View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Okayama University Scientific Achievement Repository

Acta Medica Okayama

Volume 13, *Issue* 1 1959 *Article* 2

APRIL 1959

Studies on prevention of infection (II) The enzymologic traits and pro-tective ability of the fractions obtained from Sal. typhi by high speed centrifugation

Sakae Murakami[*] Tatsuji Yoshioka[†]

Yoshikazu Oka[‡] Yoshiyuki Matsuura[**]

[*]Okayama University,

[†]Okayama University,

[‡]Okayama University,

[**]Okayama University,

Copyright ©1999 OKAYAMA UNIVERSITY MEDICAL SCHOOL. All rights reserved.

Studies on prevention of infection (II) The enzymologic traits and pro-tective ability of the fractions obtained from Sal. typhi by high speed centrifugation*

Sakae Murakami, Tatsuji Yoshioka, Yoshikazu Oka, and Yoshiyuki Matsuura

Abstract

By physically destroying typhoid bacilli and centrifuging at a high speed, an insoluble granular fraction (P1) and soluble fraction (S1) were obtained. Chemical and enzymologic properties of these substances as well as their influences on the protective ability against infection were studied; and the following results were attained: 1. P1 contains an extremely small amount of proteins when compared with S1. 2. The enzymologic activity of P1 is entirely different from that of S1. In P1 the respiratory enzyme system of only lactate and succinate is localized. 3. Although both P1 and S1 possess the antibody-producing ability in serum of rabbit to the same high degree, P1 imparts to mice a markedly high protective ability against infection. 4. By the heat-treatment of P1 its antigenicity is lost at the same time.

*Copyright ©OKAYAMA UNIVERSITY MEDICAL SCHOOL

Acta Med. Okayama. 13, 15—26 (1959)

STUDIES ON PREVENTION OF INFECTION (II)

THE ENZYMOLOGIC TRAITS AND PROTECTIVE ABILITY OF THE FRACTIONS OBTAINED FROM *Sal. typhi* BY HIGH SPEED CENTRIFUGATION

Sakae MURAKAMI, Tatsuji YOSHIOKA, Yoshikazu OKA and Yoshiyuki MATSUURA

Department of Microbiology, Okayama University Medical School Okayama, Japan (Director : Prof. S. Murakami)

Received for publication, December 29, 1958

The methods for imparting the protective immunity *in vivo* can roughly be divided into two categories; namely the one by dead virulent bacteria[1–3], and the other by living non-virulent bacteria[4]. However, if the former method is supposed to owe its immunizing power to some components of bacteria bearing the virulence, then it would be difficult to explain the effectiveness of the immunity in the latter. Furthermore, the gram-negative bacilli, especially in the *Samonella* and *Shigella* groups, it is said that S form possesses a superior immunogenicity than R form ; and this fact indicates that the presence or absence of O-antigen has an important bearing on the protective immunity. That the protective antigen is chemically not a single substance is obvious from the fact the O-antigen (toxin within the body of bacillus) is identified as lipid-polysaccharides-peptides[5–7].

On the other hand, there is no concrete evidence to prove that the antigen-antibody reaction such as the agglutination reaction and the precipitation reaction as observable *in vitro* would play any role in the mechanism of the immunologic resistance[8]. DUBOS[9] likewise states that in whatever manner the toxin in the body of bacilli may be inoculated, the immune state of the animals is never high and does not last any length of time either. Despite this he contends that the precipitin values against the toxin can easily be attained in a high degree.

The recent advance in the enzyme chemistry is gradually elucidating the fine structures and the physiological significance of the bacterium. This has especially an important bearing on the heredity and bio-synthesis, but the report in which it is introduced into the immunologic phenomena

Produced by The Berkeley Electronic Press, 1959

is amost negligible.

As long as proteins play a leading role in the revelation of immunity, the authors are of the opinion that it must have an intimate relationship with the mechanism of biosynthesis. Therefore, in the present study of the protective immunity, the authors have investigated the role that will be played by enzyme active substances within the body of bacteria.

MATERIALS AND METHODS

Test strains. The test bacilli are all typhoid bacilli consisting of S 57, S 58, S 57-R, A-2, and A-63, the last two possessing Vi-antigen. S 57-R is the R form derived from S 57 in our laboratory.

After culturing on agar medium 20 to 24 hours, they are collected in the mortar and are ground hard (at O°C) with quartz sand one hour. To this thick paste 0.02 mol phosphate buffer solution (pH 7. 0) is added in the proportion of 2 ml to 1 g of the wet bacilli, and after stirring thoroughly, the mixture is centrifuged 30 minutes at 10000 rpm. This supernatant is again centrifuged at the high speed of 15000 rpm 30 minutes and then a transparent supernatant is obtained. This transparent supernatant is still further centrifuged at the high speed of 40000 rpm one hour, and the supernatant (hereafter abbreviated as S_1) and the precipitate (P_1) are separated. For the study of the enzyme activity and the antigenicity P_1 is diluted in ratio of 1 ml phosphate buffer solution to P_1 from 1g the wet bacilli; and S_1 as it stands is used as the standard.

The enzyme activity has been estimated by Warburg's apparatus. For the test animals normal mice weighing 15 to 18g are selected.

EXPERIMENTAL RESULTS

A. *The general chemical properties and enzymatic activity of* S_1 *and* P_1

S_1 is a light yellowish transparent substance and seems to be a soluble portion, While P_1 is a reddish brown substance and it belongs to an insoluble granular fraction. Their chemical properties are shown in Table 1.

In Table 1 the characteristic traits of S_1 and P_1 of A-2 are shown, and S_1 demonstrated both the Biuret and Molish reactions quite strongly and the amount of N is as high as ten times that of P_1.

Next, Table 2 shows the enzymatic activity of S_1 and P_1 in each

Table 1. Chemical Traits of Fractions

fraction	reaction		
	Biuret	Molish	Kyeldahl
S_1	##	##	N 600 mg/dl
P_1	±	+	N 60 mg/dl

S_1 is prepared by adding 0.02 mol phosphate buffer solution (pH 7.0) in proportion of 2 ml to 1 g of the original wet bacilli

P_1 is prepared by adding 0.02 mol phosphate buffer solution in the proportion of 1 ml to 1 g of the original wet bacilli

Table 2. Enzymological Characteristics (O_2-consumption) of S_1 and P_1 of Various Typhoid Bacilli

strain / fraction / substrate	S 57-R		S 57		S 58		A-63		A-2	
	S_1	P_1	S_1	P_1	S_1	P_1	S_1	P_1	S_1	P_1
endogenous	128	0	120	4	122	1	124	4	116	3
pyruvate	112	0	110	6	130	7	117	6	120	9
lactate	206	63	104	147	232	162	144	107	154	84
acetate	123	5	129	14	126	8	120	7	124	4
citrate	211	6	123	5	121	0	173	3	131	6
succinate	192	44	140	72	152	62	162	69	157	51
malate	144	6	136	3	99	2	137	4	127	5
glucose	167	3	132	5	136	3	134	1	141	3
catalase	##	±	##	±	##	±	##	±	##	±

Content: S_1 (P_1)···2 ml, substrate···0.3 ml of 0.1 mol substrate. To the above 0.7 ml of 0.02 mol phosphate buffer solution (pH 7.0) is added, so that the final volume will be 3 ml.

The estimated values are for the duration of two hours.

strain with addition of various substrates.

As shown in Table 2, S_1 of each strain shows a high endogenous respiration, making it difficult to judge the efficacy of the addition of substrate, and it is especially true of all S_1 with an exception of S 57-R, show a fairy marked oxidation of lactate, citrate, and succinate.

Now, looking at the activity of P_1, in all strains of typhoid bacilli lactate and succinate are specifically oxidized, and other substrates are not at all oxidized. And it is also interesting to note that catalase of every S_1 is strongly positive, while that of P_1 is almost negative. This result

is exactly identical with the result obtained by Millman[10] in his observations of tubercle bacilli.

B. *The antigenicity of S_1 and P_1*

In the first experiment, instead of attacking with the typhoid bacilli loaded with mucine, the protective ability against septicemia has been determined by inoculating a large dose of bacilli. Table 3 shows the result obtained in the groups of mice treated by the heat-killed S 57, S_1 or P_1 and later attacked by 1.5 mg of S 57.

Table 3. Comparison of the Protective Ability against Infection

immunization treatment	mouse groups									
contol	●	●	●	●	●	●	●	●	●	○
S 57 (heated one hour at 56 C) 5 mg	●	●	●	●	●	●	●	●	●	●
	●	●	●	●	●	○	○	○	○	○
S57……S_1 0.25 ml	●	●	●	●	●	●	●	●	●	●
	●	○	○	○	○	○	○	○	○	○
S57……P_1 0.25 ml	●	○	○	○	○	○	○	○	○	○
	○									

Attacked ip with 1.5 mg S57 strain two weeks after the inoculation

Namely, the order of their protective ability is the heat-killed bacilli $< S_1 < P_1$, and the protective ability of P_1 is especially of a high degree. In addition, S_1 likewise shows an ability as shown in Table 3, but this treatment brings about comparatively strong side effect. This seems to be due to a high concentration of proteins as already mentioned.

From these results it has become clear that P_1 of S 57 possesses a marked protective antigenicity. Therefore, with the purpose to study to what extent of dilution this might be efficacious, the protective ability of the mice has been observed by treating the animals with diluted P_1 of S 57, S 57-R in various multiples. One and half milligrams of S 57 is used for the attack. The results are shown in Table 4.

P_1 of S 57 demonstrates the protective power completely up to the 8-fold dilution, but P_1 of S 57-R does not at all show any effect against the attack of S 57. Of course, S_1 of S 57-R is ineffective, and the heat-killed S 57-R shows a slight effect.

Next, ten mice are treated with P_1 of S 58, and the five of them are attacked with S 57 as in the former case, while the other five are attacked

Table 4. Influences of P_1 and S_1 of the S-, R- form Strains on Protection agaist Infection

material for vaccination and doses	dilution multiples				
	1	2	4	8	16
S 57, P_1 0.25 ml	5/5	5/5	5/5	5/5	3/5
S 57, heat-killed bacilli 5 mg			(0/5)		
untreated control			(1/5)		
S 57-R, P_1 0.25 ml	0/6	1/5	0/5	0/5	
S 57-R, S_1 0.25 ml	0/5	1/5			
S 57-R, heat-killed bacilli 5 mg			(2/5)		
untreated control			(0/7)		

Attacked i p with 1.5 mg S57 strain 2 weeks affter treatment.
Denominator indicates number of mice attacked.
Numberator indicates number of mice survived.

with S 58. The results, as shown in Table 5, reveal that the animals can not resist against the attack of S 57, but they can protect themselves against the attack of the similar strain, S 58.

Table 5. Comparison of the Protective Ability against S 57 and S 58 in Mice treated with P_1 of S 58-Strain

materials for vaccination	dilution multiples	group attacked with 1.5 mg S 57-strain	group attacked with 1.5 mg S 58-strain
S 58	2	● ● ● ● ●	● ○ ○ ○ ○
P_1	4	● ● ● ● ○	
dose of 0.25 ml each	8	● ● ● ● ○	
untreated control		● ● ● ● ●	● ● ● ● ●

As the vaccine now commonly used contains Vi-antigen, P_1 of A-2 strain that contains this antigen has been prepared. Then P_1 is diluted in various steps up to 14, 336-fold dilution, and after treating mice with the diluted P_1, on the second week the animals are attacked with A-63 strain possessing Vi-tantigen and loading it with 5% mucine. The results are shown in Table 6.

It has been found that up to the 28-fold dilution P_1 shows a protective power against 10^4 MLD though slightly; against 10^3 MLD up to the 112-fold dilution completely; against 10^2 MLD as high as to the 1,792-fold dilution; and against 10 MLD it shows almost completely the protective ability up to the 3,584-fold dilution. In addition, against 1 MLD P_1 shows

Table 6. Estimation of the Protective Ability of P_1-A-2

dilution multiples of P_1 of A-2	14	21	28	32	42		
A-63 attack dose (MLD) 10^4	2/5	1/5	2/5	1/6	0/6		
dilution multiples of P_1. A-2	56	84	112	168	224	336	448
A-63. Attack dose (MLD) 10^3	6/6	4/6	6/6	4/6	4/5	2/5	1/5
dilution multiples of P_1. A-2	336	448	672	896	1344	1792	
A-63. attack dose (MLD) 10^2	5/6	6/6	6/6	4/5	3/5	3/5	
dilution multiples of P_1. A-2	896	1344	1792	2688	3584		
A-63. attack dose (MLD) 10	6/6	6/6	6/6	3/6	6/6		
dilution multiples of P_1. A-2	5288	7168	10576	14336			
A-63. attack dose (MLD). 1	6/6	6/6	5/5	3/4			
control attacked with 1 MLD	(1/9)						

Mice treated with variously diluted P_1 of A-2 were attacked with $1\sim10^4$ MLD of A-63 strain two weeks after the treatment.

Denominator signifies number of mice attacked.

Numerator indicates number of mice survived.

almost complete protection up to the 14,366-fold dilution.

The result of the study on the protective ability by the attack method of 10 LD_{50} is shown in Table 7.

Table 7. Evaluation of the Protective Aility by Attack Methods of LD_{50}

method of treatment	survivals	attacking strain
No. 1 A-2, P_1 at the dil. of 10^3 dose···0.5 ml	14/20	A-63
No. 2 A-2, P_1 at dil. of 10^4 dose···0.5 ml	12/20	A-63
untreated control	0/5	A-63

Denominator···number of test mice

Numerator···number of mice survived

As shown in the above table, each P_1 of A-2, diluted 10^3-fold and 10^4-fold, is inoculated to the groups of 20 mice each, and their protective ability against A-63 has been studied. Fourteen mice of the group treated with the dose of 0.5 ml of the 10^3-fold dilution survived, while 12 mice of the group treated with the 10^4-fold dilution were able to survive. In other words, in both groups over 50 per cent of the animals survived, showing a marked protecitive ability. This proves that the effective concentration of vaccine may be as dilute as 100-fold.

P_1 of A-2, as is clear from the above results, shows a marked protective antigenicity, the degree of efficacy of S_1 has been compared with the vaccine now sold in the market. The vaccine (A-58 Vi) bought in the common market is one year old; and the result is shown in Table 8.

Table 8. Protective Ability of S_1 and Vaccine sold in the Market

method of vaccination	dose of attack	survivals
0.5 ml of vaccine in market (A58 vi) diluted 7-fold No. 1	10^4	0/6
	10^3	3/6
	10^2	5/6
0.5 ml of S_1 of A-2 diluted 35-fold No. 2	10^4	0/6
	10^3	1/6
	10^2	4/6
untreated control	1	1/6

Denominator···number of test mice
Numerator···number of mice survived

As can be seen in Table 8, the vaccine obtained in the market shows a high protective ability against 10^2 MLD, but against 10^3MLD the ability is of an intermediate degree.

The mice treated with S_1 of A-2 diluted 35-fold show a lower protective ability than in the case of the mice treated with the market vaccine (since S_1 which contains a large amount of protein shows a strong side effect at the dilution around 7-fold, it is used at the 35-fold dilution). Therefore, the protective ability of S_1, when compared with that of P_1, is indeed trival.

Our next step is to see what influences heating will have on P_1 of A-2, which showed such a remarkable effect. Namely, P_1 of A-2 is divided into the 50-fold dilution and the 150-fold dilution, and each diluted solution is heated 30 minutes at 100 C or 56 C. The protective ability against infection in the mice treated with P_1 heated variously as above is shown in Table 9.

Namely, regardless of whether heated at 100 or 56 C, P_1 whose enzymatic activity thus being inactivated loses completely its protective ability.

P_1 of A-2 demonstrates, as mentioned above, a high degree of resistance against the bacilli possessing Vi-antigen, but the result of the study in which its reaction against *S. paratyphi B* which has different agntigen

Table 9. Effect of Heating on Protective Ability

method of vaccination		attack dose (MLD)	survivals
heat 30 min at 100 C	P_1 diluted 50-fold (0.5 ml)	10^2	0/5
		10	1/5
	P_1 diluted 150-fold (0.5 ml)	10^2	0/5
		10	0/5
heated 30 min at 56 C	P_1 diluted 50-fold (0.5 ml)	10^3	1/5
		10^2	1/5
	P_1 diluted 150-fold (0.5 ml)	10^3	0/5
		10^2	0/5
untreated control		1	0/5

P_1 used for vaccination is derived from A-2 strain.
the attack strain···A-63
Denominator···number of test mice
Numerator···number of mice survived

Table 10. Protective Ability of A-2 against *S. paratyphi B*

method of vaccination	attack dose	survivals
0.5 ml of P_1. A-2 diluted 84-fold	10	0/5
0.5 ml of P_1. A-2 diluted 112-fold	1	1/5
untreated control	1	0/5

structures is shown in Table 10.

As shown in Table 10, P_1 in the dilution of 84-fold and of 112-fold shows no protective ability at all, revealing a striking specificity.

The next step is to see the grades of the local responses in the case where the intra-cutaneous inoculation of respective S_1 and P_1 of living and dead A-2 bacilli is administered to normal rabbits. The result of this observation is illustrated in Table 11.

As can be understood from Table ll, the grades of responses to such inoculations are in the order to $P_1 > S_1 =$ dead bacilli $<$ living bacilli. In other words, in the case with living bacilli a marked response can be recognized even 5 days after the treatment, but in the case with dead bacilli and S_1 even after 5 days solidification can still be seen though slight. In contrast to these in the case with P_1 three days after treatment no local reaction can at all be observed. This is true in the case of the sub-

Table 11. Comparison of Intra-Cutaneous Responses in Rabbits treated with Various Substrates

days after inoculation		3 days		5 days	
inoculum	local responses / dose	sed. test	consoli-dation	sed. test	consoli-dation
living bacilli	0.1 mg	10 mm	##	15 mm	##
	0.01 mg	5	#	10	##
dead bacilli	0.1 mg	5	+	−	±
	0.1 mg	2	?	−	±
S_1	0.1 ml ot 10-fold dilution	4	+	−	±
	0.1 ml of 100-fold dilution	2	?	−	±
P_1	0.1 ml of 10-fold dilution	−	−	−	−
	0.1 ml of 100-fold dilution	−	−	−	−

Vaccines are all derived from A-2 strain.

cutaneous inoculation as well.

The final step is to find out what responses would mice present when they are treated with P_1 of A-2, and this is performed as a safety measure. Likewise a similar experiment has been conducted with S_1.

Table 12. Safety Tests of P_1 and S_1

inoculum / dilution	A-2. P_1				A-2. S_1			
4-fold	●	●	●	●				
5-fold	●	●	○	○				
6-fold	○	○	○	○	●	●	○	○
7-fold					○	○	○	○

Dose is 0.5 ml in each case.

When P_1 in the dilution up to 5-fold is given, it presents strong side effects, even proving to be fatal to some, but it is perfectly safe in the dilution of over 5-fold. However, S_1 is not safe even at the dilution of 6-fold, and it is only safe at the dilution over 7-fold.

Briefly, P_1 can impart a sufficient protective power to mice even as high a dilution as over 7-fold, while S_1 even at the concentration where side effect appears can give only a slight protective ability.

SUMMARY AND DISCUSSION

Immunity, the term commonly used today, gives an impression of including the entire range of the antigen-antibody reactions. Such phenomena as the resistance of host against pathogenic bacteria and antibodies being produced in the body of animal as specific responses against the injection of all kinds of antigenic substances are all interpreted as the results of the immunization treatment. Moreover, the aspect of antibody production against a pathogenic antigen is interpreted as the resistance against infection, and the same is true with respect to an antibody benefitial to the host or to a harmful antibody eliciting allergic conditions. Would this not be due to the fact that the interpretation of the fluid immunity is based on antibody as its core? On the other hand, cell immunity seems to depend upon phagocytosis but this does not always mean immunity[11,12].

Recently KOBAYASHI et al. state that in all likelihood the tissue mechanism is responsible for the protection against infection in experimental typhus of animals. This seems to mean the protection is not solely dependent upon fluid antibody. ANDO and his co-workers[2] likewise explain more concretely the opinion of KOBAYASHI et al.[13] in their mouse experiments with *S. enteritidis* and *S. cholerae suis*. Namely, they propose that the fractions such as mitochondria and microsomes are the cell factors controlling the susceptibility and resistance of a host.

The authors have pursued to see what active fractions of bacterial cells are most deeply involved in the protection against infection, but not from the standpoint of tissues of a host.

The reason that P_1 has much less side effect than S_1 seems to be due to its markedly low protein contents as already shown in Table 1. Furthermore, as shown in Table 2 from the fact that P_1 specifically oxidizes only lactate and succinate and possesses the electron transport system intact, P_1 has traits quite similar to mitochondria of animal cells. Those mice treated with this P_1 escaped death from septicemia due to the attack of a large dose of bacilli (Tables 3, 4, 5), but S_1 yielded far poorer results (Tables 3,). Whereas in the previous experiments both S_1 and P_1 demonstrated antibody in a high degree. In other words, this is an interesting fact in that antibody can be differentiated from the protective ability as two separate entities. In addition, the specificity of the protective ability is extremely marked; for instance, those mice treated by P_1 of S 58 or S 57-R could not endure the attack of S 57 given in a large dose (Tables 4, 5).

In order to clarify these preliminary experiments still further the

protective antigenicity has been studied with lethal dose. Mucine was added to all the bacilli used in attack. Namely, by diluting P_1 of the A-2 strain possessing Vi-antigen into various dilutions its protective power imparted to test animals was studied while being attacked by the A-63 strain (bacilli possessing Vi-antigen), and the results obtained are as excellent as shown in Tables 6 and 7. For example, the mice treated with P_1 diluted 224-fold could resist the attack of 10^3MLD in a high proportion; and even when treated with P_1 diluted as high as 672-fold they coud completely endure the attack of 10^2 MLD.

On the other hand, the protective power of the mice treated with S_1 of the A-2 strain was extremely low, even being inferior to the one year old vaccine sold in the market. It has also been demonstrated that Vi-antibody is formed in serum when rabbit is treated with this S_1. Therefore, to what extent the evidence proving the presence of Vi-antibody is associated with the protection against infection is open to question.

P_1, that seems to show enzymologically a characteristic activity and to have an intimate relation to the antigenicity, is inactivated by heating and this inactivated P_1 is inoculated to mice to see whether or not the mice would show any protective ability. As the result the ability has been found almost completely inactivated as shown in Table 6. This seems to suggest that the antigenicity has some relationship with the enzymologic activity of the antigen introduced. This point will be looked into in our future study. It is clear from Table 10 that the treatment with P_1 of A-2 imparts a strong protection against the A-63 attack, but the same treatment can not at all impart any protective power against the attack of *S. paratyphi B.* possessing different antigen structures. Again, the reaction at the site of inoculation is the mildest as shown in Table 11.

CONCLUSIONS

By physically destroying typhoid bacilli and centrifuging at a high speed, an insoluble granular fraction (P_1) and soluble fraction (S_1) were obtained. Chemical and enzymologic properties of these substances as well as their influences on the protective ability against infection were studied; and the following results were attained:

1. P_1 contains an extremely small amount of proteins when compared with S_1.

2. The enzymologic activity of P_1 is entirely different from that of S_1. In P_1 the respiratory enzyme system of only lactate and succinate is localized.

3. Although both P_1 and S_1 possess the antibody-producing ability in serum of rabbit to the same high degree, P_1 imparts to mice a markedly high protective ability against infection.

4. By the heat-treatment of P_1, its antigenicity is lost at the same time.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Dr. Anzai, Director of the Kitazato Research Institute for his generous supply of test bacilli.

REFERENCES

1. MITSUHASHI : Studies on the experimental typhoid. 1. A comparative study of live and killed vaccine against the infection of mice with Salmonella enteritidis. Saikingaku Zasshi (Japan. J Bact.). **13**, 323, 1958
2. ANDO : Studies on the nature of immunity in experimental typhoid, application of chrome vaccine in mouse typhoid. IV. Cellular factor in susceptibility and resistance, Japan. J. Exp. Med., **24**, 27, 1954
3. ANDO, TADOKORO, SHIMOJO : A new preparation Method of vaccine by chrome treatment. Igaku Tsushin (Medical Communications), **7**, 307, 1953
4. LI, C.: Studies on the dissociation of the hog cholera bacilus. III. Active immunization with R forms. J. Exp. Med., **50**, 767, 1929
5. GOWEN, W. T. J. & PARTRIDGE, S. M.: Studies in immunochemistry. 4. The fractionation aud nature of antigenic material isolated from Bact. dyseuteriae (Shiga). Biochem. J., **34**, 169, 1940
6. KUROYA & KOIZUMI: Studies on the endotoxine of Bact. typhoid. Tohoku-Ishi, **29**, 231, 1941
7. YAGI : Toxic Proteins, Iwanami Publish. Inc. (Tokyo), 38
8. LANDSTEINER, K.: rev. ed. Harvard Univ. Press, 324, 1944
9. DUBOS, R. J.: Biochemical Determinantion of Microbial Diseases, translated Ishida & Himura (in Japanese), 68, 1957
10. MILLMAN, I. & DARTEA, R. W.: Interacellular localization of enzymes in Mycobacterium tuberculosis var hominis. Prof. Soc. Exp. Biol. Med., **91**, 271, 1956
11. DURAN-RAYNALS, F.: The effect of extracts of certain organs from normal and immunized animals on the infecting power of vaccine virus. J. Exp. Med., **50**, 327, 1929
12. DURAN-REYNALS, F. & MURPHY, J. B.: Properties of the causative agent of a chicken tumor. 1. The specific fixation by tissues of susceptible animals. J. Exp. Med., **50**, 315, 1929
13. KOBAYASHI, & USHIBA, Studies on the Immunity of the experimental typhoid. I. A review of experiments carried out in our laboratry during the past twenty years. Keio J. Med., **1**, 35, 1952