Neutralization of Bacterial Viruses by Antibodies of Young Animals

III. The Development of the Avidity of 19S and 7S Neutralizing Antibodies in the Course of Primary and Secondary Response in Young Rabbits Immunized with ΦX 174 Bacteriophage

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ABSTRACT. The development of the avidity of 19S and 7S neutralizing antibodies was studied after the immunization of young rabbits with ΦX 174 bacteriophage. Both 19S and 7S antibodies formed during the entire course of the immunization process were able to neutralize bacteriophage ΦX 174 without the participation of thermolabile serum components: neither heat inactivation at 56° C nor addition of piglet complement into the neutralizing system influenced the titer in the neutralization test. In both immunoglobulin classes (IgM and IgG) the first antibodies formed possessed a low avidity that increased in the course of the immunization process so that in the secondary response antibodies of both 19S and 7S type were formed possessing a high binding activity towards the specific antigen. Following the immunization with a high dose of the phage antigen (10^{10} PFU of ΦX 174 phage) 19S antibodies were formed at a relatively fast rate, the avidity of which did not further increase during immunization. When a low antigen dose was used for immunization the avidity of antibodies significantly increased in the course of immunization.

The avidity of antibody, i.e. the extent of its ability to form stable complexes with the antigen has been studied by various authors using all kinds of antigen-antibody systems. Thus Jerne (1951) found that diphtheria antitoxin formed during the primary response, formed complexes with the antigen that dissociated much more easily than antitoxin produced during the secondary response. Similar observations were made by Jerne and Avegno (1956) with respect to neutralizing antibodies formed during the antibody response to bacteriophage T 4. Further antigens used in model studies on the avidity of antibodies and dissociability of antigen-antibody complexes included sheep erythrocytes (Taliaferro *et al.*, 1959; Goodman & Massaites, 1960) and bovine serum albumin (Talmage, 1960; Grey, 1964). Eisen and Siskind (1964) studied the avidity of antibodies to haptens in the course of the primary and secondary response to dinitrophenyl (DNP)-protein conjugates in rabbits. From all these results it follows that the avidity of antibodies increases in the course of immunization and is enhanced by repeated antigen stimulation.

In recent work the avidity of antibodies belonging to different classes of immunoglobulins has also been studied. Most of this work claims that natural 19S antibodies of normal sera and early 19S antibodies formed during the primary response possess a low avidity. This finding was obtained with 19S macroglobulin antibodies to sheep erythrocytes by Taliaferro *et al.* (1959) and by Svehag (1964) in both natural and early antibodies during the primary response to poliomyelitis virus. On the contrary, antibodies of the 7S type formed stable,

hardly dissociable complexes with the virus antigen in the secondary reaction. Grey (1964) found a significantly lower association rate of 19S antibody with serum albumin as compared with 7S antibody even though the dissociation kinetics of both types of antibodies was approximately of the same order. On the other hand, Goodman and Massaites (1960) drew attention to the fact that γ haemolysin (19S) showed a lower intercellular transfer between the 2nd and 3rd week after immunization with sheep erythrocytes than γ_2 haemolysin (7S antibody). Similarly Greenbury *et al.* (1963) found that 19S haemolysin was less dissociable than 7S antibody to human erythrocytes in hyperimmune rabbit sera.

In our previous papers (Hájek, 1968; Hájek, 1969) the development of neutralizing activity of 19S and 7S antibodies in young rabbit sera against T2 phage was studied in correlation with the potentiating role of complement on the neutralization of that particular phage. We found that the ability of antibodies of newborns to neutralize T2 phage without the aid of complement increases in the course of the immunization process both in 19S macroglobulin antibodies and in 7S gamma globulins (Hájek, 1969).

In our present work we studied the development of the avidity of 19S and 7S neutralizing antibodies in the course of primary and secondary response to ΦX 174 bacteriophage in newborn rabbits. The experimental model used seems to be suitable for studies on the avidity of antibodies to viruses since the neutralization of ΦX 174 bacteriophage by an antibody molecule is most probably a one-step process which does not require the participation of some other "adjuvant" serum thermolabile factor (Styk *et al.*, 1964; Hájek & Mandel, 1966; Hájek, 1966; Hájek, 1967) and thus provides the possibility of taking advantage of experimental techniques many times used in the studies on the firmness of the bond between the virus particle and the antibody molecule (Jerne & Avegno, 1956; Svehag, 1964; 1965a, b; Bowman & Patnode, 1964; Hájek, 1967).

MATERIALS AND METHODS

Bacteriophage ΦX 174 (host strain *Escherichia coli* C) was prepared according to the method of Tromans and Horne (1961) and further purified according to Hall *et al.* (1959) using a DEAE cellulose column. The stock suspension of purified bacteriophage (2×10^{10} PFU) in physiological saline was used throughout all immunization and neutralization experiments.

Immunization of experimental animals. The phage antigen was injected intraperitoneally to 5-day-old rabbits in amounts of 10^{10} PFU in 1 ml of saline. The blood samples were withdrawn by cardiac puncture on day 7, 14 and 28 following the first injection of antigen. Revaccination was carried out four weeks after the primary immunization and the serum was obtained on the 7th day after the secondary injection of antigen. The revaccination dose was of the same magnitude as the dose used for primary immunization (i.e. 10^{10} PFU of ΦX 174 phage).

The isolation of 19S and 7S antibodies was performed using the Sephadex G 200 column as described in a previous communication (Hájek, 1969).

The determination of neutralizing antibodies using the 50% neutralizing test and the 50% neutralizing test in the presence of complement is described in detail in the work of Hájek (1966).

Reactivation of bacteriophage ΦX 174 from the antigen-antibody complex in solutions at different ionic strength was performed as described in our previous work (Hájek, 1967).

Dissociation of antigen-antibody complexes was studied using the method of

Jerne and Avegno (1956). The number of reactivated phage particles was expressed graphically in absolute values or as a per cent of reactivation (Finkelstein & Uhr, 1966). That value expresses in per cent the ratio of the number of phage particles reactivated at 57° for 30 min (Jerne & Avegno, 1956), related to the original number of phage particles at zero time at the beginning of the reaction which represents the value of 100%.

RESULTS

The influence of heating at 56° C for 30 min and the addition of complement on the neutralizing activity of 19S and 7S antibodies in the course of the primary and secondary response of rabbits immunized with ΦX 174 phage

In these experiments we attempted to influence the 50% neutralizing titer of antibodies of young rabbits against ΦX 174 phage by heating at 56° C for 30 min (a decrease of the neutralizing titer was expected) and further, by addition of piglet complement into the neutralizing system (an increase of the titer expected). However, this treatment did not cause the changes in the titer either in early antibody of the 19S type (on the 7th day after immunization) or in early antibodies of the 7S type (on the 14th day after immunization). Also, the heat-inactivation or addition of complement did not change the neutralizing titer of 7S antibodies produced in young rabbits on the 28th day after immunization or in 7S antibodies obtained in the secondary reaction.

The development of avidity of 19S and 7S antibodies in the course of the primary and secondary response in young rabbits immunized with two different doses of bacteriophage ΦX 174

First, the firmness of binding between the phage particle and the molecule of 19S neutralizing antibody was tested using the dilution method in solutions of different ionic strength. The 19S type antibody was obtained on the 7th day after the injection of 10^{10} PFU of ΦX 174 phage to newborn rabbits. The antibody was used in a dilution to obtain approximately a 90% neutralization of the original phage suspension ($P_0 = = 1$ to 2×10^5 PFU of ΦX 174). After the 20 minutes of the neutralization reaction had passed, the sample of the neutralization mixture was diluted in solutions with increasing ionic strength in the ratio 1 : 100 (0.1 ml of the sample + 9.9 ml of a prewarmed solution), i.e. in physiological saline and 1 M, 2 M and 4 M NaCl solution. Afterwards, in the course of further incubation at 37° C, samples were withdrawn every 10 min from the prediluted sample and tested for reactivation. Similar samples were withdrawn from the control neutralization test tube. The results are depicted in Fig. 1. It is obvious that the increasing concentration (ionic strength) of the diluting solution results in higher dissociation of complexes between phage ΦX 174 and molecules of 19S newborn antibody, so that by diluting the neutralized virus in 4 M NaCl solution a 80% reactivation of a live virus could be obtained.

The dissociation of phage particles from complexes with molecules of 19S antibody at the early stage of the primary reaction (7th day after an intraperitoneal immunization of young rabbits with 10^{10} PFU of bacteriophage ΦX 174) was studied using the method of reactivation at 57° C proposed by Jerne and Avegno (1956). The dissociation of ΦX 174 phage from the complex with 19S antibody in rabbits 7 days after immunization with a large dose of antigen (10^{10} PFU) is shown in Fig. 2. Using the reactivation at 57° C, 31% of the original number of phage particles could be dissociated. Bacteriophage ΦX 174 could still be dissociated from

the complex with 19S antibody that was produced on the 14th day after immunization by 10%, and that value was almost unchanged until the 28th day after immunization (Table 1).

data in Table 1 it follows that the avidity of quickly formed 19S antibody increased approximately 3 times between the days 7—14 after immunization with a large dose of antigen without

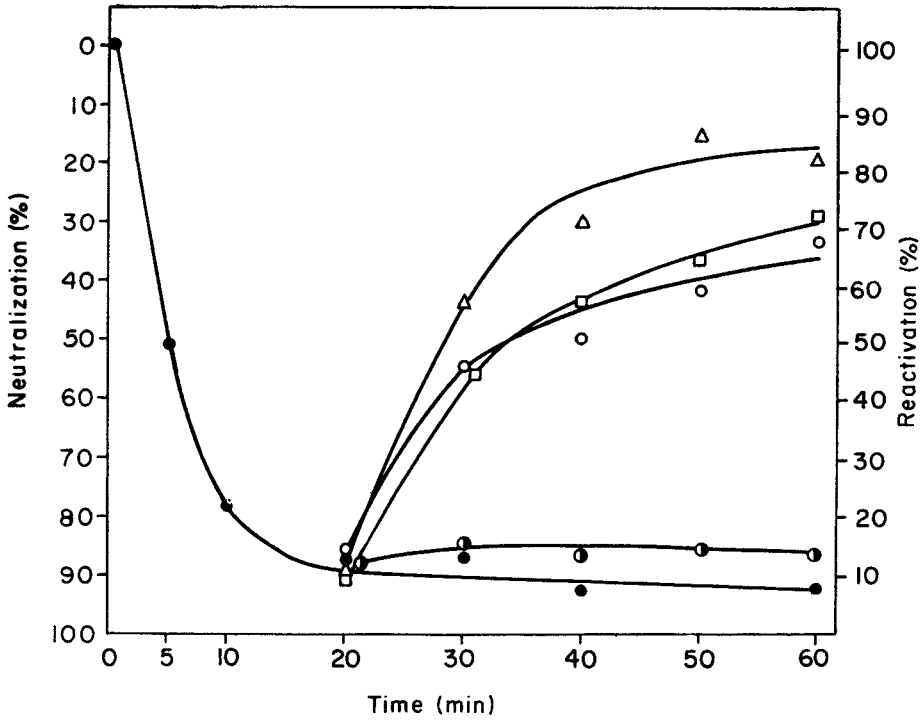

Fig. 1. Reactivation of phage ΦX 174 from the complex with early 19S antibody in solutions with different ionic strength. ◑ — physiol. saline; ○ — 1 M NaCl solution; □ — 2 M NaCl solution; △ — 4 M NaCl solution.

Following the immunization of young rabbits with a low dose of antigen (5×10^7 PFU of ΦX 174 phage) the dissociation of 19S antibody 14 days after immunization amounted to 28%, on the 4th week after primary immunization only to 3%. From the comparison of

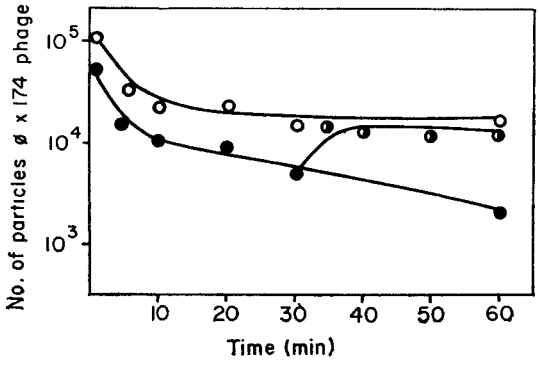

Fig. 2. Reactivation of ΦX 174 phage from the complex with early 19S antibody according to Jerne and Avegno (1956). ○ "regular titration"; ● — "decission-tube titration"; ◑ — "reaction titration".

further increase. (After the second week the content of 19S antibodies in the sera of young rabbits immunized with a large dose of phage ΦX 174 decreased.) On the contrary, in a group of young rabbits immunized with a low antigen dose (5×10^7 PFU), where the synthesis of 19S antibodies increased between the 14th and 28th day, the avidity of these antibodies increased about 9 times. During the secondary response a highly avid 19S antibody was produced the dissociation of which amounted to just a fraction of a per cent.

Table 1. The comparison of % of reactivation of bacteriophage ΦX 174 from the complexes with antibodies of young rabbits immunized with different doses of ΦX 174 phage

Sample	19 S		7 S	
	10^{10}	5×10^7	10^{10}	5×10^7
7th day of primary reaction	31	—	—	—
14th day of primary reaction	10	28	59	—
28th day of primary reaction	11	3	4	—
7th day of secondary reaction	—	0.1	0.3	—

The antibody of the 7S type produced 14 days after the primary immunization

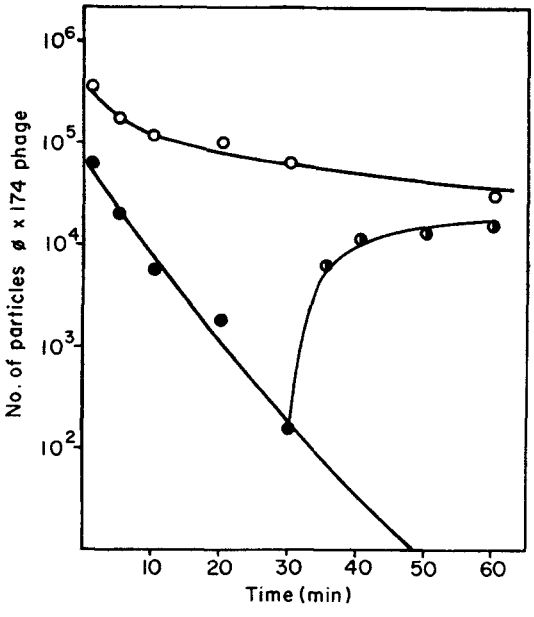

Fig. 3. Reactivation of ΦX 174 phage from the complex with early 7S antibody according to Jerne and Avegno (1956). ○ — "regular titration"; ● — "decission-tube titration"; ◑ — "reactivation titration".

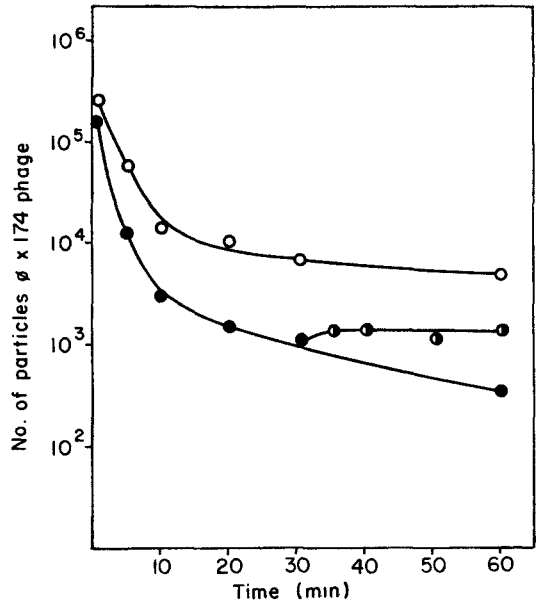

Fig. 4. Reactivation of ΦX 174 phage from the complex with secondary 7S antibody according to Jerne and Avegno (1956). ○ — "regular titration"; ● — "decission-tube titration"; ◑ — "reactivation titration".

of bacteriophage ΦX 174 with 10^{10} PFU possessed a low avidity (59% of reactivation) that increased significantly towards the 28th day after immunization (4% of reactivation). During the secondary response a highly avid 7S antibody was produced. The results are shown in Fig. 3 and 4 and Table 1.

DISCUSSION

Following the immunization of newborn rabbits with bacteriophage ΦX 174 the avidity of neutralizing antibodies increases in the course of the immunization process both in the IgM class (19S γ_1-macroglobulin) and in the IgG class (7S γ_2-globulin) of immunoglobulins. The first antibodies that are produced in both immunoglobulin classes possess a low avidity which gradually increases during immunization so that in the secondary response 19S and 7S antibodies are formed with a high avidity and binding (i.e. neutralizing) capacity. Similar results were obtained with other antigen-antibody systems (Taliaferro *et al.*, 1959; Svehag, 1965b; Šterzl *et al.*, 1965; Finkelstein & Uhr, 1966; Hájek, 1968, 1969; Webster, 1968).

Some recent works (Finkelstein & Uhr, 1966; Webster, 1968) point out that early 19S antibodies possess a significantly higher avidity than early antibodies of the 7S type. Early 19S antibodies of the macroglobulin type against bacteriophage ΦX 174, produced on the 7th day following immunization were found in the experiments of Finkelstein and Uhr (1966) to be in average twice as avid as early 7S antibodies produced on the 14th day after immunization. Similar differences could be observed even in our model of neutralizing antibodies against bacteriophage ΦX 174 in newborn rabbits. Webster (1968) also described that early antibodies of the 19S type against influenza virus were highly avid contrary to early 7S antibodies with low avidity. Even the study on the intercellular transfer of molecules of immunoglobulin classes (Goodman & Massaites, 1960; Greenbury *et al.*, 1963) favours the higher avidity of 19S haemolytic antibodies as compared with 7S haemolysins. The conclusion, namely that γM antibody possesses a higher binding capacity (avidity) than γG antibody at the same stage of the immunization process may be explained by different capacity to bind antigen by 19S (γM) or 7S (γG) molecules of immunoglobulins. These differences are given by different patterns of the combining sites on the molecules of these immunoglobulins. Unlike the IgG molecule, the molecule of IgM antibody contains 5 to 6 combining sites (Miller & Metzger, 1964; Onoue *et al.*, 1965) so that the multiple binding with antigenic sites on the surface of the virus particle may be responsible for the higher avidity of 19S antibody. These results necessarily imply that the mutual comparison of the avidity of 19S and 7S antibodies without appropriate correction is not possible because for the above mentioned reasons it cannot be the same at the same stage of the immune process. However, an important finding is, and all the above mentioned authors agree with it, that the avidity of single types of antibodies when observed separately, increases in parallel with the length of the immunization process together with the increasing number of antigenic stimuli (Svehag, 1965b; Finkelstein & Uhr, 1966; Hájek, 1968, 1969; Webster, 1968).

The basis of an increase in avidity of antibodies during the antibody response, the value of which often amounts to even several orders of magnitude (Eisen & Siskind, 1964), may be best explained by differentiation processes that take place in the competent cell population following the antigen stimulation (Šterzl & Silverstein, 1967). By gradual differentiation and proliferation of immunologically competent cells, cells arise

which are capable of forming antibodies with an increasing affinity towards the antigen. An interesting study in this respect was made by Siskind *et al.* (1968) attempting to explain the cellular basis of the development of avidity of antibodies. When they immunized using a high dose of a hapten antigen, an antibody very soon occurred the avidity of which soon reached the peak and did not change further. However, when a low antigen dose was injected, the avidity of antibody continued to increase significantly in the course of the immunization process. In the first case one may assume that following a high antigen dose a rather fast differentiation without larger proliferation directly into antibody-forming cells took place, whereas in the second case an intensive proliferation process could be expected (Šterzl, 1966; Šterzl & Silverstein, 1967). Similar dependence was observed by Steiner and Eisen (1967). In our experiments in young rabbits we also found similar dependence of the development of the avidity of neutralizing antibodies following the injection of two different doses of bacteriophage ΦX 174 (10^{10} and 5×10^7 PFU). An original explanation of the whole process of development of avidity of antibodies in the dependence on the dose of antigen was given by Siskind *et al.* (1968). The increase of affinity in time and the higher increase of the avidity following the low antigen dose is explained by an enhanced selective pressure on the antibody-forming cells with a high affinity towards the antigen ("high affinity cells") at the low antigen concentration with respect to the cells with low affinity ("low affinity cells") that are stimulated only after an injection of a large amount of antigen.

References

Bowman, B. U., Jr., Patnode, R. A.: *Neutralization of bacteriophage ΦX 174 by specific antiserum.* J. Immunol. 92 : 507, 1964.

Eisen, H. N., Siskind, G. W.: *Variations in affinities of antibodies during the immune response.* Biochemistry 3 : 996, 1964.

Finkelstein, M. S., Uhr, J. W.: *Antibody formation. V. The avidity of γM and γG guinea pig antibodies to bacteriophage ΦX 174.* J. Immunol. 97 : 565, 1966.

Goodman, H. S., Massaites, L.: *The dissociation of hemolytic antibody from sensitized cells as measured by cell to cell transfer.* J. Immunol. 85 : 391, 1960.

Greenbury, C. L., Moore, D. H., Nunn, L. A. C.: *Reaction of 7S and 19S components of immune rabbit antisera with human group A and AB red cells.* Immunology 6 : 421, 1963.

Grey, H. H.: *Studies on changes in the quality of rabbit bovine serum albumin antibody following immunization.* Immunology 7 : 82, 1964.

Hájek, P.: *Properties of the neutralizing factors against T2 and ΦX 174 phages present in normal sera.* Fol. microbiol. 11 : 290, 1966.

Hájek, P.: *Properties of natural 19S antibodies in normal pig serum against the ΦX 174 and T2 phages.* Fol. microbiol. 12 : 551, 1967.

Hàjek, P.: Dependence of the neutralization activity of 19S and 7S antibodies on complement in the primary and secondary antibody response in infant rabbits to phage T2. Fol. microbiol. 13 : 557, 1968.

Hájek, P.: *Neutralization of bacterial viruses by antibodies of the young animals. I. Dependence of neutralizing activity of 19S and 7S antibodies on complement in the course of the primary and secondary response of young rabbits immunized with T2 phage.* Fol. microbiol. 14 : 165, 1969.

Hájek, P., Mandel, L.: *Antibody response of young animals to bacteriophages of different immunological behaviour: ΦX 174 and T2.* Fol. microbiol. 11 : 282, 1966.

Hall, C. E., Maclean, E. C., Tessman, I.: *Structure and dimension of bacteriophage ΦX 174 from electron microscopy.* J. mol. Biol. 1 : 192, 1959.

Jerne, N. K.: *Study of avidity based on rabbit skin response to diphtheria toxin-antitoxin mixtures.* Acta path. microbiol. Scand., suppl. 2, 87 : 3, 1951.

Jerne, N. K., Avegno, P.: *The development of the phage neutralizing properties of serum during the course of specific immunization of animal: reversible and irreversible inactivation.* J. Immunol. 76 : 200, 1956.

Miller, F., Metzger, H.: *Characterization of a human macroglobulin. I. The molecular weight of its subunits.* J. biol. Chem. 240 : 3325, 1964.

Onoue, K., Yagi, Y., Grossberg, A. L., Pressman, D.: *Number of binding sites of rabbit macro-*

globulin antibody and its subunits. Immunochemistry 2 : 401, 1965.

Siskind, G. W., Dunn, P., Walker, J. C.: *Studies on the control of antibody synthesis. II. Effect of antigen dose and suppression by passive antibody on the affinity of antibody synthetized.* J. exptl. Med. 127 : 55, 1968.

Steiner, L. A., Eisen, H. N.: *Sequential changes in the relative affinity of antibodies synthesized during the immune response.* J. exptl. Med. 126 : 1161, 1967.

Styk, B., Závada, J., Hána, L.: *The role of non-specific serum factors in the antibody response to ΦX bacteriophage.* Fol. microbiol. 9 : 263, 1964.

Svehag, S. E.: *The formation and properties of poliovirus-neutralizing antibody. IV. Normal antibody and early immune response of rabbit origin: a comparison of biological and physicochemical properties.* J. exptl. Med. 119 : 517, 1964.

Svehag, S. E.: *The formation and properties of poliovirus-neutralizing antibody.* Regent Boktryckeri, Stockholm, 1965a.

Svehag, S. E.: *The formation and properties of poliovirus-neutralizing antibody. V. Changes in the quality of 19S and 7S rabbit antibodies following immunization.* Acta path. microbiol. Scand. 64 : 103, 1965b.

Šterzl, J.: *Immunological tolerance as the result of terminal differentiation of immunologically competent cells.* Nature 209 : 416, 1966.

Šterzl, J., Silverstein, A. M.: *Developmental aspects of immunity.* Adv. Immunol. 6 : 337, 1967.

Šterzl, J., Mandel, L., Miler, I., Říha, I.: *Development of immune reactions in the presence or absence of an antigenic stimulus.* In: Molecular and Cellular Basis of Antibody Formation, p. 351, Publ. House Czech. Acad. Sci., Prague 1965.

Taliaferro, W. H., Taliaferro, L. G., Pizzi, A. K.: *Avidity and intercellular transfer of hemolysin.* J. infect. Dis. 105 : 197, 1959.

Talmage, D. W.: *The kinetics of the reaction between antibody and bovine serum albumin using Farr method.* J. infect. Dis. 107 : 115, 1960.

Tromans, W. J., Horne, R. W.: *Structure of bacteriophage ΦX 174.* Virology 15 : 1, 1961.

Webster, R. G.: *The immune response to influenza virus. III. Changes in the avidity and specificity of early IgM and IgG antibodies.* Immunology 14 : 39, 1968.