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Q VALUE IN BACTERIAL HEMAGGLUTINATION

BY

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ABSTRACT

A series of thermodynamic studies on the mechanism of bacterial hemagglutination and on the inhibition of this reaction by mannose were carried out by the use of twin conduction microcalorimeter.

Interaction between erythrocytes and aldoses used in the present study yielded practically no significant Q value. On the contrary, of the five aldoses tested, four aldoses (D-glucose, D-galactose, D-mannose and D-xylose), the fifth being L-arabinose, were observed to yield exothermic heat by interaction with all strains of *Shigella flexneri,* irrespective of the presence or absence of fimbriation. Of particular note, however, is that D-mannose reacted with the fimbriate strains, yielding a significant Q value qualitatively peculiar and characteristic of an exothermic interaction.

Monosaccharide absorption studies on the cells of *Sh. flexneri* or on the red blood cells revealed that D-mannose was intensely absorbed by only the fimbriate strains of *Sh. flexneri.*

The Q value yielded by the interaction between the non-fimbriate strain and mannose is of the exothermic type, whereas that observed by that between the fimbriate strain and mannose is conversely of the endothermic type. And the reaction of D-mannose with the purified fimbriae is characteristically of the endothermic type, and such was also true in the reaction system with the purified fimbriae and the erythrocytes.

The fimbriate strains of *Sh. flexneri* reacted with mannan, polymer of mannose, to exhibit agglutination of the bacterial cells (Shigella-Mannan agglutination), yielding a Q value of the endothermic type, and this type of agglutination was intensely inhibited by D-mannose.

It has been suggested from the results that the mannose molecule would be situated as the terminal sugar at the binding sites or receptor on the surface of the erythrocytes, and the fimbriae of the mannose-sensitive type interact with this receptor.

INTRODUCTION

A number of studies published demonstrate that the agglutination of

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the erythrocytes by some strains of *Shgella flexneri* occurs as a characteristic phenomenon mediated by the pericellular filamentous appendages of the organisms, called fimbriae or pili (Duguid, Smith, Dempster and Edmunds[1]; Duguid and Gillies[2]; Brinton[3] and Hashimoto et al.[4]). However, our knowledge about the physico-chemical properties of the active factors associated with these filamentous structures and those of erythrocytes, contributing to the bacterial hemagglutination, as well as the mode of interaction between these factors is still sparse. In view of this, a series of studies were conducted in this laboratory to attempt to theoretically elucidate the underlying thermodynamic mechanism whereby *Sh. flexneri* agglutinates the erythrocytes of the guinea pig. The results indicate the reversible property of bacterial hemagglutination (Takada[5]), and the agglutination of the guinea pig erythrocytes with the fimbriate strain of *Sh. flexneri* proceeds as an exothermic reaction yielding the amount of heat equivalent to 12.3 Kcal. per mole (Yaoi[6]).

In the study herein described, thermodynamic measurements of the Q value were made using a microcalorimeter to clarify any inhibitory effect of mannose on the bacterial hemagglutination by the cells endowed with the fimbriae of mannose-sensitive type and examine the interactivity between mannose and the active factors involved in this type of hemagglutination.

It has been demonstrated that in bacterial hemagglutination the bacterial species or strains insensitive to the inhibitory action of mannose can be distinguished from those species or strains which are susceptible to its action, the fimbriae of the former being called mannose-resistant (MR type) and those of the latter being designated mannose-sensitive (MS type). These findings suggest the important relationship of mannose to the bacterial hemagglutination mediated by the fimbriae of the MS type. It was considered interesting, therefore, to determine at least which factor or factors of the fimbriae or the erythrocytes might react with mannose, so that, by measuring the Q value yielded in the systems involving various combinations of mannose and bacterial cell, and of mannose and red blood cell, the mechanism of the bacterial hemagglutination would become clearly known from the thermodynamic point of view.

Moreover, in the course of the experimental studies, mannan, a polymer of mannose, was found to be capable of causing agglutination of the fimbriate cells of *Sh. flexneri* and that the mannan-induced agglutination of the bacterial cells was observed to be inhibited in the presence of D-mannose. Microcalorimeteric analysis of the underlying mechanism was carried out, accordingly, by comparing the bacterial hemagglutination and the

agglutination of the bacterial cells by mannan.

Materials and Methods

Bacterial strains: Heart infusion broth cultures of four strains of *Sh. flexneri* group B, after incubation for 24 hours at 37°C., were spun down at 6,000 g for 30 minutes to collect the bacterial cells. After being washed twice with sterile physiological saline, the cells were resuspended in saline and the concentration was adjusted with saline to an optical density of 0.5 at a wave length of 660 nm. Unless otherwise mentioned, the suspensions of viable cells were used in the study. Wherever the suspensions of killed bacterial cells in saline were required, the heart infusion broth cultures were heated at 120°C. for ten minutes before the preparation of bacterial suspensions.

The four strains of *Sh. flexneri* were Reference Numbers Sh-1, Sh-3, Sh-4, and Sh-X, respectively, and of these strains, two (Sh-1 and Sh-3) were of the serotype 1a, one (Sh-X) was serotype 1c, and one (Sh-4) was serotype 2a. The strain Reference No. Sh-4 was devoid of fimbriation while all the rest were demonstrably capable of agglutinating the red blood cells and, on electron microscopic examination, possessed peritrichous fimbrial structures. From Ref. No. Sh-1, a variant strain incapable of agglutinating the erythrocytes was obtained through a serial cultivation of more than ten subcultures on heart infusion agar. The variant selected proved to be stable as long as it was maintained by serial cultivation on heart infusion agar; it consistently failed to demonstrate hemagglutination with the red blood cells of the guinea pig and lacked any demonstrable fimbriation. In the present study the prototype of this strain (Ref. No. Sh-1), fimbriate and capable of agglutinating the erythrocytes, was designated as the HA^+ strain to distinguish it from the non-fimbriate variant which will hereinafter be referred to as the HA^- strain.

Preparation of purified fimbriae: According to the methods described originally by Brinton et al.[7] and later modified by Takada[5], a suspension of the strain Sh-1 HA^+ in physiological saline was agitated by means of the Waring Blendor to mechanically remove the fimbriae, and the mixture was centrifuged, followed by multiple cycles of extraction of the supernatant by salting out with the 0.1 M $MgCl_2$ solution to obtain a purified preparations of fimbriae.

Erythrocytes: Guinea pig and bovine red blood cells were used. The guinea pig blood was washed three times with physiological saline and the washed cells were resuspended in saline at a concentration of either one

per cent for use in the observation of hemagglutination with *Sh. flexneri* or at a concentration of 750,000 RBC's per cubic millimeter which was found to be optimal for the calorimetric measurement of the Q value (Takada[5] and Yaoi[6]). Bovine erythrocyte suspensions in physiological saline were prepared in the same manner but using the defibrinated blood.

Sugar solutions: Solutions of five different aldoses, three aldohexoses (D-mannose, D-glucose, and D-galactose) and two aldopentoses (D-xylose and L-arabinose), were used. Concentrations of the sugar solutions were in all instances 0.1 M in physiological saline and the solutions were sterilized individually by filtration through the Millipore filters (pore size 0.45 μm, type HA). Solutions in saline of three polysaccharides (mannan, xylan and dextran), at a concentration of w/v per cent and sterilized by filtration in the same manner, were also employed.

Absorption of monosaccharides: Determinations of bacterial and erythrocytic absorption of three monosaccharides (mannose, galactose and xylose) were performed. To assay the monosaccharide uptake by the bacterial cells, the cell suspensions, after thorough washing with saline to completely eliminate the sugars from the culture media, were centrifuged, and 0.3 g per ml (as moist cell weight) of the resultant sediment was added to 5 ml of sugar solution. To determine the sugar uptake by the red blood cells, on the other hand, a sugar solution, 120 μg sugar per ml, was added to an equal volume of erythrocyte suspension containing 750,000 cells per cu. mm. The sugar-cell mixture was agitated at room temperature for ten minutes to allow for the reaction to occur, followed immediately by centrifugation and filtration through the Millipore filter to eliminate all cell particles.

The filtrate, in 1 ml aliquots, was poured into the test tubes and examined calorimetrically by the phenol-sulfuric acid reaction method to determine the content of the sugar remaining in the samples, using a calibration curve established by preliminary assays of known standard solutions. The amount of sugar absorbed by the bacterial cells or the red blood cells was determined accordingly.

Bacterial hemagglutination and Shigella-polysaccharide agglutination: A half ml of a bacterial suspension at a concentration of 0.5, O.D. 660 nm and 0.5 ml of 1% RBC suspension were pipetted into the wells of a hemagglutination plate, and the mixture was rotated for five minutes horizontally at the rate of 60 rev/min. The mixture in the wells was then examined for agglutination.

Slide agglutination technique was also used to detect the reaction of *Sh. flexneri* with the polysaccharides. One drop of a bacterial suspension (at

an O.D. of 0.5 at 660 nm) and that of a polysaccharide solution containing 5 mg of sugar per ml were placed on a glass slide and mixed together and a macroscopic observation of the agglutination reaction was made immediately. In this segment of experiments, yeast mannan, xylan and dextran (products of Tokyo-Kasei K.K., Tokyo, Japan) were used.

Inhibition of Shigella-mannan agglutination by monosaccharides: Serial two-fold dilutions of a 0.1 M monosaccharide solution in physiological saline were prepared, and they were poured in 0.5 ml aliquots in a series of test tubes, followed by the addition of 0.25 ml of bacterial suspension containing a specified concentration of cells in each tube. The tubes containing the mixtures were allowed to stand at room temperature for ten minutes. A quarter of a ml of the mannan solution, 10 mg per ml, was then added to each tube and the mixture was agitated sufficiently. The tubes were then allowed to stand at room temperature for 24 hours before reading for inhibition of agglutination.

Determination of Q value: The calorimetric measurement was conducted by the use of a twin conduction calorimeter (model: RCM-1N, manufactured by Institute of Applied Electricity, Tokyo). Cuvettes were thoroughly rinsed with distilled water, soaked in 70 per cent ethanol for sterilization, and finally rinsed with sterilized physiological saline immediately prior to use to avoid contamination by other microorganisms. In addition, attempts were also made to maintain an intracuvettal temperature constant by circulating water continuously around the cuvette to stabilize the base line.

Subsequently, the standard Q value was determined. After placing the reacting agents separately into two cells of one cuvette, in volume of 5 ml in each cell (one cuvette serving as reagent blank), they were allowed to stand for 10 to 15 minutes for equilibration of the intracuvettal temperature. Then an electrical current was passed for a certain unit of time to record the Joule heat thereby generated as a corresponding electromotive force, which being taken as the standard Q value.

Upon confirmation of the base line and the ensuing measurement of the standard Q value, the calorimeter was turned 130°, and the two reacting agents poured into the cells of each cuvette were combined, mixed thoroughly and allowed to stand for reaction for a specific length of time. The consequently recorded temperature difference in the time course was converted to a value in weight units to institute comparison with the standard Q value for every reaction. The Q value thus determined was expressed as the value per mole in the case of the monosaccharide solutions, or per number of the bacterial cells or of the erythrocytes, or per amount of nitrogen in mg of purified fimbriae, respectively.

RESULTS

Exothermic Reaction of Erythrocytes with Aldoses

Table 1 summarises the Q values obtained in regard to the reaction systems comprising the respective combinations of the five aldoses with the erythrocytes of the guinea pig or cattle. The aldoses fall under the category of either group 2 or group 3 according to the classification described by Mäkelä[8] (Fig. 1); they have been shown to be capable of specifically inhibiting the agglutination of the erythrocytes of the domestic and laboratory animals by the lectins (Boyd et al.[9-11]).

As seen from the table, all the sugars tested including D-mannose, which was known to be capable of inhibiting hemagglutination by the fimbriate strains, were found to react only so slightly with the erythrocytes of the guinea pig, as the Q value was as low as about −10 cal. per mole, thus indicating that the slight reactions involved were invariably of the exothermic or thermogenic type. D-mannose has similarly proven to react with

Table 1. Q values by reaction of aldoses withred blood cells

Aldose	Q values yielded with red blood cells of	
	Guinea pig	Cattle
d-Mannose	0 cal	−2∼3 cal
d-Gleucose	−10 〃	−12 〃
d-Xylose	−10 〃	−12 〃
l-Arabinose	−10 〃	− 1.2 〃
d-Galactose	−11 〃	−12 〃

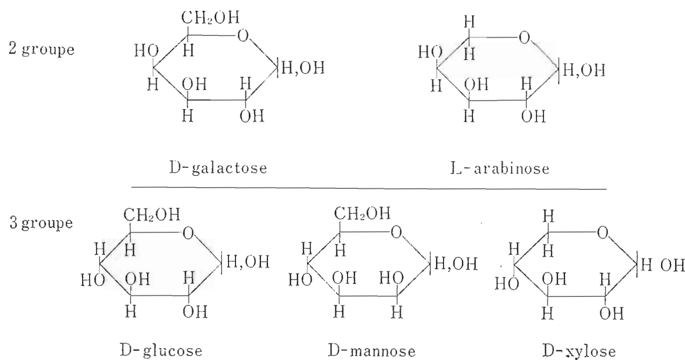

Fig. 1. Configuration of aldose to be tested.

the bovine erythrocytes, which have been demonstrated to fail to agglutinate even with the fimbriate strains (Duguid et al.[2]; and Hashimoto et al.[4]), yielding only −2 to −3 cal. per mole. All the remaining aldoses were also observed to react with the bovine red blood cells with the Q value being only −12 cal. per mole at the most.

It seems to follow from these findings that none of the monosaccharides therein studied reacted with the guinea pig or bovine erythrocytes to yield any significant Q value measurable with a microcalorimeter.

Q Value in Reaction Between Aldoses and Fimbriate or Non-fimbriate Strain

Q value in the reaction of aldoses with fimbriate strain Sh-1 and with non-fimbriate strain Sh-4 were measured microcalorimetrically, and the results obtained are summarized in Table 2.

Of particular note in regard to the results is the conspicuous difference between the fimbriate and non-fimbriate strain in the pattern of reaction with D-mannose. A considerably higher exothermic Q value of −264 cal. per mole was noted to result from the reaction between D-mannose and the fimbriate cells, which are capable of agglutinating the red blood cells. Furthermore, D-glucose, D-xylose and D-galactose were also observed to react with the fimbriate cells used, yielding rather greater Q values of the exothermic type ranging from −305 to −332 cal. per mole. These results indicate that it is not D-mannose alone which react with the fimbriate strains of *Sh. flexneri*. As shown in Fig. 2, however, a fairly distinct difference can be noted to exist between mannose and each of the other sugars with respect to the length of time required for attaining the peak levels of heat production.

That is to say, peak levels were accomplished with D-glucose, D-xylose or D-galactose after more than 30 minutes had elapsed, whereas with D-mannose the peak level was reached within a few minutes after the reaction

Table 2. Q values by reaction of aldoses with bacterial cells of *Sh. flexneri*

Aldose	Fimbriate cells		Non-fimbriate cells	
	Q value	Time (min)	Q value	Time (min)
d-Mannose	−264 cal	few	0.0cal	70
d-Glucose	−329 〃	30	−545 〃	70
d-Xylose	−305 〃	40	−156 〃	35
l-Arabinose	− 5.5 〃	8	0.0 〃	70
d-Galactose	−332 〃	40	−1.270 〃	70

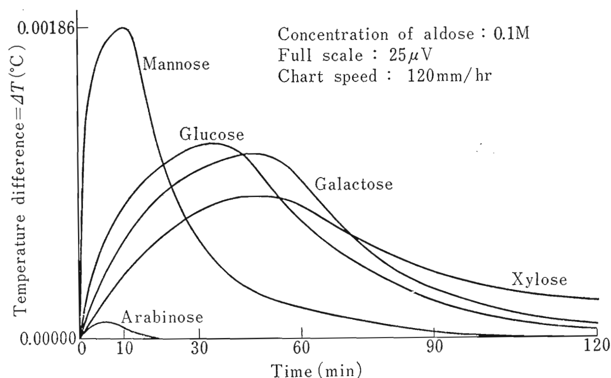

Fig. 2. Temperature difference-time curve by reaction of aldoses with fimbriate strain.

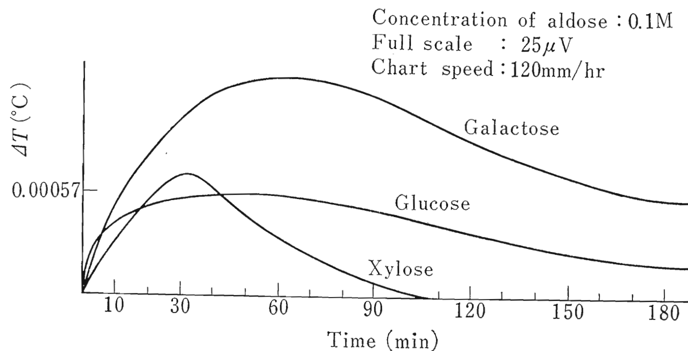

Fig. 3. Temperature difference-time curve by reaction of aldoses with non-fimbriate strain.

had been initiated. Calorimetric measurements failed to reveal any significant Q values from the reaction of Sh-1 with L-alabinose, however.

When the non-fimbriate cells of Sh-4, lacking the ability of agglutinate the erythrocytes, were allowed to react with D-glucose, D-xylose and D-galactose, considerably high exothermic Q values were recorded while the mixture of L-arabinose with the non-fimbriate cells yielded no significant amount of heat (Table 2). The reaction curves are shown in Figure 3. As may be seen, an exothermic Q value as much as −1,270 cal. per mole was recorded in the reaction of Sh-4 with D-galactose, exhibiting a fairly low-pitched slope of the reactoin curve and requiring 70 minutes to attain the peak level of the exothermal reaction. By contrast, the mixture of Sh-4 with D-mannose, which proved to yield a notably greater Q value in the reaction with the fimbriate cells, did not yield any significant detectable amount of Q value at all; hence a remarkable difference was noticed be-

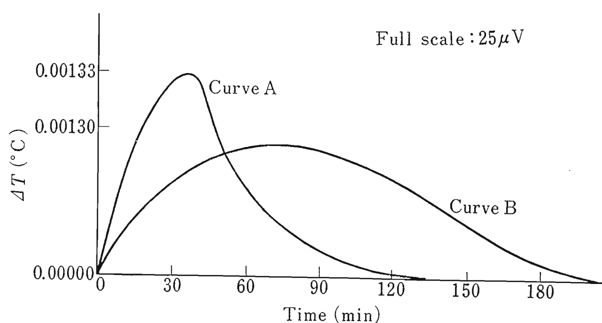

Fig. 4. Temperature difference-time curve by reaction of bacterial cells with red blood cells of guinea pig. Curve A represents the reaction of the fimbriate strain and curve B shows the reaction of non-fimbriate variant of the former strain.

tween the fimbriate strain and the non-fimbriate strain as to the production of heat in the reaction with D-mannose.

In association with the experiment just described, the Sh-1 strain (HA^+) was compared with its non-fimbriate variant, (HA^-) strain, to determine whether a similar difference in the reaction with D-mannose exists. The results obtained are shown in Figure 4. The reaction between the fimbriate Sh-1 (HA^+) strain and D-mannose yielded exothermic Q value of −264 cal. per mole, and displayed a sharply pointed peak about 5 to 10 minutes after the initiation of the reaction while the reaction between D-mannose and the non-fimbriate Sh-1 (HA^-) strain showed a gently sloping curve characteristic of an exothermic type of reaction *in toto* with a Q value of −420 cal. per mole. This finding appeared to suggest a substantial difference in the pattern of reaction between the fimbriate strain and the non-fimbriate variant of Sh-1. Therefore, it is likely that difference might exist between the originally fimbriate strain and the non-fimbriate variant derived from the fimbriate strain as to the reactivity against D-mannose.

Whatever the difference in reactivity, it may be deduced from the results that D-mannose reacts with the fimbriate cells at a fairly high rate (of a few minutes) with a consequent release of a considerably large amount of heat whereas the non-fimbriate cells are practically devoid of such exothermic reactivity. With the exception of L-arabinose, the three other monosaccharides were observed to react with either strain, invariably yielding considerably a large amount of heat though the reaction proceeded more slowly; the finding seemed to indicate that these sugars react rather non-specifically with the bacterial cells regardless of the presence or absence of fimbriation.

Table 3. Quantitative absorption test of monosaccharides by bacterial cells of *Sh. flexneri* or red blood cells of guinea pig

Monosaccharides	Amount of monosaccharides absorbed by		
	Fimbriate cells*	Non-fimbriate cells*	R. B. C
Mannose	45 γ/ml	0	0
Galactose	5	0	0
Xylose	0	0	0

* Absorbing doses: 0.3 g (in wet weight)

Absorption of Monosaccharides by Bacterial Cells or Erythrocytes

To determine which of the two principal factors involved in bacterial hemagglutination might react with mannose, which has been suggested as acting specifically upon the fimbriae in the foregoing thermodynamic analysis, absorption of monosaccharides by each of the cellular principles was studied.

Table 3 shows the results of the assay for residual monosaccharides in the supernatants of the reaction mixtures, comprising the sugar solution with each of the agglutinating facters added to it, and accordingly of the assay for the monosaccharide uptake by the agglutinating factors. As seen from the table, only 5 mcg of D-galactose per ml were absorbed by the fimbriate cells while the amount of D-mannose taken up by the same fimbriate cells was 45 mcg per ml. These cells failed to absorb any significant amount of xylose, however. None of the sugars tested was found to be absorbed by the non-fimbriate cells. Erythrocytes similarly failed to absorb any of the monosaccharides. These results seem to provide an indirect evidence for the specific reactivity of D-mannose with the fimbriate strains of *Sh. flexneri*.

Q Value by Reaction Between Fimbriae and Erythrocytes.

Calorimetric measurement of the Q value revealed a considerable exothermic production of heat even by the reaction between the non-fimbriate strain of *Sh. flexneri* and the erythrocytes, the combination being known to show no agglutination at all (Nakajima[12]). Then, it was considered necessary to determine whether any significant Q value might be obtained from the reaction of the fimbriae themselves with the surface of the red blood cells, which are thought to be the genuine cause of hemagglutination, and, if at all, to determine the type and mode of heat production. As a result of making the purified fimbriae react with the guinea pig erythrocytes, the

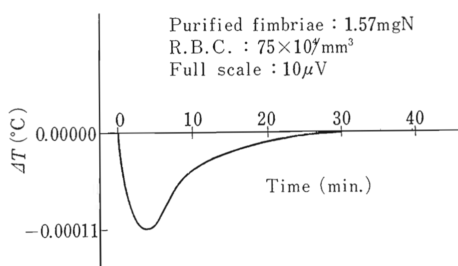

Fig. 5. Temperature difference-time curve by reaction of purified fimbriae with guinea pig erythrocytes. Q value is estimated to be 0.024 cal. per 25 mg N.

Table 4. Q values by reactions of purified fimbriae with red blood cells

Red blood cells of	Q value
Guinea pig	+0.024 cal/mgN
Cattle	0.000 〃

time-course curve of temperature difference shown in Figure 5 was recorded.

It is evident from these results that the fimbriae of *Sh. flexneri* react remarkably fast with the guinea pig erythrocytes exhibiting a considerably profound endothermic Q value of +0.024 cal. per mg N of fimbriae. By contrast, no Q value of significance could be estimated from the reaction between the purified fimbriae and bovine erythrocytes. When the preparations, obtained by the treatment of the non-fimbriate strains in the same purification procedure, were allowed to react with the guinea pig erythrocytes and with the bovine erythrocytes, none of the combinations yielded any traceable Q value. These findings, therefore, were considered to provide direct evidence for the specific reactivity of the bacterial fimbriae to the red blood cells. To confirm these findings, the following experiments were carried out.

Figure 6 illustrates the calorimetric measurements of the time-course of temperature difference in the reactions carried out under the conditions permitting hemagglutination and under those not permitting it, using the same samples. When the red blood cells were incubated together with a suspension of the fimbriate strain (Sh-1 HA^+) in a 0.1 M mannose solution, a curve characteristic of the exothermic reaction with a Q value of −1.442 cal. was obtained, particularly at an erythrocyte concentration of about 4×10 cells per ml. An exothermal reaction curve comparable to it was also

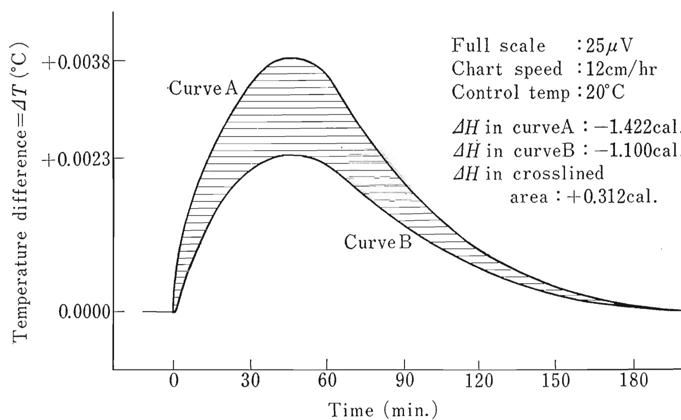

Fig. 6. Temperature difference-time curve by reaction of bacterial suspension of *Sh. flexneri* with suspension of red blood cells of guinea pig. Curve A represents the reaction of fimbriate strains [Sh–1 (HA^+)] with mannose, and curve B the reaction of fimbriate strains [Sh–1 (HA^+)] without mannose. Both strans were used in a concentration of O.D. 660 nm 0.5, and the concentration of RBC was 375×10^7 cells/ml. The horizontal-line area is assumed to be the true Q value of bacterial hemagglutination.

observed with the same reaction mixture minus the mannose, thereby permitting hemagglutination, wherein a lower Q value was obtained, Q value being −1.100 cal. with the same concentration of erythrocytes. The balance, +0.312 cal., is considered to present the Q value generated through the union of the bacterial fimbriae with the erythrocytes, and it may be said that the union of the fimbriae with the surface of the erythrocytes is intrinsically endothermic.

Q Value by Reaction Between Fimbriae and Mannose

As the results of the foregoing experiments indicated a specific reactivity of mannose to the fimbriate cells, further study was pursued on the interaction of the fimbriae with mannose with particular reference to the Q value generated from the reaction.

When the fimbriate strain (Sh-1 HA^+) was made to react with mannose, a curve characteristic of the exothermic reaction, yielding a Q value of −0.264 cal. per mole, was recorded (Curve A in Fig. 7). Interaction between its non-fimbriate variant (Sh-1 HA^-) and mannose exhibited a considerably different curve (Curve B in Fig. 7) but yet characteristic of the exothermic reaction with a Q value of −0.420 cal. per mole. As the structural difference between the two strains used in these experiments was solely the

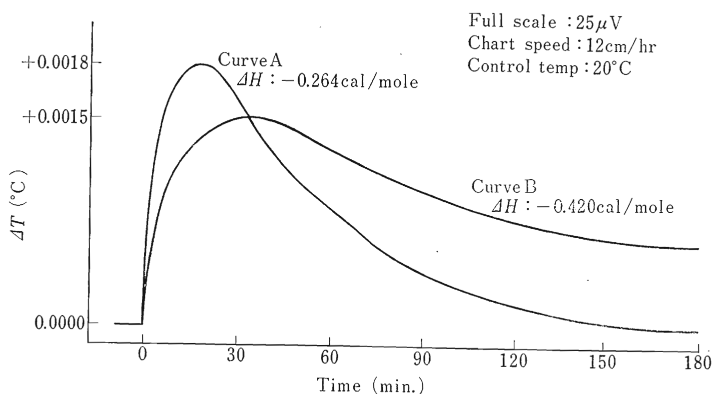

Fig. 7. Temperature difference-time curve by reaction of bacterial suspension of *Sh. flexneri* with mannose solution. Curve A represents the reaction using the fimbriate cells [Sh–1 (HA^+)] in a concentration of O.D. 660 nm 0.5, and curve B the reaction using the non-fimbriate cells [Sh–1 (HA^-)] at the same concentration of mannose solution was 0.1M.

presence or absence of the fimbriae while the two possessed entirely the same O antigenic structure, the difference in the Q values observed to exist between the two systems may be considered to represent a difference totally ascribable to the interaction between the fimbriae and mannose. The difference in the Q value was found to amount to +0.156 cal. per mole, thereby suggesting at least that the fimbriae-mannose interaction or union is of the endothermic type.

On the basis of these findings, the purified fimbriae of the fimbriate strain Sh-1 were allowed to react with mannose and the interaction displayed a curve characteristic of the endothermic reaction where the Q value

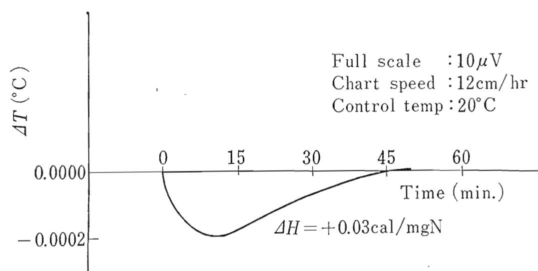

Fig. 8. Temperature difference-time curve obtained by interaction of purified fimbriae with mannose solution. Purified fimbriae were suspended in normal saline in a concentration of 1.0 mgN, and the concentration of mannose was 0.1M.

was determined to be +0.03 cal. per mgN of the fimbriae (Fig. 8). By contrast, the interaction between the purified preparation of the fimbriae and the sugar incapable of inhibiting hemagglutination, e.g. glucose, showed no generation of heat at all.

Interaction of Shigella Flexneri with Mannan

The reactivity of the fimbriate strains of *Sh. flexneri* (Sh-1 HA^+, Sh-3 and Sh-X) and the non-fimbriate strains (Sh-1 HA^- and Sh-4) to the high polymer sugars such as mannan, dextran and xylan was studied, taking their hemagglutination with the guinea pig erythrocytes as control. The results thus obtained are summarized in Table 5. The fimbriate strains demonstrated, as a matter of course, a profound hemagglutinability with the guinea pig erythrocytes and also showed an intense agglutination only in the presence of mannan. However, these fimbriate strains were found to become devoid of such reactivity with the guinea pig erythrocytes and with

Table 5. Agglutination of red blood cells of guinea pig or polysaccharides by bacterial cells of *Sh. flexneri*

1. Agglutination with red blood cells

Bacterial suspension of		Agglutination of red blood cells by bacterial cells	
		Without heating	With heating
Fimbriate	Sh–1 (HA^+)	3	0
	Sh–3	3	0
	Sh–X	3	0
Non–fimbriate	Sh–1 (HA^-)	0	0
	Sh–4	0	0

2. Agglutination with polysaccharides

Bacterial suspension of		Agglutination by					
		Mannan		Dextran		Xylan	
		of bacterial cells					
		Without heating	With heating	Without heating	With heating	Without heating	With heating
Fimbriate	Sh–1 (HA^+)	3	0	0	0	0	0
	Sh–3	3	0	0	0	0	0
	Sh–X	3	0	0	0	0	0
Non–fimbriate	Sh–1 (HA^-)	0	0	0	0	0	0
	Sh–4	0	0	0	0	0	0

Table 6. Agglutination of bacterial suspension* of *Sh. flexneri* by mannan

Bacterial cells of		Agglutination by mannan in concentration of (mg/ml)								
		9	2.3	0.6	0.3	0.15	0.08	0.04	0.02	0.01
Fimbriate	Sh-1 (HA+)	3	3	3	3	3	3	3	2	1
	Sh-3	3	3	3	3	2	1	0	0	0
	Sh-X	3	3	3	3	2	1	0	0	0
Non-fiimbriate	Sh-1 (HA-)	0	0	0	0	0	0	0	0	0
	Sh-4	0	0	0	0	0	0	0	0	0

* Concentration of suspension: O. D. 660 nm 0.5

mannan after had been heated for ten minutes at 120°C. The non-fimbriate strains invariably failed to exhibit agglutination with the guinea pig erythrocytes or with any of the high polymer sugars regardless of thermal treatment or no thermal treatment.

The fimbriate strains were incubated with the serial two-fold dilutions of mannan in physiological saline to make a quantitative estimation on agglutination with mannan. As seen from Table 6, which summarizes the results, agglutination of the fimbriate strains at a concentration of O.D. 0.5 at a wave length 660 nm was found to occur in the presence of not less than 0.02 mg of mannan per ml with Sh-1 HA^+ strain, and not less than 0.15 mg of mannan per ml with Sh-3 and Sh-X strains.

Q Value by Reaction Between Shigella flexneri and Mannan

It has been strongly suggested from the foregoing experiments that the union of the fimbriae with the erythrocyte surface as well as the interaction between the fimbriae and mannose molecule was of the endothermic type. Then, studies were carried out to clarify the thermodynamic pattern of an interaction between the bacterial cells of *Sh. flexneri* and mannan. Curve A in Figure 9 represents the reaction of a non-fimbriate variant strain (Sh-1 HA^-) with mannan, which, as can be seen, is characteristic of the exothermic reaction and the Q value in this reaction was estimated to be -0.078 cal. per 25 mg of mannan. When mannan was allowed to react with the fimbriate prototype strain (Sh-1 HA^+), on the other hand, a characteristic endothermic type of reaction was observed to occur (Curve B below the base line), and the Q value was estimated to be $+0.005$ cal. per 25 mg of mannan.

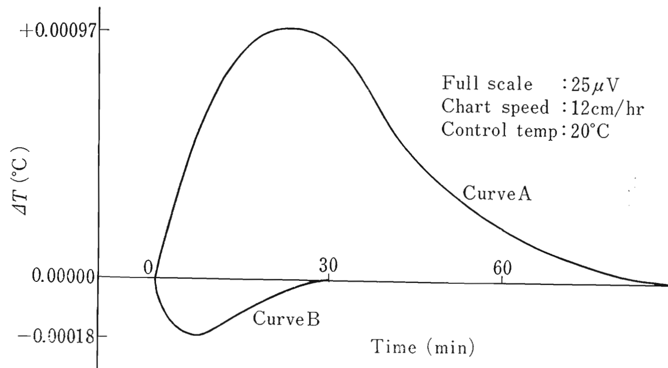

Fig. 9. Temperature difference-time curve by Shigella-mannan agglutination. In the experiment recorded in curve A, the non-fimbriate strains [Sh–1 (HA^-)] were mixed with mannan solution at a concentration of 25 mg/ml, and in the experiment recorded in curve B, the fimbriate strains [Sh–1 (HA^+)] were used in the same concentration. Q value was −0.078 cal/25 mg mannan in curve A and +0.005 cal/25 mg mannan in curve B.

Inhibition of Shigella-Mannan Agglutination by D-mannose

Serial two-fold dilutions of 0.1 M solutions of D-mannose, D-glucose, D-xylose, L-alabinose and D-galactose of up to 1:128 were prepared and the inhibitory effect, if any, of these dilutions of monosaccharides on the agglutination of the fimbriate cells with mannan was studied. The results obtained with Sh-1 HA^+ are shown in Table 7. As evident from the data presented, the agglutination of the fimbriate cells with mannan was in-

Table 7. Inhibition of Shigella-mannan agglutination by monosaccharides

Monosaccharides	Shigella-mannan agglutination in the presence of monosaccharides in							
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
d–Galactose	3	3	3	3	3	3	3	3
l–Arabinose	2	2	3	3	3	3	3	3
d–Xylose	2	2	2	3	3	3	3	3
d–Glucose	1	1	1	2	3	3	3	3
d–Mannose	0	0	0	0	0	1	2	3
Normal saline solution (control)	3	.	.	.	.	.	.	.

Concentration of bacterial suspension: O.D. 660 nm 0.5
Diluent: normal saline solution
Strain: Sh–1 (HA^+)

hibited most intensely by D-mannose up to a dilution of 1:32, followed in the decreasing order of inhibition by D-glucose up to only a dilution of 1:4. The inhibitory effect of the latter, therefore, was notably modest as compared with that displayed by D-mannose. No such suppressive effect on the agglutination was observed at all with D-xylose, L-alabinose and D-galactose.

Summary and Comment

Since the observation of a conspicuous agglutination of the erythrocytes taking place in a mixture of *Escherichia coli* and red blood cells from certain animal species (Kraus and Ludwig[13]; Guyot[14]; and Fukuhara[15]), the phenomenon called the bacterial hemagglutination has come to be generally recognized to occur not only with the erythrocytes but also with such other kinds of animal cells as spermatozoa as well as with plant cells such as yeast cells and pollen (Rosenthal[16]; and Keogh et al.[17]).

While the ability to agglutinate such cellular factors had initially been largely limited to the members of *E. coli,* the property of agglutinating animal and plant cells has been characteristic of the specific strains of this species, and accordingly, the possible existence of some factors involving the bacterial hemagglutination had been presumed. In 1950, electronmicroscopic evidence was presented that the peritrichous filamentous appendages arising from the surface of the cells of *E. coli* could clearly be distinguished from the flagella (Houwink and van Iterson[18]). Subsequently, these filamentous structures designated fimbriae (Duguid, Smith, Dempster and Edmunds[1]) or pili (Brinton[3]) were found to be possessed by many species of the family *Enterobacteriaceae* including *E. coli* (Duguid et al.[1]; Duguid and Gillies[2]; and Okamoto[19]). Studies on the biological significance of these structures revealed their irrelevance to bacterial pathogenicity but presented an evidence that they function as a factor to cause bacterial hemagglutination. From the findings showing that the filamentous appendages show a marked adherence to the cells of the intestinal mucose, it was also pointed out that they might also function to facilitate infestation onto the solid surface (Duguid and Gillies[2]; and Toba[20]).

Regarding the ability of these filamentous structures to agglutinate the red blood cells, it was pointed out that hemagglutination was inhibited by certain monosaccharides, especially by mannose (Duguid et al.[1]). The fimbriae of this sort was called the mannose-sensitive (MS) type, and the fimbriae of the bacterial species, the hemagglutination of which is unaffected by mannose, were designated as mannose-resistant (MR) type. Consider-

able differences were presumed to exist in the antigenic structures and in the composition of the components even among the fimbriae that are comparable in electronmicroscopic appearance and in hemagglutinating activity (Duguid and Gillies[2]; and Hashimoto et al.[4]), and it has been found that there are at least three distinct antigenic types of fimbriae demonstrable by cross-agglutination test and by absorption test with specific antisera against fimbriae (Hashimoto et al.[21]). Meanwhile, electronmicroscopic studies on the fine structure of the fimbriae as to length, width and pitch have suggested difference in the three-demensional structure among the various bacterial strains (Brinton[7]).

Certain strains of *Pseudomonas aeruginosa* are known to possess fimbriae which adhere onto the erythrocytes but are incapable of agglutinating them (Marx and Heumann[22]; Heumann and Marx[23]; and Kosugi[24]). Observations were also made that *Acromonas hydrophila* has fimbriae which intensely agglutinated non-cellular or cellular particles other than the red blood cells but showed only a very modest hemagglutinability (Kameyama[25]). The functional difference displayed by the fimbriae of various species or strains of bacteria are now generally believed to be derived from differences intrinsic in the physico-chemical properties of the structural constituents, especially of the subunits or pilin, of the fimbriae.

From the fact that, in cases of many of the members of *Enterobacteriaceae,* the fimbriae are considered to function as a factor contributing to cellular agglutination, it is believed that, at least, there exist on the surface of the erythrocytes what may be called combining sites or receptors which unite with the fimbriae, and hemagglutination occurs as a result of the union of these receptors with the subunits of the fimbriae, pilins. It appears also, however, that there are several different patterns of fimbriae-erythrocyte union, as may be suggested from the observations that the union of erythrocytes with the fimbriae of the MS type of *Sh. flexneri* is inhibited by mannose whereas the hemagglutination by the MR type of fimbriae is affected little by the sugar.

As an effective means for the study directed toward the clarification of the pattern of union in bacterial hemagglutination mediated by the fimbriae, thermodynamic measurement of the binding energy thereof has been introduced. While extreme difficulties are inherent in attempts to demonstrate the reversibility or irreversibility of the biological reactions or to measure the binding energy or free energy associated with them, a few groups of investigators have made thermodynamic analysis on the isohemagglutination of human erythrocytes with noteworthy results (Wurmser et al.[26]; and Wilkie and Becker[27]). By the application of their theories,

reversibility of bacterial hemagglutination was demonstrated (Takada[5]) and the heat of reaction was also calculated on a theoretical basis (Yaoi[6]), and based on these results, the practicability of the thermodynamic study on the bacterial hemagglutination was pointed out.

In view of this, an attempt was made to clarify the mechanism of bacterial hemagglutination and of its inhibition by mannose using some strains of *Sh. flexneri,* representative of the MS type of the fimbriae, by means of a twin conduction microcalorimeter.

It was found by the studies herein described that when the agglutinating agent, whether it is a bacterial cell or an erythrocyte, was made to react with each of the five aldoses in various combinations, a specific generation of heat was evident only with the reaction system consisted of the fimbriate cells and mannose. That is to say, the aldoses tested were found to fail to react with the guinea pig erythrocytes or with the bovine erythrocytes. Combinations of the fimbriate cells with mannose were noted to yield very large Q value by the reaction whereas no appreciable production of heat was observed in the combination of the non-fimbriate cells with mannose. As for the three (glucose, xylose and galactose) of the four remaining aldoses, the fourth being arabinose, fairly large Q values were obtained in the reactions with the non-fimbriate cells or with the fimbriate cells although some qualitative difference was recorded as compared with the reaction with mannose. However, there was no evidence of qualitative difference in their pattern of reaction between the fimbriate and the non-fimbriate strains. It follows that a peculiar interaction, notably in quality, occurred between mannose and the fimbriate strains. The phenomenon seems not merely to be closely related to the inhibition of bacterial hemagglutination and the disintegration or dissociation of an agglomerate by mannose, but also it may possibly be construed as meaning the reactivity of mannose not with the red blood cells but with the bacterial cells, particularly with their fimbriae. Furthermore, the fact that no traceable Q value was obtained in the interaction between mannose and the heat-killed fimbriate cells, corresponds to the well-known observation that the thermal treatment of the fimbriate cells caused the fimbriae to come off or to disintegrate with the consequent loss of the ability to agglutinate the red blood cells.

To obtain an experimental evidence in support of the viewpoint just described, we incubated the solutions of several monosaccharides with known concentrations of the bacterial cells to see if and to what extent the sugars might be absorbed by the bacterial cells. From the results of these experiments demonstrating that the bacterial strains with the fimbriae alone actively absorbed mannose from the incubation mixture, we came to the conclusion that even indirect evidence has been obtained for the specific

reactivity of mannose with the fimbriate cells, whereas mannose interacts little with the red blood cells.

The specificity of the interaction between bacterial fimbriae and mannose was in evidence even with a system consisting of purified fimbriae and aldose or of erythrocytes and aldose. That is, to be noteworthy, a significant change in the Q value, invariably characteristic of an endothermic reaction, was noticed to take place solely by the interaction of the fimbriae with mannose.

Further experimental analysis was performed on the interaction between *Sh. flexneri* and mannan, a polymer of mannose, using dextran and xylan, polymers of glucose and xylose, as the reference compounds. The experiments revealed an intense agglutination of the fimbriate strains to occur as in the case of the combination of the fimbriate cells and the guinea pig erythrocytes. The agglutination of the fimbriate strains of *Sh. flexneri* in the presence of mannan is, to our knowledge, a phenomenon which has never been described, and we would like to designate it as "*Shigella-Mannan agglutination*". Among the five aldoses examined, mannose was found to most profoundly inhibit the agglutination of the fimbriate cells with mannan, thereby again providing evidence to strongly support the viewpoint that mannose specifically combined with the fimbriae.

In view of this, attempts were made to estimate actually the true Q value in consequence of the union of the bacterial fimbriae with the surface of the erythrocytes, using a microcalorimeter. The data thus obtained, using as the control a non-fimbriate strain derived from a fimbriate strain and hence antigenically identical to the former, suggested the endothermic nature of the interaction directly involving the agglutination of the fimbriate strain with the red blood cells. Concurrently, it was also demonstrated that the binding reaction of the purified fimbriae with the surface of the red blood cells, too, proceeds as an endothermic reaction.

It follows from these findings that similarity exists between the two systems of reaction, i.e. the fimbriae and mannan as well as the fimbriae and erythrocytes, in regard to the endothermic nature of reaction. Supreme importance, therefore, may be said to be implicit in the analysis of the role played by mannose in bacterial hemagglutination in order to elucidate the underlying mechanism.

To simplify by diagrammatization the conceptualization of the mechanism whereby mannose inhibit the agglutination of *Sh. flexneri* with mannan and the related phenomenon heretofore observed, a hypothesis that mannose occurs as critical therminal structural unit at a combining site on the surface of the guinea pig erythrocytes and, corresponding to the subunit

of the bacterial fimbriae, this sugar eventually combines with the fimbriae to produce hemagglutination, would be considered tenable from the experimental results. Granted that this hypothesis works, the binding sites of the bacterial fimbriae would become saturated with the mannose molecule when the latttr were present sufficiently in the reaction mixture to consequently render to fimbriae incapable of combining with the terminal site of the erythrocytes. Moreover mannan, particularly being a high polymer possessing the terminal mannose molecules as in the case of the binding sites of the erythrocytes, would react with the bacterial fimbriae to ultimately proceed to agglutination.

References

1) Duguid, J. P., Smith, I. W., Dempster, G., and Edmunds, P. N.: Non-flagellar filamentous appendages and hemagglutinating activity in Bacterium coli. J. Pathol. Bacteriol., 70: 335–348, 1955.
2) Duguid, J. P., and Gillies, R. R.: Non-flagellar filamentous appendages ("fimbriae") and hemagglutinating activity in dysentery bacilli. J. Gen. Microbiol., 15: vi, 1956; Fimbriae and adhesive properties in dysentery bacilli. J. Pathol. Bacteriol., 73: 397–411, 1957; Fimbriae and hemagglutinating activity in Salmonella, Klebsiella, Proteus and Chromobacterium. J. Pathol. Bacteriol., 75: 519–520, 1958.
3) Brinton, C. C., Jr.: Non-flagellar appendages of bacteria. Nature, 183: 782–786, 1959.
4) Hashimoto, M. et al.: Studies on structure and function of bacterial fimbriae. Rep. I. Demonstration of fimbriae in Gram-negative enteric bacteria. Bull. Tokyo Med. Dent. Univ., 10: 181–203, 1963; Rep. II. Adhesive properties of fimbriae. Ibid., 10: 493–506, 1963.
5) Takada, R.: Thermodynamic studies on the bacterio-hemagglutination with special reference to the reversibility of the reaction. (in Japanese, with English abstract). Ochanomizu Med. J., 16: 93–102, 1968.
6) Yaoi, S.: Quantitative assay on bacterio-hemagglutination from thermodynamic point of view. (in Japanese, with English abstract). Ochanomizu Med. J., 17: 189–195, 1969.
7) Briton, C. C., Jr.: Contribution of pili to the specificity of the bacterial surface, and a unitary hypothesis of conjugal infectious heredity. *In* Specificity of cell surface, ed. by Davis, B. D. and Warren, L., Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 1967, pp. 37–70.
8) Mäkelä, O.: Studies in hemagglutination of Leguminosae seeds. Welin und Göös, Helsinki, 1957.
9) Boyd, W. C., and Reguera, R. M.: Hemagglutinating substances for human cell in various plant. J. Immunol., 62: 333–339, 1949.
10) Boyd, W. C., and Shapleigh, E.: Antigenic relations of blood group antigens as suggested by tests with lectins. J. Immunol., 73: 226–231, 1954; Diagnosis of subgroups of blood group A and AB by use of plant agglutinins (lectins). J. Lab. Clin. Med., 44: 235–237, 1954; Separation of individuals of any blood group into sectors and non-sectors by use of a plant agglutinin (lectin). Blood, 9: 1195–1198, 1954.
11) Boyd, W. C., Everhart, D. L., and McMaster, M. H.: The anti-N lectin of Bauhinia purpurea. J. Immunol., 81: 414–418, 1958.

12) Nakajima, M.: Thermodynamic analysis of bacterio-hemagglutination with the aid of calorimeter. (in Japanese, with English abstract). Ochanomizu Med. J., 18: 1–9, 1970.
13) Kraus, R., und Ludwig, S.: Ueber Bakterio-Hämagglutinine und Antihämagglutinine. Wien. Klin. Wochenschr., 15: 120–121, 1902.
14) Guyot, G.: Ueber die Bakterielle Hämagglutination (Bakterio-Hämagglutination). Centralbl. Bakteriol. Parasitenk., 47: 640–653, 1908.
15) Fukuhara, Y.: Ueber Hämagglutinierende Eigenschaften der Bakterien. Z. Immun-Forsch., 2: 313–322, 1909.
16) Rosenthal, L.: Agglutination properties of Escherichia coli: Agglutination of erythrocytes, leucocytes, thrombocytes, spermatozoa, spores of molds and pollen by strains of E. coli. J. Bacteriol., 45: 545–550, 1943.
17) Keogh, E. V., North, E. A., and Warbuton, M. F.: Hemagglutinations of the Hemophilus group. Nature, 160: 63–70, 1947.
18) Houwink, A. L., and van Iterson, W.: Electron microscopical observations on bacterial cytology. II. A study on flagellation. Biochim. Biophys. Acta, 5: 10–44, 1950.
19) Okamoto, T.: Bacterial hemagglutination by Escherichia coli. (in Japanese). Ochanomizu Med. J., 6: 1530–1541, 1958. KKKK
20) Toba, T.: Agglutination of Candida albicans by Escherichia coli and Shigella flexneri. (in Japanese). Ochanomizu Med. J., 7: 2735–2744, 1959.
21) Hashimoto, M.: Studies on bacterial fimbriae. (in Japanese). Igaku No Ayumi, 54: 577–648, 1965.
22) Marx, R., und Heumann, W.: Ueber Geisserfeinstruktur und Fimbrien bei Zwei Pseudomonas-Stämmen. Arch. Mikrobiol., 43: 245–254, 1962.
23) Heumann, W., und Marx, R.: Feinstruktur und Funktion der Fimbrien bei der Stern bildender Bakterium Pseudomonas echinoides. Arch. Mikrobiol., 47: 325–337, 1964.
24) Kosugi, J.: Studies on function and structure of fimbriae in Pseudomonas aeruginosa with observation of stalk-like appendages. (in Japanese, with English abstract). Ochanomizu Med. J., 17: 217–224, 1969.
25) Kameyama, Y.: Observations on the fimbriae and stalk-like structure in Aeromonas hydrophila. (in Japanese, with English abstract). Ochanomizu Med. J., 18: 131–140, 1970.
26) Wurmser, S. F., Jacquot, Y., et Wurmser, R.: Sur la combinaison des iso-hémagglutinines avec les groupes agglutinogénes. J. Chim. Phys., 47: 419–431, 1950; Sur l'agglutinogéne B et les isoagglutinines β (A_1O) et β (OO). Ibid., 49: 550–556, 1952; Sur l'iso-agglutinine β (A_1A_1). Ibid., 50: 240–242, 1953.
27) Wilkie, M. H., and Becker, E. L.: Quantitative studies in hemagglutination. 1. Assay of anti-B iso-hemagglutinins. J. Immunol., 74: 192–198, 1955; 2. Effect of certain variables upon the iso-agglutinin assay. Ibid., 74: 199–204, 1955.