Japan. J. Microbiol.
Vol. 13 (4), 359–366, 1969

# Heat-Labile Toxin of *Bordetella Pertussis* Purified by Preparative Acrylamide Gel Electrophoresis[1]

Yasukiyo NAKASE, Kuniyoshi TAKATSU, Masayoshi TATEISHI, Kachiko SEKIYA, and Tadayoshi KASUGA

*Department of Bacteriology, The Kitasato Institute, Tokyo*

(Received for publication, May 20, 1969)

ABSTRACT

The heat-labile toxin (HLT) of *Bordetella pertussis* was purified by column chromatography on DEAE-cellulose, salt fractionation and preparative acrylamide gel electrophoresis. The toxin obtained was confirmed to be free from hemagglutinins, protective antigens, histamin sensitizing factors, and K and O agglutinogens, and was shown to be homogenous by agar gel diffusion tests, ultracentrifugation, and electrophoresis. The minimal necrotic dose of the HLT in guinea pig was 0.01 $\mu$g dry weight. It was a protein in nature, contained a sugar moiety and possessed S value of 1.4. The toxicity of purified HLT was increased by mixing with a protein, such as rabbit serum.

A considerable number of reports [1, 2, 12, 15, 17, 20, 21] have been accumulated on the purification and characterization of the heat-labile toxin (HLT) of *Bordetella pertussis*.

We have also attempted to purify the toxin as completely free from contamination of other biologically active substances as possible, e.g. agglutinogens, protective antigen, histamin sensitizing factor and hemagglutinin. In a previous paper [11], we separated the HLT fraction from a sonic extract of the organisms by column chromatography on DEAE-cellulose. The HLT obtained, however, was still contaminated slightly with hemagglutinin, histamin sensitizing factor and protective antigen. It also showed several bands with thin-layer acrylamide-gel electrophoresis. This paper presents the results of further purification of the HLT by preparative acrylamide-gel electrophoresis, and reports on the chemical and biological properties of the HLT so recovered.

[1] This paper was presented at the 21st Meeting of Kanto Branch, the Japan Bacteriological Society, held in Tokyo on June 14, 1968.

## MATERIALS AND METHODS

*Organisms.* Strain Maeno (phase 1) of *B. pertussis* was grown for 48 hr on a modified charcoal agar medium [8]. The cells were washed with phosphate-buffered saline, and were then resuspended in distilled water to a concentration of 1,500 billion per ml.

*Sonic extraction.* The suspension was disrupted in a Otake model 150, 10 KC sonic oscillator for 10 min at 1–2 C, and centrifuged at 10,000×g for 60 min. This supernate was used as a starting material for column chromatography on DEAE-

cellulose.

*Column chromatography on DEAE-cellulose.* According to previous reports [11], 50 ml of sonic lysate was applied onto a DEAE-cellulose column (3×50 cm) which had been equilibrated with 0.005 M phosphate buffer (pH 8.0). The column was washed with each 1,000 ml of 0.01 M and 0.02 M phosphate buffer (pH 7.6) to remove K agglutinogens, and then, the HLT fraction (F2) was collected by eluting with 1,000 ml of 0.05 M phosphate buffer (pH 7.6). The eluate was concentrated to approximately one fiftieth of the original volume by ultrafiltration. Ammonium sulfate was added to the fluid to a concentration of 10% w/v, and the precipitate removed. Additional ammonium sulfate was added to the supernatant to a final concentration of 20% w/v, and the precipitate collected by centrifugation. This salt-fractionation step was repeated twice, and the final precipitate was dialized against 0.01 M phosphate-buffered saline for 3 days, and then freeze-dried. This product was used as the material for preparative acrylamide-gel electrophoresis.

*Preparative acrylamide-gel electrophoresis (PAE).* The apparatus and technique described by Gordon and Louis [6] were used with slight modifications. A column (40×8 mm) of acrylamide-gel with 0.3 M borate buffer (pH 8.2) was prepared. Fifty mg of the derived material was dissolved in 3.3 ml of 0.3 M borate buffer (pH 8.2), was mixed with a small amount of Sepharose 4B gel, and then, applied onto the acrylamide-gel column. Electrophoresis was carried out in the same buffer at 60–70 V, 40 mA for 35–48 hr at 1–2 C. Effluents were continuously collected into sample tubes, 3 ml each, to a total of approximately 600–800 ml. Flow rate was approximately 18 ml per hr.

*Agglutinin-absorption test.* Agglutinin-absorbing activity was determined by the method of Flosdorf and Kimball [5]. Both anti-K and anti-O sera were used.

*Agar gel diffusion test.* The method of Ouchterlony was employed [16].

*Animal tests.* The dermatonecrotic effect was tested in guinea pig by injecting 0.1 ml of sample intracutaneously. The reaction was observed for 48 hr, and the necrotic lesion, larger than 5 mm in diameter, was read as a positive reaction. To find the antitoxin producing-ability, rabbits weighing 2–2.5 kg were injected subcutaneously with a total of 2.5 mg of the formalized HLT, both with and without Freund's adjuvant. The rabbits were bled at the end of the 4th and 8th weeks. The sera were mixed with varying concentration of toxin, and after standing at 4 C overnight, the mixtures were injected intracutaneously into guinea pigs for titration of antitoxin. Toxicity tests were performed using 4-week-old mice (ddN strain), which had been injected intraperitoneally with a saline suspension (0.5 ml) of the sample. Deaths occurring before 48 hr after injection were regarded as being due to the HLT. For histamin sensitizing test, the method of Niwa [14] was used. Active protection was assayed by the method of the National Institute of Health of Japan for standarding the potency of pertussis vaccine, and was described previously [11].

*Chemical and physicochemical tests.* For electrophoretic analysis, the method of Tachibana [19], a thin-layer acrylamide-gel electrophoresis, was used. Ultracentrifugal analysis was conducted with a Beckman Spinco model E. Ultraviolet absorption was measured with a Hitachi model 139 spectrophotometer. Quantitative analysis of neutral sugars was performed using phenol-sulfuric acid methods [3]. A modification of Rondle and Morgan's technique was used for the amino-sugar determination.

Lipid was estimated by the method of Salton [18]. Ninhydrin, Biuret and Lowry methods for protein were employed. Total nitrogen was determined by a modification of the micro-Kjeldahl technique. Phosphorus determinations were done using a modification of Lowry's method [3]. Sugars and amino-acids were analyzed on paper chromatography after the sample was hydrolyzed in 6N sulfuric acid.

## RESULTS

The HLT fraction (F2) obtained by column chromatography on DEAE-cellulose and described in a previous paper [11], was further purified by precipitating with 20% (w/v) ammonium sulfate. Preliminary experiments showed that the ammonium sulfate precipitated toxin gave two distinct, well separated bands on the cathodic side after thin-layer acrylamide-gel electrophoresis (Fig. 1). No activity of the HLT, however, was extracted from these. The toxin was extracted from a trace band which had migrated approximately 1.2 cm toward the cathode. A possible method to

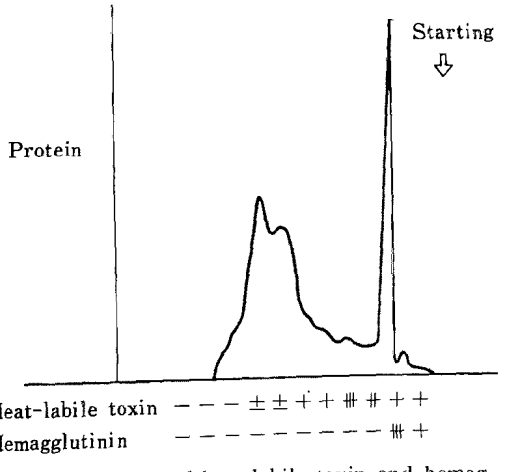


Fig. 1. Activities of heat-labile toxin and hemagglutinin in strip of acrylamide-gel electrophoresis. A piece of gel, 5 mm×10 mm, was extracted in 1 ml of buffered saline, pH 8.2.

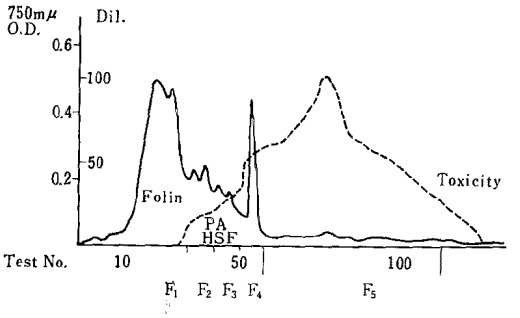


Fig. 2. Preparative acrylamide-gel electrophoresis.
PA: Protective antigen.
HSF: Histamine sensitizing factor.

separate the HLT from hemagglutinin and protective antigen was found. Accordingly, the ammonium sulfate-precipitated HLT was applied onto a column of PAE, and was then eluted. As shown on Fig. 2, following two protein peaks without biological activities, faint peaks which contained protective antigen and histamin-sensitizing factors were eluted. Subsequently, the HLT activity appeared but without a significant protein peak. All the hemagglutinin was retained on the column. Effluents in the tubes (numbers 62 to 90) were pooled, dialized and freeze-dried. The recovery of HLT in the salt-precipitation step was about 11–22% of F2 by column chromatography on DEAE-cellulose based on dry weight. The recovery of the purified HLT as a final product was about 60% of HLT obtained by salt-precipitation from F2. Although some portion of the HLT was inactivated during purification, the final product showed a minimal necrotic dose (MND) of about 0.01 μg in dry weight. When the F2 fraction of column chromatography on DEAE-cellulose was precipitated with ammonium sulfate, about 81–97% of the total HLT activity in the fraction was recoverable in the precipitate, and the activity was increased about 10–40 times the F2. The total recovery of the HLT activity in the toxin fraction on PAE was usually

Table 1. Effect of protein on heat-labile toxin

| Injected with | MND in dilution |
|---|---|
| Heat-labile toxin (0.2 μg) | 1:4 |
| +rabbit serum No. 1 | 1:16 |
| +rabbit serum No. 2 | 1:8 |
| +K-agglutinogen (0.5 mg) | 1:8 |
| +phase I sonic extract heated at 60C for 30 min | 1:16 |

70–80%, and the activity was increased about 2 times the salt fraction, and about 80 times that of the sonic extract. We found that the activity of HLT obtained from PAE was further significantly increased by about 4 times when it was injected with a protein such as rabbit serum, bovine albumin or the K agglutinogen of *B. pertussis*. (Table 1)

The toxin purified by PAE, was analyzed for some of its chemical constituents. It gave slightly positive tests for protein and amino-acids (Biuret, Folin and ninhydrin), and sugar (phenol-sulfuric acid, Molisch and anthrone), and yielded negative tests for nucleic acids (Orcinol HCl and diphenylamine). It contained 14.6% nitrogen and 1.4% reducing sugar. No lipids or phosphorus were detected. The samples hydrolyzed with sulfuric acid were analyzed by both paper and thin-layer chromatography. Mannose and an unknown reducing substance were found. Amino-acids detected on a paper chromatography were as follows: asparatic acid, lysine, arginine, proline, alanine, glutamic acid, leucine, phenylalanine, valine, histidine, glycine, isoleucine, and tyrosine. The ultraviolet-absorption of the toxin is shown in Fig. 3, which is a protein absorption spectra. The Schlieren pattern on the ultracentrifugation is shown on Fig. 4. The toxin was homogeneus with a 1.4 S value. On the thin-layer acrylamide electrophoresis, a faint single band was found at 1.2 cm from the starting line toward the anode, while tracer

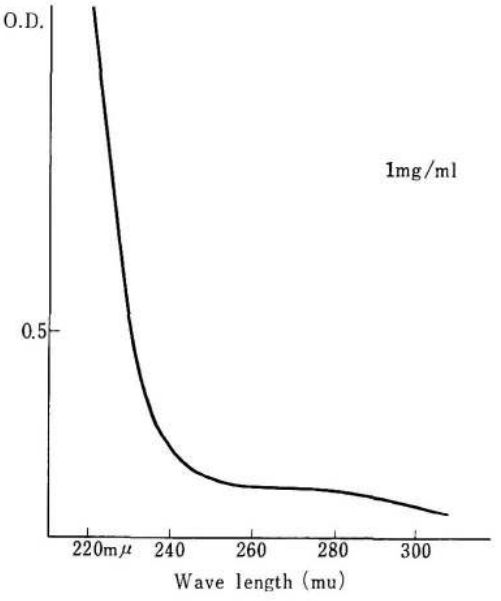


Fig. 3. UV-absorption of heat-labile toxin.

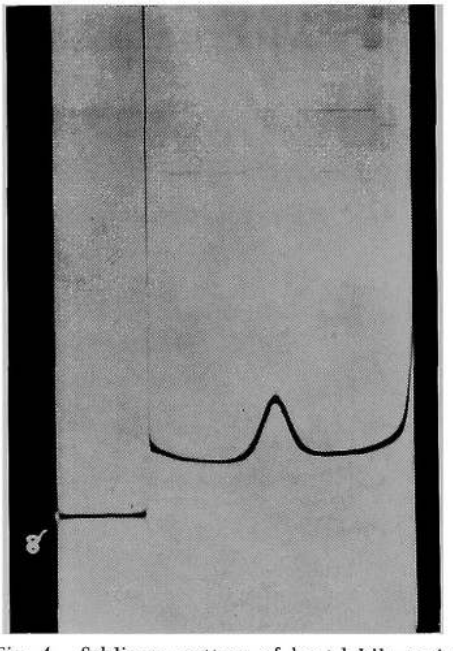

Fig. 4. Schlieren pattern of heat-labile toxin. Ultracentrifugation of the HLT by the valve type synthetic boundary cell in schlieren diaphragm 70C. Solution (0.5%) in 0.1 M sodium phosphate buffer, pH 8.0, $\mu$=0.14, photographed at 8 min after operating speed was reached (60,000 rpm, 20.4C).

stain (amidoblack) migrated 5 cm toward the anode. Homogenous aggregated particles were observed in electron-micrographs of the HLT by use of a negative stain technique with phosphotungstic acid (Fig. 5).

In agar-gel diffusion precipitation, HLT showed a single line of precipitation against both anti-toxin and anti-*B. pertussis* phase I which contained anti-toxin (Fig. 6). No line was detected between HLT and anti-K or anti-O sera. HLT absorbed neither K or O agglutinins.

No contamination with hemagglutinin or histamin-sensitizing factor, was noted on examining 1 mg of HLT.

An anti-toxin which neutralized dermatonecrotic activity, was produced by injection of HLT detoxified with formalin into rabbits (Table 2). The K agglutinin titer in the antitoxin was 1:40 or lower.

Immunization of mice with 1 mg of the alum-precipitated HLT, which had been detoxified by formalin, failed to protect mice against intracerebral challenge with 200 LD50 of *B. pertussis* phase I organisms, 18–323.

A sharp increase in blood leucocyte count about 3–4 fold of normal, and a significant decrease of the spleen weight, to about

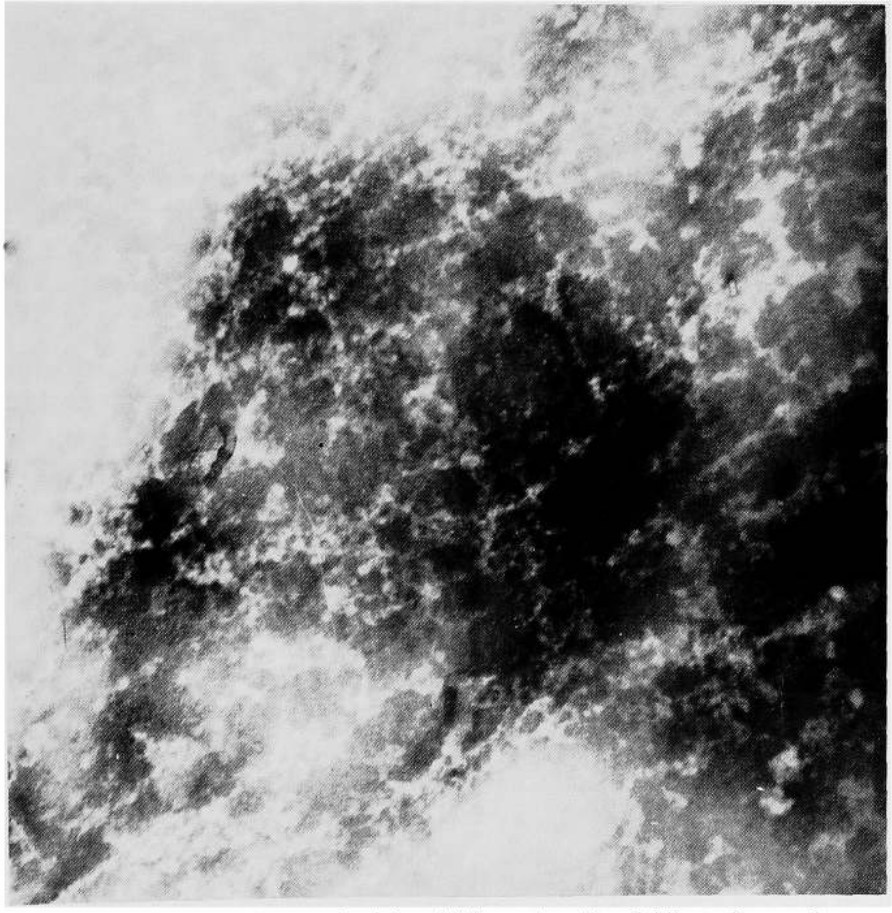

Fig. 5. Electron micrograph of heat-labile toxin. Heat-labile toxin negative stained with phosphotungstic acid (×125,000).

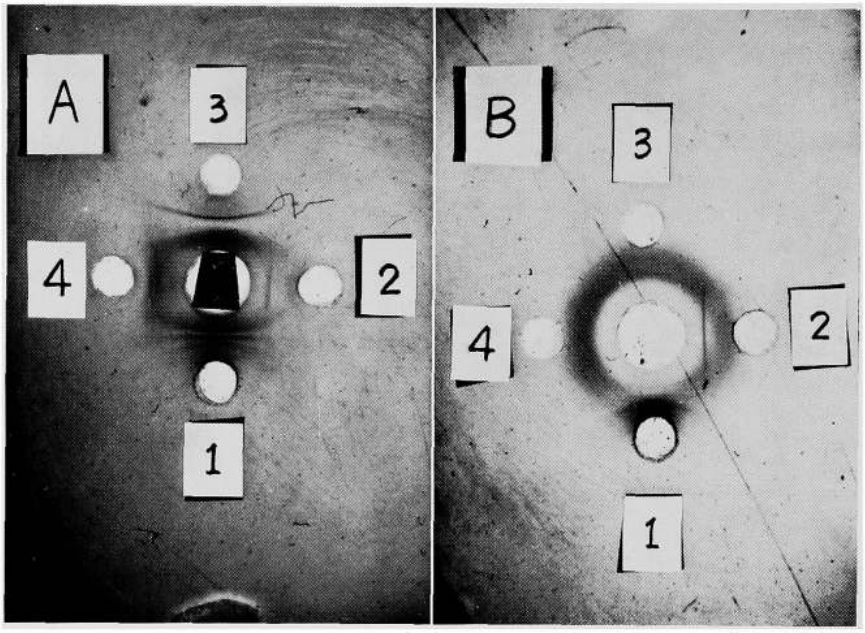


Fig. 6. Gel-diffusion precipitation of heat-labile toxin.
Ouchterlony gel diffusion tests with antiserum to toxin,
Center well, A: anti-phase I *B. pertussis*
B: anti-toxin
Outer well, 1: phase I sonic extract
2: heat-labile toxin
3: K-antigen
4: phase III sonic extract

Table 2. Anti-toxin and agglutinin titers of rabbits immunized with heat-labile toxin

| Rabbit No. | Immunized with | Neutralizing titer at 4W | Neutralizing titer at 8W | K-agglutinin at 8W |
|---|---|---|---|---|
| 681 | 2.3 mg toxin with adjuvant | 2 MND | >8 MND | 20 x |
| 682 | " | 2 MND | >8 MND | 40 x |
| 683 | 2.3 mg toxin without adjuvant | 2 MND | 2 MND | <10 x |
| 684 | " | 1 MND | >8 MND | <10 x |
| 685 | " | 1 MND | >8 MND | <10 x |
| 686 | " | 2 MND | >8 MND | 20 x |

50% of normal, were observed in the mice which had been injected with 0.5 to 2 MND of HLT intravenously (Fig. 4). Injection with 4 MND of HLT caused a significant decrease of body weight in mice.

The animals, both mice and guinea pigs, were injected the HLT intravenously and intraperitoneally to observe histopathological changes. A significant necrosis, hemorrhage, congestion and degeneration were found in liver, spleen and kidney of these animals.

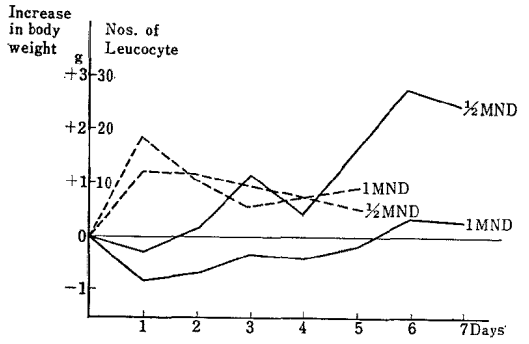


Fig. 7. Toxicity test of heat-labile toxin to mice.
solid line: body weight of mouse.
broken line: numbers of circulating blood leucocyte.

## DISCUSSION

Since Robbin and Pillemer separated the heat-labile toxin from *B. pertussis* organisms by methanol-precipitation [17], several workers [1, 2, 12, 15, 20, 21] have attempted to obtain a pure toxin which was free of other biological activities or substances.

Yamamoto et al. [20] obtained an agglutinogen-free HLT by salt precipitation and zone-electrophoresis, but the toxin still contained protective antigen. Kuroya, et al.[12] obtained HLT with a 20 S value, by ultracentrifugation and electrophoresis. Their toxin was still contaminated with K agglutinogen. Billaudelle et al. [2] isolated HLT by column electrophoresis, and the toxin was found to be free from protective antigen but was non-homogenous in agar gel diffusion. Onoue et al. [15] purified the HLT by calcium phosphate-gel treatment, salt fractionation, potassium phosphate precipitation and finally by the column chromatography on DEAE-cellulose. The toxin obtained still contained both K agglutinogen and protective antigen, and it revealed three precipitin bands by agar-gel diffusion. Sato et al. obtained a toxin with a 13 S value, by sugar-gradient ultra-centrifugation (Unpublished). Their toxin also had three components in gel diffusion test. These authors, and Banerjea and Munoz [1] found that the chemical nature of HLT was a protein.

In a previous paper [11], the present authors obtained an HLT fraction by column chromatography on DEAE-cellulose, which contained traces of both protective antigen and histamin-sensitizing factor. Consequently, an attempt was made to further purify the HLT fraction by preparative acrylamide-gel electrophoresis. The HLT finally obtained was free of protective antigen, histamin-sensitizing factor, hemagglutinin and agglutinogens. It was confirmed to be homogenous by electrophoresis, ultracentrifugation and agar-gel diffusion tests. Spectrophotometric readings of the toxin were similar to that described by Kuroya et al. [12], who found that it was a protein containing traces of sugar and lipid. The toxin obtained from PAE, was a protein with sugar. Its hydrolysate on paper chromatography showed mannose and an unknown reducing substance. The toxin had a 1.4 S value. Molecular weight of the HLT was considered to be smaller than that of the HLT values thus far reported [12, 15, 20, 21]. By injection of the HLT with adjuvant into rabbits, an anti-toxin which neutralized the dermatonecrotic activity was easily produced. Its activity increased in the presence of a foreign protein, such as rabbit serum, bovine albumin, and K agglutinogen. The purified HLT was denatured in vitro easier than the crude in buffered saline. The HLT mixed with a large molecule protein, might give a more prolonged action in tissue than the purified HLT itself. There is a possibility that purified HLTs of high molecular weight so far reported, might really be HLTs mixed with proteins such as K agglutinogen, protective antigen or the inert pro-

tein of the cells.

ACKNOWLEDGEMENTS

We are grateful to Dr. T. Arai, Department of Bacteriology, school of medicine, the Keio University, for his help in taking electron micrographs, to Dr. Y. Nishiyama for his help in pathological observation, and to Dr. M. Yoshioka, for valuable help and advice.

This work was supported in part by a grant from the Ministry of Education.

REFERENCES

[1] Banerjea, A., and Munoz, J. 1962. Antigen of *Bordetella pertussis* II. Purification of heat-labile toxin. J. Bacteriol. **84**: 269–274.

[2] Billaudelle, H., Edebo, L., Hammarsten, E., Heden, C. G., Malmgren, B., and Palmstierma, H. 1959. Biologically active fractions from *Hemophillus pertussis*. Acta Pathol. Microbiol. Scand. **50**: 208–224.

[3] Chen, P. S., Jr., Toribara, T. Y., and Warner, H. 1956. Micro-determination of phosphorus. Annal. Chem. **28**: 1756–1758.

[4] Dubois, M., Gilles, K. A., Hamilton, J. K., Rabers, P. A., and Smith, F. 1956. Colorimetric method for determination of sugar and related substances. Anal. Chem. **28**: 350–356.

[5] Flosdorf, E. W., and Kimball, A. C. 1940. Comparison of various physical means of liberation of the agglutinogen from *H. pertussis* phase I. J. Immunol. **39**: 287–295.

[6] Gordon, A. H., and Louis, L. N. 1967. Preparative acrylamide electrophoresis; A single system. Anal. Biochem. **21**: 190–200.

[7] Kabat, E. A., and Mayer, M. M. 1964. Experimental immunochemistry, p. 476–482 and p. 527–528. Charles C Thomas, Publisher, Springfield, Ill.

[8] Kagami, K. 1960. Properties of B. pertussis grown on charcoal agar. Kitasato Exp. Med. **33**: 29–40. (in Japanese)

[9] Kasuga, T., Nakase, Y., and Takatsu, K. 1953. Studies on *Heamophillus pertussis*. I. Antigenic structure of *H. pertussis*. Kitasato Arch. Exp. Med. **26**: 121–134.

[10] Kasuga, T., Nakase, Y., and Takatsu, K. 1953. Studies on *Heamophillus pertussis*. II. Resistance of each antigen to heat, alcohol and HCl. Kitasato Arch. Exp. Med. **26**: 135–142.

[11] Kasuga, T., Nakase, Y., nad Takatsu, K. 1962. Studies on the constituents of *Bordetella pertussis*, especially on the protective substance. Kitasato Arch. Exp. Med. **35**: 1–11.

[12] Kuroya, M., Amano, Y., Kurokoshi, T., Ito, M., Shiratori, O., and Ota, R. 1959. Studies on *Haemophillus pertussis* toxin. Japan J. Med. Progr. **46**: 436–439. (in Japanese)

[13] Masamura, H., and Yoshizawa, Z. 1957. Biochemical studies on carbohydrate. Tohoku J. Exp. Med. **15**: 169–176.

[14] Niwa, M. 1962. Histamine Sensitizing Factor of *Bordetella pertussis*. J. Biochem. **51**: 222–230.

[15] Onoue, K., Kitagawa, M., and Yamamura, Y. 1963. Chemical studies on cellular components of *Bordetella pertussis*. III. Isolation of high potent toxin from *Bordetella pertussis*. J. Bacteriol. **86**: 648–655.

[16] Ouchterlony, O. 1948. Antigen-antibody reactions in gels. Arch. Kem. Mineral Gel. **26**: 1–9.

[17] Robbins, K. C., and Pillemer, L. 1950. The separation of a protective antigen from a toxin-producing strain of *Hemophilus pertussis*. J. Immunol. **65**: 393–406.

[18] Salton, M. R. J. 1953. Studies of the Bacterial cell wall. Biochem. Biophys. Acta **10**: 512–523.

[19] Tachibana, T. 1965. Thin-layer Polyacrylamide gel electrophoresis. Anal. Inst. **3**: 9–17. (in Japanese)

[20] Yamamoto, A., Iida, T., Komatsu, N., Ogata, Y., Komase, Y., Saito, M., Taguchi, K., Yoshida, T., Kusano, N., Kawai, K., and Arahama, M. 1959. Studies on *Hemophilus pertussis* toxin. Japan J. Med. Progr. **46**: 384–388. (in Japanese)

[21] Yamamoto, A., Zenyoji, H., Tanaka, T., and Uchida, T. 1953. *Hemophilus pertussis*. VII. The chemical properties of thermolabile toxin. Gunma J. Med. Sci. **2**: 85–92.