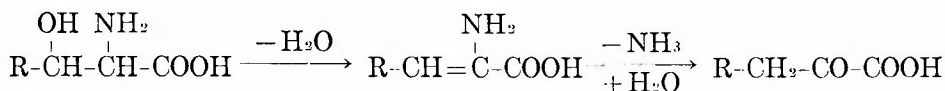
Next, the effect of ATP on the efficiency of amino acid metabolism was paper-chromatographically investigated. In Chapter 3 serine and threonine were shown to be most involved in metabolic changes. In this experiment ATP had a rather inhibiting effect on the utilization of serine by myosin-adapted, muscle-adapted and myositis strain, but on the contrary, a promoting effect on the same by bone-marrow-adapted and osteomyelitis strains. As to the utilization of threonine, ATP nearly always promoted it, especially that by myosin-adapted, bone-marrow-adapted, and osteomyelitis strain. ATP rather inhibited the formation of α -amino-n-butyric acid (Fig. 23-25).

It is clear from the foregoing results that ATP does not always promote the amino acid metabolism of tissue-adapted strains; and that its action varies with each amino acid. In this experiment, contrary to MASAKI's report, ATP was found to act rather inhibitingly on staphylococcal growth. As it is, the significance of ATP in the amino acid metabolism is still clouded in obscurity, and its clarification is left for future investigations.

EXPERIMENT II. THE SERINE- AND THREONINE-DEHYDRASE ACTIVITIES OF EACH STRAIN

By the paper-chromatographical screening test of the amino acid metabolism of the staphylococcus it was ascertained that the metabolism of serine and threonine was partially connected with staphylococcal affinity for skeletal muscle. In this

connection the present author was aware of the fact that serine and threonine, both belonging to β -hydroxyamino acid, were not essential amino acids of staphylococci, and conducted the following experiment on serine- and threonine-dehydrase. By the way, the existence of this kind of dehydrase is now established in *Neurospora* (YANOFSKY & REISSIG 1952, 1953) and *Escherichia coli* (DAVID & ESMOND 1952)



I. Material

(a) Substrate. DL-serine, DL-threonine (RIKEN)

(b) Coenzyme. Pyridoxine (aderoxine.....SONNEBOD Co.)

(c) Test strains. The myosin-, muscle- and bone-marrow-adapted strain; the control strain; the myositis, osteomyelitis and carbuncle strain; and the F. D. A. 209-P mother strain.

(d) Enzyme Sources. It seemed desirable to use casa-amino acid semi-synthetic media for this purpose, as for subculture, but as a large quantity of staphylococci was needed, broth agar media were used instead. It was taken for granted that the adaptive enzymes were genetically-fixed. Staphylococci cultured at 37°C for 24 hours were collected, and washed three times with icy distilled water. One volume was suspended in five volumes of icy distilled water. This bacterial suspension was put to experimental use as an enzyme solution.

(e) 0.1 M sodium pyrophosphate buffer solution.

(f) Reagents for the quantitative measurement of pyruvic acid.

(i) 10% trichloroacetic acid solution (chemical pure).

(ii) Xylol (chemical pure).

(iii) 2% dinitrophenylhydrazin (DNP-reagent). A quantity of 0.5 g of 2,4-dinitrophenylhydrazin was added to 100 cc of a 2 N HCl solution, and dissolved by heating in a reflux condenser.

(iv) 10% sodium carbonate solution.

(v) 4 N NaOH.

(vi) Sodium pyruvate.

II. Methods

With reference to YANOFSKY's method, the reaction mixture was prepared by mixing together 1 cc of 6×10^{-2} M DL-serine (or 3×10^{-2} M DL-threonine), 10 γ of pyridoxine, 0.5 cc of a 0.1 M sodium pyrophosphate buffer solution, and 4 cc of enzyme suspension. This reaction solution was adjusted to have pH 10, and incubated at 37°C for 1.5 hours. Five cc of a 10 % trichloroacetic acid was then added to the same amount of this reaction solution to arrest the progress of reaction. After centrifugation and deproteinization 8 cc of the supernatant was withdrawn, and the quantities of pyruvate and keto butyrate were measured after the FRIEDMANN-HAUGEN-SHIMIZU's method. A solution consisting of pyridoxine and substrate (1), and the enzyme suspension (2) concurrently incubated as control. The control value was deducted from the total reaction value.

The quantitative measurement of pyruvate and keto butyrate was done as follows: The supernatant 8 cc in volume placed in a warm bath at 25°C is added to 0.7 cc of DNP reagent, and after reaction had been allowed to take place for 5 minutes, further added to 8 cc of xylol. Air is sent through a capillary tube into the supernatant, which is then shaken actively for 3 minutes. By centrifugation the supernatant is divided into two layers. The lower layer being eliminated, the upper xylol layer is washed three times with distilled water, and added with 3 cc of distilled water. The pH of the water layer is adjusted to 2-3 by the addition of HCl, followed by storage in a refrigerator for 24 hours. Subsequent procedures are the removal of the water layer, the addition of 6 cc of a 10% sodium carbonate solution, sending of air through a capillary tube, and active shaking for 3 minutes, and centrifugation into two layers. After all these procedures, 5 cc of the water lalyer is withdrawn, and added to 2 cc of a 4 N NaOH solution. The developing color is quantitatively measured within 5-20 minutes, using BECKMANN's photoelectric colorimeter with a wave length of 470 m μ .

Pyruvic acid and α -keto butyric acid were confirmed as such with paper chromatography. That is, the test material previously treated with DNP reagent was added to 10 cc of ethyl acetate, and abstructed by aeration. After removal of water layer, ethyl acetate was volatilized by decompression. The test material thus condensed was placed on a sheet of Toyo filter paper No. 50 at the site previously bedaубed with a N/100 hposphate buffer solution of pH 7.2. Water-saturated butanol solution was used as solvent for development. Purified materials were similarly

developed, and their RF's studied for the sake of checking up on experimental results.

Fig 27 PH and DL-serine-dehydrase activity of the myosin-adapted strain.
reaction time 30 minutes, bacterial amount 0.4 g, temperature 37°C

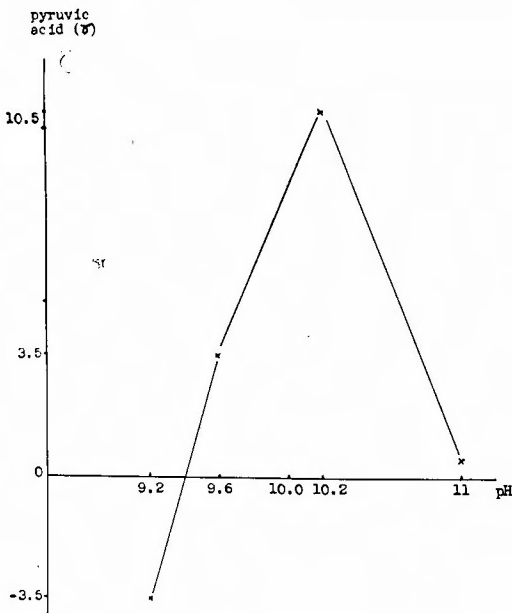
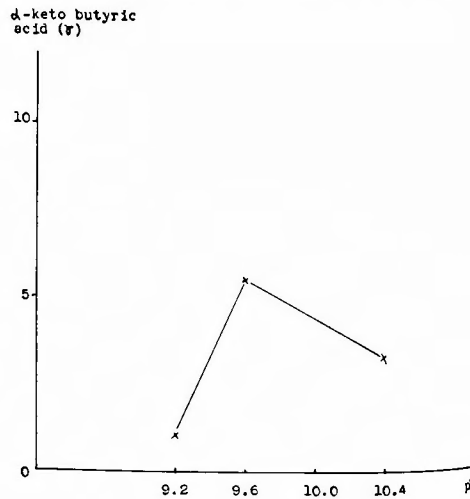


Fig. 28 PH and DL-threonine-dehydrase activity of the myosin-adapted strain.
reaction time 1.5 hours, bacterial amount 0.5 g, temperature 37°C



III. Results

(a) Optimum pH (Fig. 27-28). WOOD and GUNSALUS (1949) reported that the optimum pH for the L-serine- and L-threonine-deaminase of *Escherichia coli* was 7.8. DAVID and ESMOND, noting that the optimum pH for D-serine-dehydrase of the same was 8.0, stated that these might be two different enzymes. YANOFSKY (1952) (1953) reported that the optimum pH for the D-serine-dehydrase of *Neurospora* was 8.2, while that for the L-serine- and L-threonine-dehydrase was 9.2. In this experiment it was ascertained that the optimum pH for deamination of DL-serine was 9.6-10.2 in the case of the myosin-adapted strain.

Fig. 29 Reaction time and DL-serine-dehydrase activity of the myosin-adapted strain.

bacterial amount 0.4 g, pH 10,
temperature 37°C

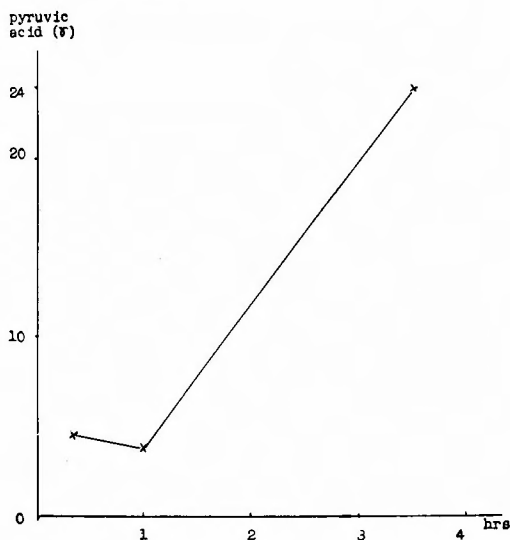
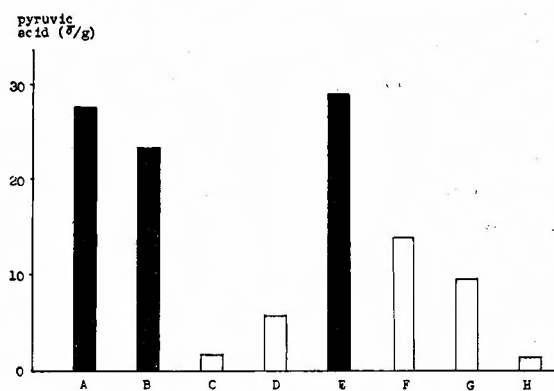


Fig. 30 DL-serine-dehydrase activities of each strain of staphylococci.

bacterial amount 1g, pH 10, temperature 37°C, reaction time 1.5 hours

A. myosin-adapted strain, B. muscle-adapted strain, C. bone-marrow-adapted strain, D. control strain, E. myositis strain, F. osteomyelitis strain, G. carbuncle strain, H. F. D. A. 209-P mother strain



(b) Reaction Time (Fig. 29). Prolongation of reaction time increased the quantity of isolated pyruvate. In this experiment, the reaction time being fixed as 1.5 hours, the enzymatic activity of each strain was comparatively examined.

(c) The DL-serine-dehydrase activity of the myosin-adapted strain varied a little with each generation, but it was increased throughout all the generation, compared with that of the control strain (Table 5).

(d) The muscle-adapted, the myosin-adapted, and the myositis strain possessed

Table 5 DL-serine dehydrase activity of the myosin-adapted strain in each generation.

generation	strain	myosin-adapted strain	control strain
19		7.2	-1.6
29		27.5	5.9
40		8.5	7

pyruvic acid (γ)

a higher serine-dehydrase activity, and produced pyruvate in definitely larger quantities than other strains (Fig. 30).

In short, serine-dehydrase activity was experimentally proved to vary with strains, while on the other hand, no clear differences among strains were noted in threonine-dehydrase activity (Table 6).

Table 6 DL-threonine dehydrase activity of each strain.

generation	strain	myosin-adapted strain	control strain	myositis strain
19		-6	2.5	
29		15.5	11	1.5

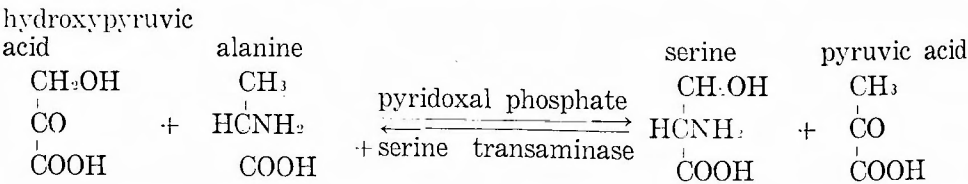
α-keto butyric acid (γ)

EXPERIMENT III. SERINE-TRANSAMINASE ACTIVITY OF EACH STRAIN

CHAPTER 1. PAPER-CHROMATOGRAPHICAL INVESTIGATION OF SERINE CONSUMPTION AND ALANINE PRODUCTION

In Experiment I it was shown that serine and threonine, both belonging to β-hydroxy-amino acid, were readily utilizable by myostrains; and in Experiment II that myostrains possessed high serine-dehydrase activity. In view of these facts it is easily conceivable that pyruvic acid is actively produced from serine. If the reverse of the above reaction were to be proved possible, Ozawa's vitamin B₁ deficiency theory on the occurrence of myositis would obtain a partial, if not complete, vindication from the possibility of synthesis of bacterial protein from pyruvic acid. But so far such a reverse reaction has not been demonstrated.

When bacterial growth or the synthesis of bacterial protein is a subject of inquiry, the synthesis of essential amino acids necessarily comes into question. SALLACH (1955) demonstrated in animal tissue the existence of transaminase whose formula is given in the following.



This serine-transaminase is capable of synthetizing alanine from pyruvic acid and serine. Alanine, by the way, is an amino acid indispensable to staphylococcal growth, and as for pyruvic acid, FUJIWARA showed that it was present in skeletal muscle in particularly large quantities, and increased in quantity in the case of vitamin B₁ deficiency. The following experiment was instituted, as it was considered that comparison among strains in the activity of serine-transaminase was important not only in clarifying the nature of skeletal-muscle-affinity of myostrains, but also in understanding the correlationship between our theory on the pathogenesis of polymyositis, and Ozawa's vitamin B₁ deficiency theory.

I. Materials

- (a) Enzymes. Staphylococci of various strains were cultured in broth agar media at 37°C for 24 hours, and washed three times with an isotonic saline solution.
- (b) SPRENSSEN's phosphate buffer solution (pH 7.6).
- (c) 0.2 M sodium pyruvate solution.
- (d) 0.2 M serine solution.
- (e) Pyridoxine.

II. Methods

A mixed solution consisting of 1 g of enzyme, 3.5 cc of phosphate buffer solution, 1 cc of a 0.2 M sodium pyruvate solution, 1 cc of 0.2 M serine solution, and 20 γ of pyridoxine was prepared, and its pH was adjusted to 7.6. Paper-chromatographical investigations were carried out immediately after the making of this solution, and 24 hours after its incubation at 37°C, using the procedures described in Experiment I.

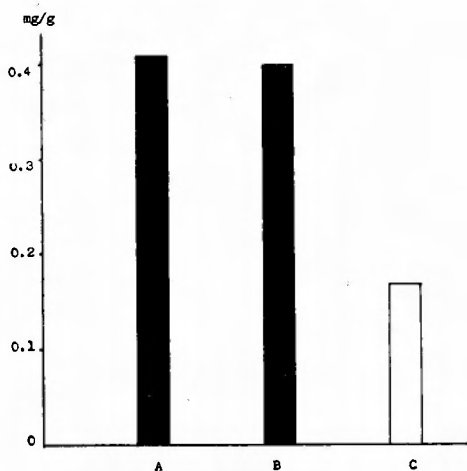
A control solution was prepared by mixing together enzymes, phosphate buffer solution and pyridoxine.

III. Results (Fig. 31-32)

Myostrains such as myosin-adapted and myositis strains showed a large production of alanine. The production of alanine on the part of the control strain was also large, but not so large as in the above case. On the other hand, the decrease of serine was more marked with the control strain than with myostrains.

Fig. 31 Serine-transaminase activities (productions of alanine) of the myosin-adapted strain, myositis strain, and control strain.

A. myosin-adapted strain, B. myositis strain, C. control strain
pH 7.6, temperature 37°C, reaction time 12 hours, bacterial amount 1g



CHAPTER 2. THE EFFECTS OF SERINE, ALANINE, PYRUVIC ACID, AND PYRIDOXINE ON THE GROWTH OF MYOSIN-ADAPTED AND CONTROL STRAINS, VIEWED FROM THE STANDPOINT OF SERINE-DEHYDRASE AND -TRANSAMINASE

The increased serine-dehydrase and -transaminase activities on the part of myostrains were shown in the preceding experiments. The following experiment was instituted to investigate the effects of serine, alanine, pyruvic acid and pyridoxine on the growth of myostrains and control strains.

I. Materials and Methods

Alanine was removed from SURGALLA's synthetic media, and in its stead, serine, alanine, pyruvic acid, and pyridoxine, coenzyme of dehydrase and transaminase, were

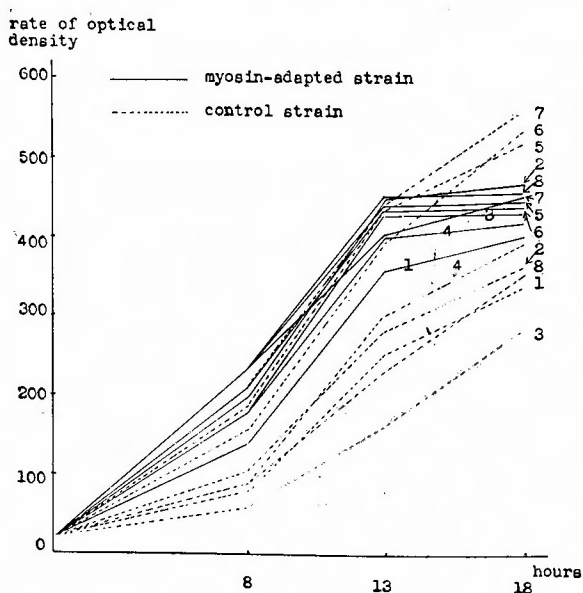
respectively added at the ratio given in the Figure 33. Staphylococci of each strain were inoculated in these media in 0.1 mg/cc quantities, and their growth was comparatively studied.

II. Results (Fig. 33) and Summary

The addition of alanine alone promoted the growth of both the myosin-adapted and the control strain, especially of the former. Serine inhibited the growth of the control strain in some degree, but rather promoted that of the myosin-adapted strain. As for pyridoxine, it had growth-promoting effects on the both strains. The addition of serine and pyridoxine had no marked effects on the control strain, but remarkably accelerated the growth of the myosin-adapted strain. Sodium pyruvate induced the growth of the both strains, but its growth-promoting effects did not differ so much from those of alanine, serine and pyridoxine. The active growth of the myosin-adapted strain in the basic medium seemed to be due to the effects of glycine, as mentioned in Chapter 4, Experiment I.

Fig. 33 Effects of serine, alanine, pyruvic acid, and pyridoxine on the growth of the myosin-adapted and control strain, viewed from the standpoint of serine-dehydrase and -transaminase.

1. S. (SURGALLA'S amino acid synthetic medium from which alanine was removed)
2. S. + alanine (0.002M), 3. S. + serine (0.002M)
4. S. + pyridoxine (20γ/cc), 5. S. + alanine (0.002 M) + pyruvic acid (0.005M)
6. S. + serine (0.002 M) + pyruvic acid (0.005 M)
7. S. + serine(0.002 M) + pyruvic acid (0.005 M) + pyridoxine (20γ/cc)
8. S. + serine (0.002 M) + pyridoxine (20γ/cc)



In the preceding experiment alanine, serine, and pyridoxine were ascertained to favor the growth of the myosin-adapted strain; in every case the growth of this strain was remarkably fostered in comparison with that of the control strain. This

fact corresponds with the increased serine-dehydrase activity of the myosin-adapted strain.

The addition of sodium pyruvate in combination with serine, alanine, and pyridoxine acted acceleratingly upon the growth of the both strains to nearly the same degree. Under the present experimental conditions the experimental results obtained were not perfectly in accord with the altered pyruvic dehydrogenase (FUJIWARA) and serine-transaminase activities of these strains.

DISCUSSION

In the above experiments the myosin-adapted, the muscle-adapted and the myositis strain were ascertained to be capable of ready utilization of serine and threonine, and to possess a highly-developed enzymatic system, namely, increased serine- (or threonine-) dehydrase and serine-transaminase activities.

As identical enzymes can work for both resolution and synthesis, it may be supposed that the reverse enzymatic reaction, namely, production of serine from pyruvate, is going on during the growing process of adapted strains. This reaction has a deep significance for the synthesis of bacterial protein, and further for the bacterial growth. Even if such a reverse reaction is in reality non-existent, the bacterium growing in the medium may still be considered to possess such reaction process as threonine $\rightarrow$ α -keto butyric acid $\rightarrow$ α -amino-n-butyric acid, because a large production of α -amino-n-butyric acid was paper-chromatographically demonstrated in spite of the relatively weak threonine-dehydrase activity revealed in the quantitative measurement of keto acid. In the experiment on staphylococcal growth, however, α -amino-n-butyric acid exerted no appreciable effects on myostrains and the control strain, and so this acid may be considered to have little significance for the synthesis of bacterial protein.

JANOFSKY stated that serine-dehydrase was identical with threonine-dehydrase. If both of these enzymes are really analogic, the reaction process (serine $\rightarrow$ pyruvate $\rightarrow$ alanine) is theoretically possible. As alanine is an amino acid indispensable to staphylococcal growth, the development of such a reaction process is very meaningful for staphylococcal growth and tissue-affinity. In this connection the present author carried out investigations into serine-transaminase first reported by SALLACH, and discovered that myostrains possessed a highly-increased transaminase activity.

LIEN and GREENBERG (1952) reported on the reaction process (threonine $\rightarrow$ α -amino-n-butyric acid), and stated that this process included two stages of reaction, namely, deamination and transamination.

FUJIWARA stated that in clarifying the frequent occurrence of myositis in the vitamin B₁ deficient individuals importance should be attached to the accumulation of pyruvate in skeletal muscle due to such deficiency and the increased pyruvic acid splitting enzyme activities, especially pyruvic dehydrogenase activity of the muscle-adapted strain.

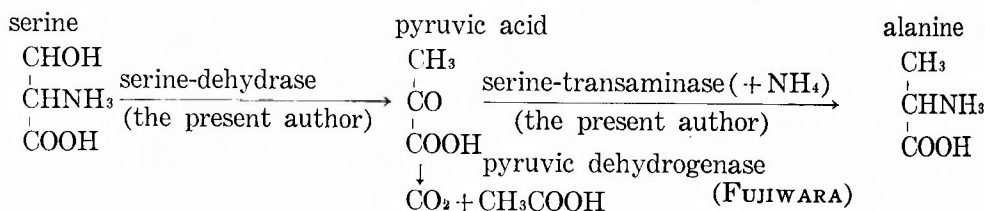
In the present investigation myostrains have been ascertained to possess increased serine-dehydrase and -transaminase activities, and the synthesis of alanine from

pyruvate has also been confirmed. It is therefore considered that skeletal muscle, especially in a vitamin B₁ deficiency condition, afford myostrains an environment quite suited for the synthesis of amino acids, or protein. This consideration connects OZAWA's vitamin B₁ deficiency theory on the occurrence of myositis with our muscle adaptation theory.

The investigation of the composition of the amino acids produced through resolution of myosin by each strain has shown that myostrains produce serine and threonine in larger quantities than other strains. This fact is worth a special attention, for this larger production on the part of myostrains is due to their original stronger tissue-affinity in which serine-dehydrase and -transaminase activities are not yet involved.

Although the change of serine into tryptophane, methionine and glycine was confirmed in *Neurospora* and *Escherichia coli*, it is said not to occur in the staphylococcus. It seems necessary, however, in order to determine whether or not these reaction processes exist, to recheck by using tissue-adapted strains.

Fig. 34 Enzymatic system which connects the vitamin B₁ deficiency theory with the muscle-adaptation theory.



CONCLUSIONS

The present author's co-workers have so far ascertained that the affinity of the staphylococcus for skeletal muscle is related not only with its peculiar enzymatic structure, but also with chemical environmental factors of tissue such as myosin-ATPase system, and ATP; and that myosin in particular has an important significance as a staphylococcal protein source. The present author, using various strains of staphylococci, conducted paper-chromatographical investigations chiefly concerning the relation between amino acid metabolism and muscle-affinity, and obtained the following results.

(1) Free amino acid compositions in rabbit skeletal muscle, and bone marrow were investigated. The skeletal muscle contains β -alanine, threonine, α -amino-n-butyric acid in large quantities; and the bone marrow aspartic acid, glutamic acid and leucine.

(2) The composition of amino acids produced through the resolving of myosin by each strain was investigated. The myositis and the myosin-adapted strain possess high enzymatic activity. They also produce serine and threonine in large quantities. As for the osteomyelitis strain, it produces α -amino-n-butyric acid in a relatively large quantity, though its production of threonine is small. The control

strain produces serine, threonine and α -amino-n-butyric acid only in small quantities.

(3) The utilization of amino acids in the synthetic medium by each strain was investigated. The myosin-adapted, the muscle-adapted and the myositis strain utilize serine and threonine particularly well, and in turn produce α -amino-n-butyric acid in a large quantity.

(4) With the addition of serine or threonine the myosin-adapted strain shows a mild growth and multiplication in the glycine-free SURGALLA's synthetic medium. Staphylococci of the control strain, on the contrary, decrease in number, and gradually disappear. The addition of glycine induces an excellent growth, especially of the myosin-adapted strain.

(5) ATP exerts rather inhibiting effects on the growth of each strain. It seems to inhibit the utilization of serine by myostrains. On the contrary, the utilization of threonine, especially by osteostrains, is promoted by it.

(6) The myosin-adapted, the muscle-adapted and the myositis strain possess rather increased serine-dehydrase activity, in comparison with other strains. As for threonine-dehydrase activity, it does not vary so much with strains.

(7) The myosin-adapted, and the myositis strain possess increased serine-transaminase activity, and accordingly produce alanine in large quantity.

(8) When alanine, serine, or pyridoxine is added to the SURGALLA's synthetic medium from which alanine has been removed, the myosin-adapted strain always shows a marked growth, in comparison with the control strain. The addition of sodium pyruvate in combination with alanine, serine or pyridoxine acts acceleratingly on the growth of the both strains to nearly the same degree.

In short, it has been ascertained in the present investigation that the myositis strain as well as the muscle- and the myosin-adapted strain is engaged in active utilization of serine and threonine; and that their serine-dehydrase and -transaminase activities are increased. In other words, strains of staphylococci with muscle-affinity, namely, myostrains such as muscle-adapted, myosin-adapted and myositis strains, have been experimentally demonstrated to possess a well-developed enzymatic system, and accordingly to be capable of producing pyruvate from serine, and utilizing pyruvate and serine for the synthesis of alanine indispensable to staphylococcal growth. This experimental result indicates the possibility of the utilization by these strains of pyruvate contained in large quantities in skeletal muscle, especially in vitamin B₁ deficiency condition, for the synthesis of amino acids needed for growth and building of bacterial protein. The present investigation has thus revealed an interesting fact which connects OZAWA's vitamin B₁ deficiency theory on the occurrence of myositis with our muscle adaptation theory.

The author wishes to express sincere gratitude to Dr. KOICHI ISHIGAMI, the instructor of our clinic, for many valuable suggestions and criticism throughout the present investigation.

REFERENCES

- 1) Akabori, S. : Koso Kenkyuho. I, II, III. Asakura shoten, Tokyo, 1957.
- 2) Alexander, N. & D. M. Greenberg : Studies on the biosynthesis of serine. *J. Biol. Chem.*, **214**, 821-837, 1955.
- 3) Awapara, J. : Application of paper chromatography to the estimation of free amino acids in tissue. *Arch. Biochem.*, **19**, 172-173, 1948.
- 4) Bellamy, W. D. & J. W. Klimek : Some properties of penicillin-resistant staphylococci. *J. Bact.*, **55**, 2, 153-160, 1948.
- 5) Cavallini, D., N. Frontari & G. Toschi : Determination of keto acids by partition chromatography on filter paper. *Nature*, **163**, 568-569, 1949.
- 6) Chargaff, E. & D. B. Sprinson : Studies on the mechanism of deamination of serine and threonine in biological systems. *J. Biol. Chem.*, **151**, 273-280, 1943.
- 7) Clegag, D. L. : Paper chromatography. *Analyt. Chem.*, **22**, 1, 48-59, 1950.
- 8) Densenbyo-Kenkyu-Gakuyukai : Saikingaku-Jissyu-Teiyo. Maruzen Co., Tokyo, 1958.
- 9) Dogura, I. : Enzymological studies on the pathogenesis of polymyositis and polyosteomyelitis, especially on the origin of the staphylococcal tissue-affinities. *Arch. Japan. Chir.*, **28**, 5, 1751-1765, 1951.
- 10) Egami, H. et al : Hyojun-Seikagaku-Jikken. Bunkodo, Tokyo, 1953.
- 11) Fischer, R. B., D. S. Parsons & G. A. Morrison : Quantitative paper chromatography. *Nature*, **161**, 764-765, 1948.
- 12) Fischer, R. B., D. S. Parsons & R. Holmes : Quantitative paper chromatography. *Nature*, **164**, 183, 1949.
- 13) Friedmann, T. E. & G. G. Haugen : Pyruvic acid, (1) Collection of blood for determination of pyruvic acid. *J. Biol. Chem.*, **144**, 67-77, 1942.
- 14) Fujiwara, N. : Experimental studies on the pathogenesis of acute suppurative polymyositis, especially enzymological supplementary investigation concerning vitamin B₁-deficiency theory (Ozawa). *Arch. Japan. Chir.*, **28**, 6, 2325-2350, 1959.
- 15) Gale, E. F. : Factors influencing the enzymatic activities of bacteria. *Bact. Rev.*, **7**, 139-173, 1943.
- 16) Gale, E. F. : The assimilation of amino acids by bacteria, VI. The effect of protein synthesis on glutamic acid accumulation and the action thereon of sulphathiazole. *J. Gen. Microbiol.*, **1**, 327-334, 1947.
- 17) Gale, E. F. : The chemical activities of bacteria. 3rd edition, London University Tutorial Press LTD, London, England. 1957.
- 18) Gale, E. F. & A. W. Rodwell : Assimilation of amino acids by bacteria, VII. The nature of resistance to penicillin in staphylococcus aureus. *J. Gen. Microbiol.*, **3**, 127-142, 1949.
- 19) Gale, E. F. & A. W. Rodwell : Amino acid metabolism of penicillin-resistant staphylococci. *J. Bact.*, **55**, 161-167, 1948.
- 20) Gale, E. F. & P. D. Mitchell : The assimilation of amino acids by bacteria, IV. The action of triphenylmethane dyes on glutamic acid assimilation. *J. Gen. Microbiol.*, **1**, 299-313, 1947.
- 21) Gladstone, G. P. : Valine to be inhibitory to leucine utilization, valine and threonine, threonine and serine. *Brit. J. Exp. Path.*, **20**, 189, 1939.
- 22) Hirano, I. : Experimental study on the pathogenesis of polymyositis (Analytical inquiry into the significance of myosin-ATPase system and ATP concerned with bacterial affinity for skeletal muscle). In press.
- 23) Holder, J. T., R. B. Wildmann & E. E. Snell : Growth promotion by keto- and hydroxyacid and its relation to vitamin B₆. *J. Biol. Chem.*, **191**, 559-576, 1951.
- 24) Ishigami, K. : Studies on the enzymatic action of penicillin-resistant staphylococci. *Arch. Antibiotics*, **5**, 1-2, 1-50, 1952.
- 25) Ishigami, K. & T. Masaki : Experimental studies on the pathogenesis of polymyositis and polyosteomyelitis. *J. Jap. Surg. Soc.*, **57**, 135-137, 1956.
- 26) Ishigami, K. & T. Masaki : On the acute suppurative myositis, especially on the pathogenesis of polymyositis. *The Saishin-Igaku*, **12**, 6, 1303-1313, 1957.

Fig. 3

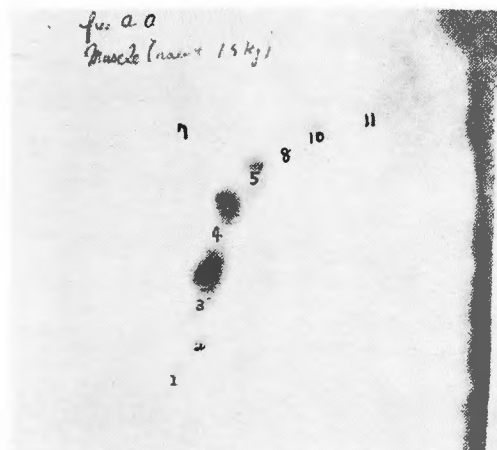


Fig. 4

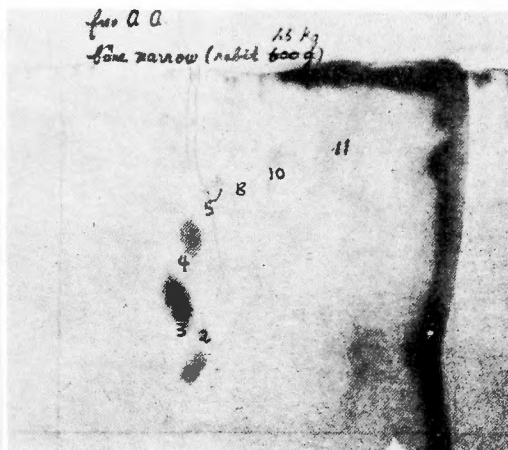


Fig. 5

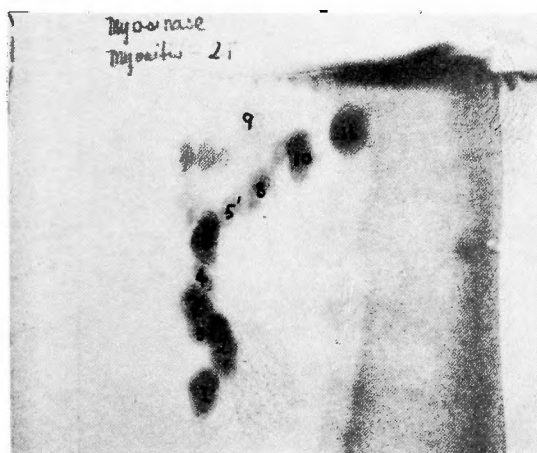


Fig. 6

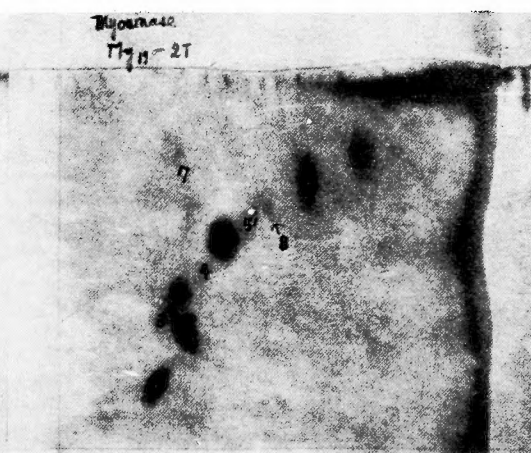


Fig. 7

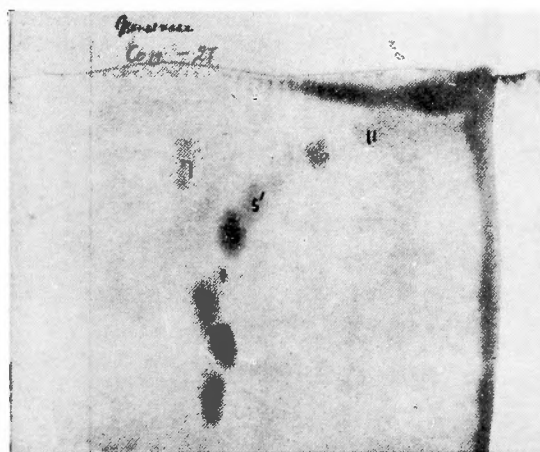


Fig. 8



Fig. 10

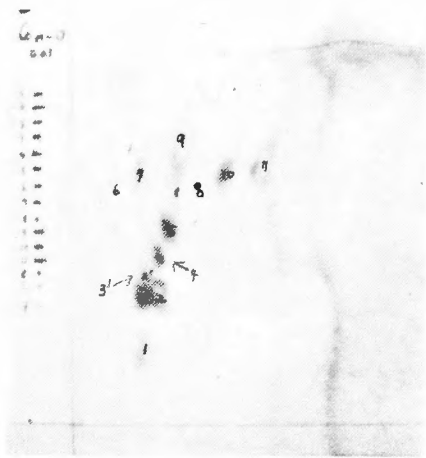


Fig. 11

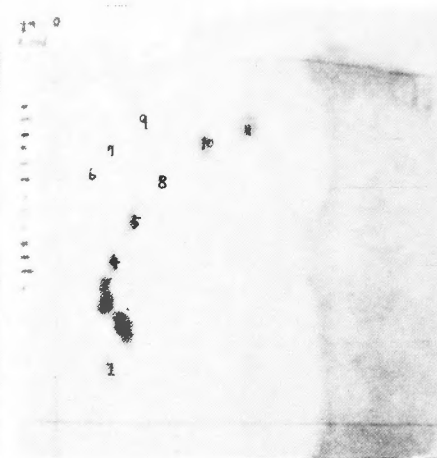


Fig. 12

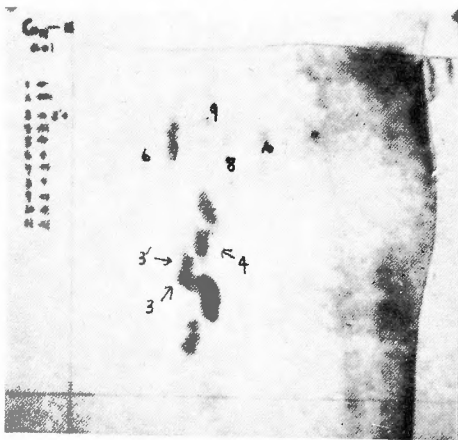


Fig. 13

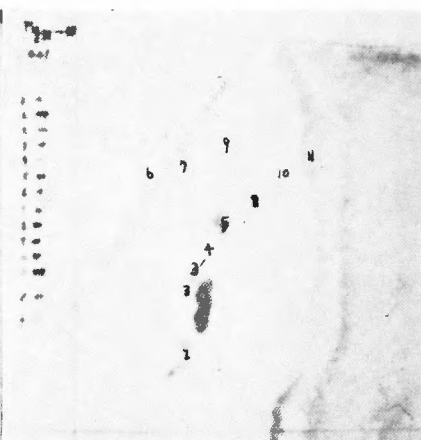


Fig. 14

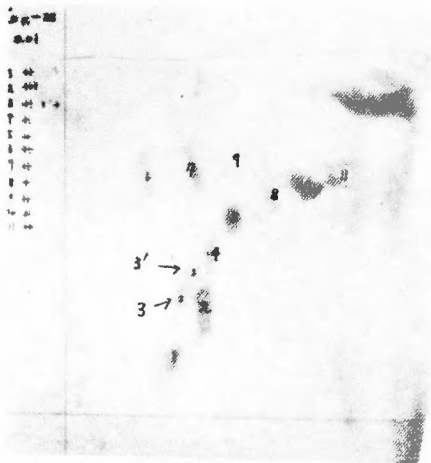


Fig. 15



Fig. 16

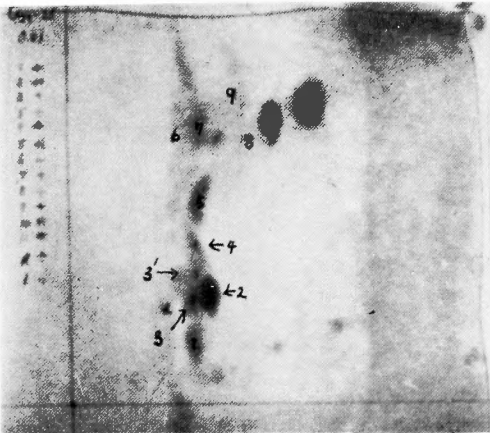


Fig. 17



Fig. 18



Fig. 19

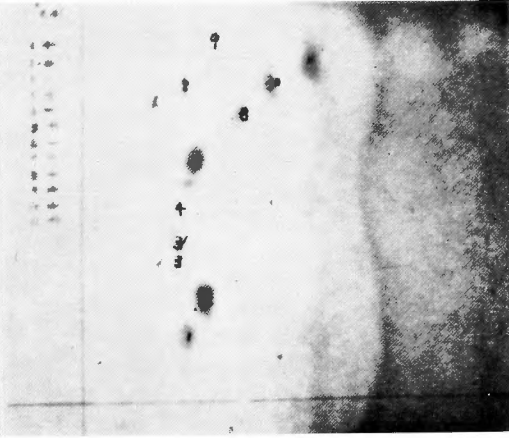


Fig. 20



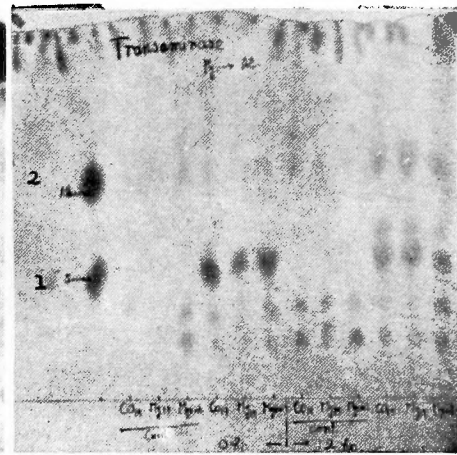
Fig. 21



Fig. 22



Fig. 32



A B C D E

EXPLANATION OF FIGURES

Part I. Paper chromatograms of free amino acids in rabbit skeletal muscle and bone-marrow.

1. aspartic acid, 2. glutamic acid, 3. serine, 3'. glycine, 4. threonine, 5. alanine, 5'. β -alanine, 6. histidine, 7. arginine, 8. α -amino-n-butyric acid, 9. proline, 10. valine and methionine, 11. phenylalanine and leucine

Fig. 3: Skeletal muscle.

Fig. 4: Bone marrow.

Part II. Paper chromatograms of amino acids formed through resolution of myosin by the myosin-adapted, control, myositis and osteomyelitis strains.

Fig. 5: By myositis strain.

Fig. 6: By myosin-adapted strain.

Fig. 7: By control strain.

Fig. 8: By osteomyelitis strain.

Part III. Paper chromatograms of amino acids in synthetic media in which tissue-adapted, control and pathogenic strains were cultured.

Fig. 10: Control strain (the 19th generation) cultured for 0 hour.

Fig. 11: Myosin-adapted strain (the 19th generation) cultured for 0 hour.

Fig. 12: Control strain (the 19th generation) cultured for 18 hours.

Fig. 13: Myosin-adapted strain (the 19th generation) cultured for 18 hours.

Fig. 14: Control strain (the 19th generation) cultured for 28 hours.

Fig. 15: Myosin-adapted strain (the 19th generation) cultured for 28 hours.

Fig. 16: Control strain (the 29th generation) cultured for 25 hours.

Fig. 17: Myosin-adapted strain (the 29th generation) cultured for 25 hours.

Fig. 18: Control strain (the 40th generation) cultured for 25 hours.

Fig. 19: Myosin-adapted strain (the 40th generation) cultured for 25 hours.

Fig. 20: Myositis strain cultured for 21 hours.

Fig. 21: Osteomyelitis strain cultured for 21 hours.

Fig. 22: Bone-marrow-adapted strain cultured for 21 hours.

Part IV

Fig. 32: Paper chromatogram showed serine consumption and alanine production by serine-transaminase of myosin-adapted, myositis and control strain.

1. alanine, 2. serine, A. pure amino acid (alanine and serine), B. control reaction (0 hour), C. main reaction (0 hour), D. control reaction (12 hours), E. main reaction (12 hours).

Co₁₉: control strain (the 19th generation)

My₁₉: myosin-adapted strain (the 19th generation)

Myosit: myositis strain

- 27) Ishigami, K. et al : On the pluralism of pyogenic staphylococci. *J. Jap. Surg. Soc.*, **56**, 574-575, 1955.
- 28) Kataoka, Z. : Experimental studies on the pathogenesis of infectious hepatorenal symptom complex. *Arch. Japan. Chir.*, **28**, 5, 1751-1765, 1959.
- 29) Kisliuk, R. L. & W. Sakami : A study of the mechanism of serine biosynthesis. *J. Biol. Chem.*, **214**, 47-57, 1955.
- 30) Kuwahara, S. : On the synthetic media of various bacteria. *The Saishin-Igaku*, **3**, 7, 337-344, 1948.
- 31) Lichtstein, H. C. & J. F. Christmann : The role of biotin and adenylic acid in amino acid deaminase. *J. Biol. Chem.*, **175**, 649-662, 1948.
- 32) Lien, O. G. & D. M. Greenberg : Chromatographic studies on the interconversion of amino acid. *J. Biol. Chem.*, **195**, 634-644, 1952.
- 33) Lien, O. G. & D. M. Greenberg : Identification of α -aminobutyric acid enzymatically formed from threonine. *J. Biol. Chem.*, **200**, 367-371, 1953.
- 34) Maeda, T. : 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. *Arch. Japan. Chir.*, **28**, 4, 1197-1220, 1959.
- 35) Masaki, T. : Experimental study on the pathogenesis of polymyositis and polyosteomyelitis, with special reference to the role of the altered staphylococcal tissue-affinities induced by adaptation to the environment where they developed, and to the role of the chemical constituents of bone-marrow. *Arch. Japan. Chir.*, **25**, 5, 464-494, 1956.
- 36) Meinke, W. W. & B. R. Holland : Threonine-serine antagonism in some lactic acid bacteria. *J. Biol. Chem.*, **173**, 535-545, 1948.
- 37) Meltzer, D. E. & E. E. Snell : Deamination of serine. I. Catalytic deamination of serine and cystine by pyridoxal and metal salts. *J. Biol. Chem.*, **198**, 353-361, 1952.
- 38) Meltzer, D. E. & E. E. Snell : Deamination of serine. II. D-serine dehydrase, a vitamin B₆ enzyme from *Escherichia coli*. *J. Biol. Chem.*, **198**, 363-373, 1952.
- 39) Mori, H. : Supplemental studies on amino acids in urine in cases of hepatic damage. *Arch. Int. Med.*, **2**, 99-108, 1945.
- 40) Myrwick, Q. N. & R. S. Weissner : Studies on antibacterial factors in mammalian tissues and fluids. I. A serum bactericidin for *Bacillus subtilis*. *J. Immun.*, **74**, 1, 9-16, 1955.
- 41) Nagai, T. : Physical chemistry of muscular construction. *Igaku Shoin*, Tokyo, 1956.
- 42) Nishino, Y. : On the statistical observation of acute primary suppurative myositis. *Hokuetsu Igk. Z.*, **48**, 8, 893-909, 1933.
- 43) Nishio, S. : On the statistical observation of acute suppurative myositis. *J. Jap. Surg. Soc.*, **23**, 307, 1922.
- 44) Ochoa, S. : Biosynthesis of ribonucleic acid. *Medicine of Japan* in 1959. **1**, 857-869, 1959.
- 45) Oota, T. : Saikingaku-Jikkensho. Hirokawa Shoten, Tokyo, 1959.
- 46) Ozawa, Y. : Über die Pathogenese der eitrigen Myositis. *Mitteil. d. Med. Gesellschaft zu Osaka*, **26**, 12, 3219-3305, 1927.
- 47) Sallach, H. J. : A symposium on amino acid metabolism. The Johns Hopkins Press, p. 782, 1955.
- 48) Satake, K. : Paper chromatography. *Kagaku no Ryoiki*, **3**, 7, 264-276, 1949.
- 49) Satake, K. : Paper chromatography in biological chemistry. *Kagaku no Ryoiki*, **4**, 8, 420-426, 1950.
- 50) Satake, K. : Chromatography. *Kyoritsu Shuppan Co.*, Tokyo, 1958.
- 51) Shibata M. : Practice of paper chromatography. *Kyoritsu Shuppan Co.*, Tokyo, 1958.
- 52) Stephenson, M. : Bacterial metabolism. 3rd edition, 1949, Longmans, Green & Co., London.
- 53) Snell, E. E. & B. M. Guirard : The inhibitory effects of glycine, serine, β -alanine and threonine on the utilization of alanine. *Proc. Nat. Acad. Sc.*, **29**, 66, 1943.
- 54) Waki, S. : Experimental studies on the mutation of staphylococci. *Zikken Igaku Z.* **27**, 5, 401-427, 1943.
- 55) Wood, W. A. & L. C. Gunsalus : Serine and threonine deaminase of *Escherichia coli* : Acti-

vators for a cell free enzyme. J. Biol. Chem., 181, 171-182, 1949.

56) Yanofsky, C. D-serine dehydrase of Neurospora. J. Biol. Chem., 198, 343-352, 1952.

57) Yanofsky, C. & J. L. Reissig: L-serine dehydrase of Neurospora. J. Biol. Chem., 202, 567-573, 1953.

和 文 抄 録

多発性筋炎ならびに骨髄炎の成因に関する実験的研究 (病原菌のアミノ酸代謝よりみた横紋筋親和性, とくに 横紋筋適応説とビタミンB₁欠乏説〔小沢〕との関連に ついて

京都大学医学部外科学教室第2講座 (指導: 青柳安誠教授)

西 野 正 弘

急性化膿性筋炎, 急性感染性骨髄炎はしばしば多発的に発生するが, この際病原ブドウ球菌がひとたび横紋筋または骨髄をおかすと, つづいて各菌はそれぞれ横紋筋または骨髄のみを撰択的に順次おかすことが知られている。この機作を解明するために, さきに教室真先は同一菌株から出発したブドウ球菌を上記各組織浸出液を加えた培地に継代培養し, それぞれの組織に対して親和性を有する適応菌を作製することに成功した。しかもこの適応菌は実験とは別に臨床的に筋炎或は骨髄炎の患者の炎症巣から直接分離された各起炎菌と類似した酵素構造を有するように変化することを明らかにした。そしてさらに, 教室土倉, 前田は横紋筋の有する Myosin-ATPase 系の化学的環境が, 横紋筋親和性を有する菌株の発育に対して大きな意味をもっていることを指摘した。また教室藤原は筋組織親和性を獲得した菌株では焦性ブドウ酸分解酵素能がとくに亢進していること, またビタミンB₁欠乏の際には焦性ブドウ酸が特に筋肉内に蓄積されることを証明し, ビタミンB₁欠乏動物の筋組織は, 筋組織親和性を有する菌株に対して, より好適な発育環境となりうるものであると結論し, 小沢の筋炎ビタミンB₁欠乏説の妥当性を強調した。

ところで以上の真先, 土倉, 前田, 藤原等一連の実験においては, 上記各組織浸出液について, それぞれの適応菌を作成したが, 著者はさらにその適応物質を解明する第一段階として, 筋の主要構成蛋白質であるミオシンを精製し, F. D. A. 209-P株を母菌株として

ミオシンに対する適応菌をも作成して, 実験に供した。

感染の前段階として, ブドウ球菌が増殖する際には, 各組織のアミノ酸, 更に組織蛋白質が菌体蛋白源として利用されることは当然考えられるが, 平野はミオシン適応菌および筋炎起炎菌についてミオシンが実際その菌体蛋白源として重要な意義を有することを明らかにしたので, 著者は各起炎菌, 各適応菌について菌発育に最も重要な意義を有するアミノ酸代謝の態度を比較検討し, 横紋筋親和性の本態を解明する為に実験を行い, 以下の成績を得た。

1) 家兎横紋筋および同骨髄の遊離アミノ酸組成に関しては, 横紋筋ではβ-アラニン, スレオニンおよびα-アミノ-n-酪酸が, 骨髄ではアスパラギン酸, グルタミン酸およびロイシンが多く含まれていることを知った。

2) 各菌株のミオシン分解によつて生ずるアミノ酸組成をしらべると, 筋炎起炎菌およびミオシン適応菌は旺盛な分解能を示し, また筋炎起炎菌およびミオシン適応菌では, セリン, スレオニンの産生が多く, 骨髄炎起炎菌ではスレオニンの産生が少ない割合に, α-アミノ-n-酪酸の産生が多く, 対照菌ではセリン, スレオニンおよびα-アミノ-n-酪酸のいずれの産生も少ないことを明らかにした。

3) 各菌株の発育に際して合成培地中のアミノ酸がいかに利用されるかを比較検討すると, ミオシン適応菌, 筋汁適応菌, 筋炎起炎菌などにおいては, セリ

ン, スレオニンがとくによく利用されており, 一方また α -アミノ-n-酪酸が多く産生されていることを認めた。

4) Surgalla合成培地組成中のグリシンを除いた培地においては, ミオシン適応菌はセリンまたはスレオニンが加えられると一応発育して, 菌も多少増加するが, これに反して, 対照菌では発育が行われないばかりか, 菌は次第に消失した。グリシンが加えられると, とくにミオシン適応菌は旺盛な発育を示した。

5) 各菌株の発育に対して ATP はむしろ抑制的に作用した。myostrain においてはセリンの利用は ATP の添加によつて抑制されたが, これに反してスレオニンの利用は ATP の添加によつて全般的に亢進し, とくに Osteostrain においてその程度が大であつた。

6) ミオシン適応菌, 筋汁適応菌および筋炎起炎菌においてはセリン・デヒドラーゼ能が他の菌株に比べてかなり亢進していた。これに反して, スレオニン・デヒドラーゼ能ははつきりした変化を示さなかつた。

7) ミオシン適応菌および筋炎起炎菌においてはセリン・トランスアミナーゼ能が対照菌に比べて亢進しており, したがつてこの反応によつて生ずるアラニンの生成量も大であつた。

8) Surgalla 合成培地中のアラニンを除いた培地においては, ミオシン適応菌はアラニン, セリン, ビリドキシンをそれぞれ加えたときに, いずれの場合に

においても対照菌に比べてより著明にその発育が促進された。一方焦性ブドウ酸ソーダとアラニン, セリン, ビリドキシンとをそれぞれ配合したときには, 両菌株の発育様相の間にはつきりした差異を認めることができず, そのうえいずれに対しても発育促進的であつた。

以上のように, 筋汁適応菌, ミオシン適応菌は勿論のこと筋炎起炎菌においても, それらの発育に際してはとくに培地中のセリンおよびスレオニンをよく利用しており, 一方またこれらの菌株においてはセリン・デヒドラーゼ能およびトランスアミナーゼ能が亢進していることが明らかになつたのである。すなわちセリンより焦性ブドウ酸を産生し, 一方焦性ブドウ酸とセリンよりブドウ球菌の発育に必要なアラニンを合成することが可能である酵素系が, 横紋筋親和性を示す菌株, すなわち筋汁適応菌, ミオシン適応菌, 筋炎起炎菌などの myostrain において亢進していることが実証されたわけである。このことは, これらの菌株が, 横紋筋, とくにビタミンB₁欠乏状態のそれにおいて多量に含まれている焦性ブドウ酸から, その発育に必要なアミノ酸であるアラニンを合成し, ひいては菌体蛋白を容易に合成しうることを示しているもので, こゝに著者は多発性筋炎の成因に関するビタミンB₁欠乏説(小沢)とわれわれの横紋筋適応説とをむすびつける興味ある成績を得たものと信ずるものである。