negatively charged heparin and thus present to the blood stream, at least initially, a surface rich in heparin.

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Production of a Polysaccharide by *Staphylococcus aureus.* III. Action of Penicillins and Polysaccharides on Enzymic Lysis. (29236)

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It was previously reported(1) that a polysaccharide component of *Staphylococcus aureus* accumulates within cells that are grown in the presence of subinhibitory concentrations of penicillin G or nafcillin. Further study(2) suggested that subinhibitory levels of the semisynthetic penicillins nafcillin, oxacillin and methicillin produce a disturbance or disorganization of the cell wall fabric of *S. aureus* resulting in an intracellular accumulation of the polysaccharide. In addition, treatment with nafcillin renders the normally lysozyme-resistant cells of *S. aureus* susceptible to partial lysis by lysozyme, and the concomitant addition of trypsin results in further dissolution of the cell bodies.

In this present work, by a comparative study of the penicillins nafcillin, oxacillin and cloxacillin, the following problems have been investigated: (a) effect of penicillin level on synthesis of the polysaccharide; (b) basis for superiority of one penicillin over another penicillin in production of the polysaccharide; (c) enzymic lysis of cells previously treated with various levels of the penicillin; and (d) effect of the polysaccharide and related polysaccharides on the enzymic lysis of nafcillin-treated cells.

Materials and methods. A penicillin resistant strain CHP of *S. aureus* was used. Conditions for growth of the organism and production and isolation of the polysaccharide have been described(1). Briefly, the organism was grown at 37°C for 18 hours on the surface of a casein hydrolysate-agar medium. Cells were harvested from the medium by centrifugation and washed twice with distilled water. They were suspended in saline that was standardized to contain 8.0×10^{10} cells/ml, were autoclaved at 120°C for 10 minutes, and were cooled and centrifuged at 3,000 rpm for one hour at 5°C. The supernatant fluid containing the crude polysaccharide was collected and titrated by a turbidimetric procedure(3).

Crystalline egg white lysozyme (Armour Laboratories), lysozyme substrate in the form of powdered, ultraviolet-treated cells of *Micrococcus lysodeikticus* (Difco Laboratories), crystalline trypsin, and soybean trypsin inhibitor (Worthington Laboratories) were used. Potassium hyaluronate prepared from a Group A *Streptococcus pyogenes* according to a method recently described(4) and chondroitin sulfate (Mann Laboratories) were also tested.

Two different preparations of staphylococcal polysaccharide were studied. Staphylococcal polysaccharide I was prepared in accordance with methods previously described(1). Staphylococcal polysaccharide II was prepared as follows: *S. aureus* CHP was grown for 18 hours in medium originally containing 0.1 μg/ml nafcillin and the cells were harvested from the medium with distilled water. Cell preparations standardized to contain 8.0 $\times$ 10^{10} cells/ml were incubated with lysozyme (20 μg/ml) and trypsin (10 μg/ml) for 18 hours at 37°C. This procedure resulted in a virtually complete dissolution of the cell bodies of *S. aureus*. Insoluble material was removed by centrifugation and the polysaccharide in the highly viscous supernatant fluid was precipitated by addition of 3 volumes of acetone. A white, voluminous clot which formed was removed and dissolved in distilled water. The crude polysaccharide was dialyzed for 72 hours at 5°C against frequent changes of distilled water and was then freeze-dried. Reconstituted preparations of polysaccharide II reacted with acidified horse serum to form a clot, and the polysaccharide was readily depolymerized by a bovine testicular hyaluronidase preparation with a potency of 840 USP units/mg.

The lytic action of lysozyme and trypsin was studied with cell preparations of (1) untreated bacteria suspended in 0.1 M phosphate buffer (pH 7.0) and (2) bacteria previously grown for 18 hours at 37°C in subinhibitory levels of the penicillins. The latter were harvested with distilled water, centrifuged, washed twice with distilled water, and suspended in 0.1 M phosphate buffer (pH 7.0).

For the turbidimetric study of lysis, suspension densities of penicillin-treated and untreated bacteria were adjusted to give final readings of 400 on the Klett-Summerson colorimeter (red filter No. 66) and the standardized bacterial suspensions were then placed in a water bath at 37°C. To these tubes, 1.0 ml of enzyme appropriately diluted in 0.1 M phosphate buffer (pH 7.0) was added and the contents were thoroughly mixed. Tubes were incubated for various intervals in a 37°C water bath and read for changes in optical density.

The effect of staphylococcal polysaccharides and related polysaccharides on enzymic lysis of *S. aureus* was studied as follows: Lysozyme (20 μg/ml) and trypsin (10 μg/ml) were mixed with each of the polysaccharides in small volumes and incubated for 30 minutes in a 37°C water bath. After incubation, the polysaccharide-enzyme solutions were added to Klett tubes containing either distilled water suspensions of *M. lysodeikticus* or standardized suspensions of *S. aureus*. Turbidity measurements were taken at various intervals after adding the polysaccharide-enzyme mixtures to the substrate suspensions. Control tubes containing the enzymes and substrates were run concomitantly with each test system.

Electron micrographs were taken with an RCA EMU-3G Electron Microscope equipped with an objective aperture. Bacterial suspensions were washed twice with distilled water and suspended in saline. A small drop of suspension suitably diluted with distilled water was mounted on formvar-coated grids and allowed to dry. Preparations were shadowed at a 30° angle with platinum-palladium. All work was done at the initial magnification of 9000 $\times$ and enlarged photographically to 18,000 $\times$.

Results. Effect of penicillin on accumulation of polysaccharide. During the early investigations it became apparent that the amount of polysaccharide accumulating within the cell was dependent on the subinhibitory concentration of the penicillin. Of the penicillins tested, nafcillin produced the highest level of the polysaccharide. The yields of polysaccharide, in general, increased progressively with the concentration of the penicillin, short of the minimal inhibitory concentration. Since nafcillin, oxacillin and cloxacillin possessed essentially identical inhibitory activity against *S. aureus* CHP *in vitro* (Fig. 1), and assuming that such inhibition was due to the accumulation of polysaccharide among other things, it was of interest to study the effect of various subinhibitory concentrations of these penicillins on the release of polysaccharide from autoclaved suspensions of cells.

Fig. 2 illustrates the results. More polysaccharide accumulated in the presence of nafcillin than in the presence of comparable concentrations of oxacillin or cloxacillin. Peak levels were attained when 0.1-0.2 μg/ml nafcillin was incorporated in the medium. An

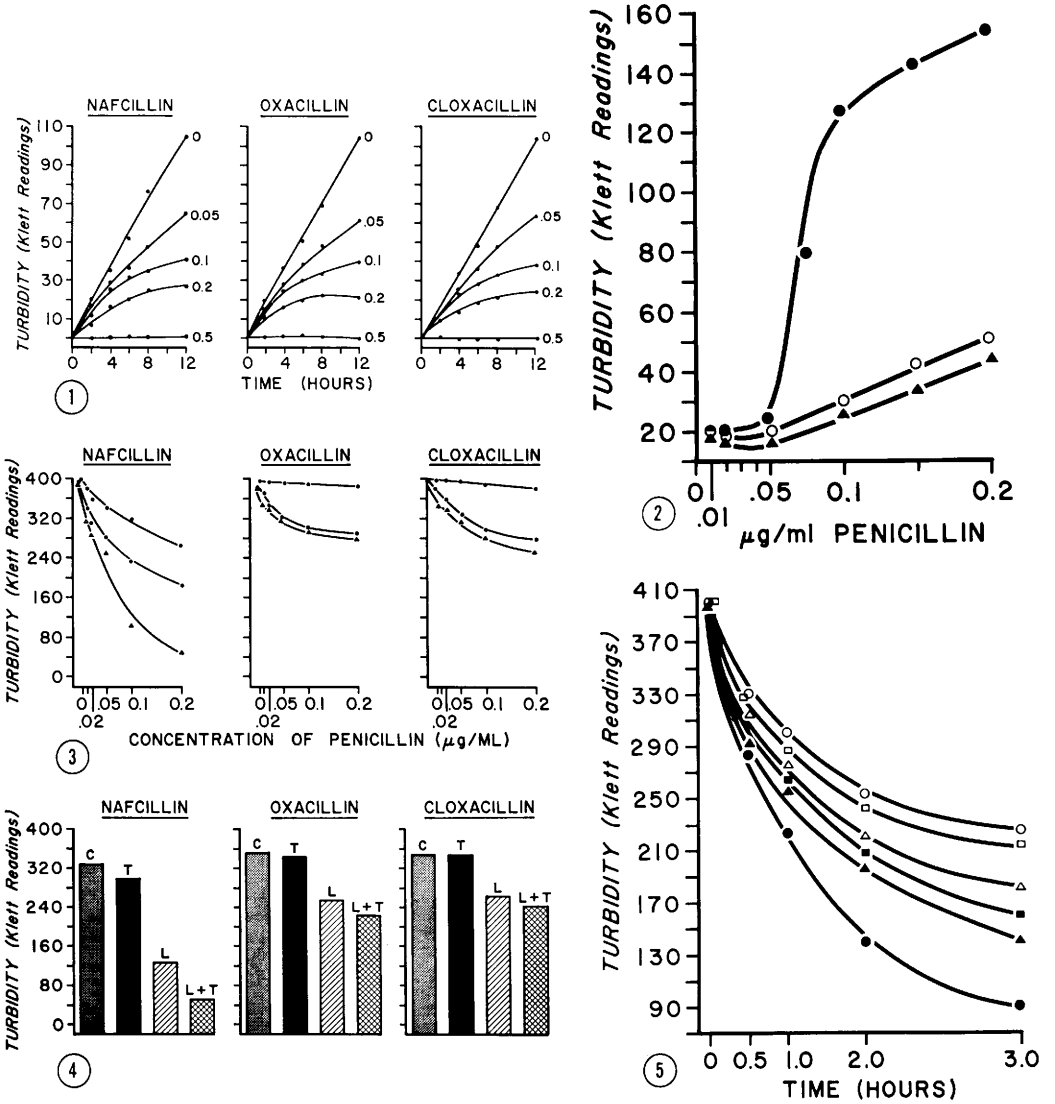

FIG. 1. Effect of penicillins on growth of *S. aureus* strain CHP. Concentrations in μg/ml.

FIG. 2. Concentration of polysaccharide in 18 hr culture of *S. aureus* strain CHP grown in medium containing subinhibitory concentrations of penicillins. ●—●, nafcillin; ○—○, oxacillin; ▲—▲, cloxacillin.

FIG. 3. Effect of previous penicillin treatment on subsequent lysis of *S. aureus* strain CHP by trypsin and/or lysozyme. ●—●, lysozyme (20 μg/ml); ○—○, trypsin (10 μg/ml); △—△, lysozyme + trypsin. Cell suspension + enzyme(s) incubated 3 hr at 37°C.

FIG. 4. Lysis of penicillin-treated suspensions of *S. aureus* after incubation for 18 hr at 37°C in presence of lysozyme (L), 20 μg/ml; trypsin (T), 10 μg/ml; enzyme-free control (C). Penicillin concentration 0.1 μg/ml.

FIG. 5. Inhibition of enzymic lysis of nafcillin-treated cells of *S. aureus* strain CHP by polysaccharides and trypsin inhibitor. ●—●, cells incubated with lysozyme (20 μg/ml) + trypsin (10 μg/ml); ▲—▲, chondroitin sulfate (2 mg/ml); ■—■, polysaccharide I (2 mg/ml); △—△, Strep. hyaluronate (2 mg/ml); □—□, polysaccharide II (2 mg/ml); ○—○, trypsin inhibitor (0.1 mg/ml).

appreciable polysaccharide response did not occur for oxacillin or cloxacillin at these concentrations.

To determine whether the remarkable difference in the accumulation of the polysaccharide by the penicillins could be attributed to the bactericidal activity characteristic for each antibiotic, extensive penicillin-bactericidal studies were carried out. Employing a method described by Roberts *et al*(5) for evaluating the bactericidal activity of antibiotics, it was found that in the concentrations studied the bactericidal activity of the 3 penicillins was not significantly different. Thus, there was no evidence that the death rate of *S. aureus* strain CHP due to nafcillin could account for the significant increase in the accumulation of the polysaccharide caused by this penicillin.

Lysis experiments with S. aureus. Penicillin-treated suspensions of *S. aureus* in 0.1 M phosphate buffer (pH 7.0) were incubated for 3 hours at 37°C with (1) crystalline trypsin (10 μg/ml); (2) crystalline lysozyme (20 μg/ml) and (3) lysozyme + trypsin. Turbidity readings of control and enzyme-treated suspensions of *S. aureus* grown in the absence of the penicillins showed no significant differences. Addition of trypsin to nafcillin-treated suspensions brought about a significant decrease in turbidity when the organism was grown in the presence of 0.2 μg/ml of the antibiotic; no such change occurred with comparable concentrations of cloxacillin or oxacillin (Fig. 3). Incubation of suspensions of penicillin-treated *S. aureus* with lysozyme resulted in a small but consistent decrease in turbidity for oxacillin and cloxacillin and enhanced lysozyme lysis of nafcillin-treated cells. The course of lysis of suspensions of *S. aureus* after treatment with cloxacillin and oxacillin and incubation with lysozyme and trypsin indicated that turbidity was not significantly affected by the presence of trypsin. In contrast, a rapid fall in turbidity resulted when these enzymes were incubated with nafcillin-treated cells (Fig. 3).

The finding that treatment of *S. aureus* with nafcillin significantly increased the lytic action of lysozyme upon the addition of trypsin whereas oxacillin and cloxacillin showed neither a potentiation nor even a simple additive effect, suggested an experiment to determine whether the relative course of lysis of the penicillin-treated cells by the enzymes could be altered following a prolonged incubation period. In Fig. 4, suspensions of *S. aureus* treated with cloxacillin and oxacillin show a low rate of lysis with lysozyme. However, repeated attempts to enhance this lysis during 18 hours' incubation at 37°C with trypsin were without success. Under conditions similar to those used with the other penicillins, nafcillin-treated cells were significantly lysed following incubation with lysozyme, and the concomitant addition of trypsin produced a potentiation of this response.

Effect of polysaccharide on enzymic lysis of S. aureus. The virtually complete loss of resistance to lysis with lysozyme and trypsin that *S. aureus* cells undergo following the growth of the organism in the presence of subinhibitory concentrations of nafcillin has been explained in terms of an interference with the cell wall synthesizing mechanism, which prevents incorporation of the polysaccharide into the wall(2). Therefore, a study was designed to determine whether enzymic lysis of *S. aureus* could be blocked by the staphylococcal polysaccharide and related polysaccharides; this would offer a possible mechanism whereby untreated bacteria overcome lysis by these enzymes. Since earlier experiments(2) showed that addition of trypsin to nafcillin-treated suspensions of lysozyme-lysed *S. aureus* resulted in a rapid fall in turbidity, lysis experiments in the presence of trypsin soybean inhibitor were also carried out.

The results of a representative experiment demonstrated that partial inhibition of enzymic lysis of *S. aureus* could be achieved with the various polysaccharides. Polysaccharide II and streptococcal hyaluronate produced a high inhibitory activity (Fig. 5). Inhibitory activity of a lesser magnitude was obtained with polysaccharide I and chondroitin sulfate. Additional studies revealed appreciable inhibition in lysis when the trypsin-soybean inhibitor mixture was incubated at 37°C for 20 minutes prior to the addition of the nafcillin-treated cell suspension. More-

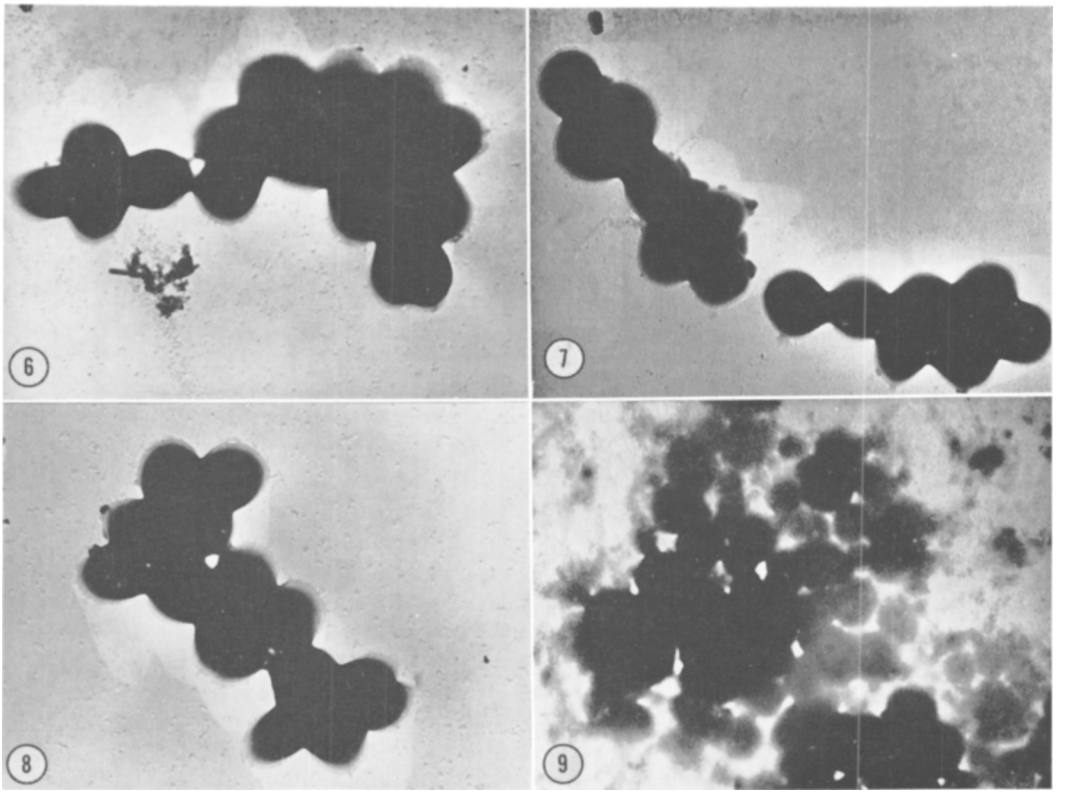

FIG. 6. Normal cells of *S. aureus* strain CHP. Magnification ×12,600.
FIG. 7. Nafcillin-treated cells of *S. aureus* strain CHP. Magnification ×12,600.
FIG. 8. Normal cells of *S. aureus* strain CHP after exposure to lysozyme (20 μg/ml) and trypsin (10 μg/ml) for 90 min at 37°C.
FIG. 9. Nafcillin-treated cells of *S. aureus* strain CHP after exposure to lysozyme (20 μg/ml) and trypsin (10 μg/ml) for 90 min at 37°C.

over, the lack of any significant effect by any of the polysaccharides on lysozyme when the enzyme-polysaccharide mixtures were added to *M. lysodeikticus* substrate would appear to indicate a direct effect of the polysaccharides on trypsin.

Effect of lysozyme and trypsin on cell structure of S. aureus. Electron microscopical observations of normal and nafcillin-treated suspensions of *S. aureus* are shown in Fig. 6 and 7, respectively. Normal cells treated with trypsin and lysozyme for 90 minutes (Fig. 8) showed no change in appearance when compared with the untreated cells. The results of typical experiments with the enzyme mixture incubated for 90 minutes with nafcillin-treated cells are shown in Fig. 9. It is apparent that such treatment altered or destroyed the cell wall, allowing the cell contents to disperse and leaving behind the cell bodies. Concurrently with a loss of electron-dense material, every field that was examined showed a large amount of debris and cell fragments.

Discussion. From previous work it has been concluded that subinhibitory levels of a number of penicillins can serve in some manner in enhancing the polysaccharide content of the cells of *S. aureus* and making this available for release when the cells are subjected to heat or sonic disruption. Preliminary enzymic lysis studies, as well as electron micrographic observations, suggested a relationship between accumulation of the polysaccharide and actual interference with cell wall synthesis. The experiments reported here support these conclusions.

The interesting differences between the properties of nafcillin and the isoxazole penicillins in accumulation of the polysaccharide

and in degree of resistance to lysis with lysozyme and trypsin remain unexplained. Since previous studies(6) showed that nafcillin possesses more effective bactericidal and bacteriolytic properties than oxacillin, it might be argued that nafcillin renders both the cell walls and protoplasmic contents of *S. aureus* more susceptible to enzymic lysis by virtue of a quantitative difference in these properties. However, it has been found that nafcillin, oxacillin and cloxacillin have identical antibacterial and bactericidal activities against this strain of *S. aureus* in the concentrations used. It would appear, therefore, that an inherent property of nafcillin inhibits synthesis of cell wall polymers sufficiently to allow the penetration or action of lysozyme molecules, with subsequent dissolution of the protoplasmic contents following addition of trypsin. A similar potentiation of lysozyme activity by trypsin was found by other investigators(7,8) when certain heated, lysozyme-resistant bacterial species were employed. The limited lytic action of lysozyme on cells treated with oxacillin and cloxacillin, as well as the resistance of these cells to further lysis by trypsin, leads to the conclusion that the extent of physical disorganization of the bacterial cells varies with the penicillin studied, thereby permitting different thresholds of accessibility or alteration of the substrate to lysozyme action. This observation is substantiated by electron microscopic results, which show extensive disruption of the cell walls and whole cells of nafcillin-treated *S. aureus* following an exposure to lysozyme and trypsin.

The resistance of living and heat-killed cells of *S. aureus* to digestion by proteolytic enzymes such as trypsin may be governed in part by the polysaccharide component of the cell walls of these organisms. In support of this view is the finding that the staphylococcal polysaccharides released from nafcillin-treated cells by heat or enzymatic treatment neutralize to an appreciable extent the trypsin effect. In addition, related polysaccharide constituents, *i.e.*, streptococcal hyaluronate and chondroitin sulfate, effectively inhibit trypsin activity. It should be pointed out that our experimental procedure did not reveal that lysozyme activity could be blocked by these large polymers.

Although caution must be exercised in interpretation or use of data from *in vitro* studies when such information is to be ascribed as analogous to that found in the host-parasite relationship, it is attractive to speculate on the significance of the marked difference between nafcillin and the isoxazole penicillins in rendering cells vulnerable to enzymic lysis. The problem of antibiotic-resistant staphylococci brings into focus the frequently overlooked concept that among the determinants in establishment of an infection, host factors may be as important as the virulence of the infecting microorganism. Penicillins, or any chemotherapeutic agent, even in subinhibitory concentrations, may damage a microbial cell to the point where host factors may successfully take over during an infection. Thus it is conceivable that the superior therapeutic activity of nafcillin against experimental infections in mice(9) may in part be attributed to a selective advantage of this penicillin, which in subinhibitory concentrations may mediate a favorable interplay between the tissue environment and the parasite.

Summary. 1. The resistance of *S. aureus* to lysis by lysozome and trypsin was abolished by exposing growing cells to subinhibitory concentrations of nafcillin. This observation, together with the results of electron microscopic studies of nafcillin-treated cells exposed to maximal enzymatic action, reflect an actual interference with cell wall synthesis. 2. Although the precise cytological action of the subinhibitory levels of the penicillins on the integrity of the cell wall is not known, the limited lytic action of lysozyme on cells treated with oxacillin and cloxacillin, as well as the resistance of these cells to further lysis by trypsin, suggest at least a quantitative difference between nafcillin and the other penicillins in the disorganization of the cell wall structure. 3. On the basis of the experiments described, evidence is presented which indicates that subinhibitory levels of nafcillin produce an alteration of cell wall structure, thereby increasing permeability and rendering the cell wall and proto-

plasmic constituents accessible to the action of lysozyme and trypsin.

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Influence of Thymectomy on Tumor Induction by Polyoma Virus in C57BL Mice. (29237)

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Polyoma virus will induce tumors in many strains of mice when inoculated in the neonatal period(1). Resistance to isogenic transplants of polyoma tumor cells is evident in adult mice which have been inoculated with virus at birth(2,3). It is also evident in mice injected with virus in adult life(2,3) or with cells of a histoincompatible polyoma tumor (4). Such a resistance is not dependent on the presence of viral antibodies since passive transfer of a heterologous immune serum will not give protection(2). Apparently resistance can be transferred to normal recipients by means of lymphoid cells from immunized donors(5). To explain these phenomena, it has been postulated that the polyoma virus transforms normal cells to tumor cells in both newborn and adult mice, that the transformed cells contain a distinct cellular antigen deficient or absent in normal host tissues, that the immunologically immature newborn tolerates this new antigen thus allowing tumor growth, and that the immunologically mature adult is capable of rejecting the antigenic tumor cells by a mechanism similar to classical transplantation immunity.

A strain resistance to polyoma virus has been observed in C57BL mice(6). Such intact mice are highly insusceptible to the oncogenic effect of all strains of polyoma virus tested, even when inoculated on the day of birth(7). However salivary gland epithelium of C57BL mice in organ culture, shows the same proliferative response as C3H tissues (8) and is readily transformed to a neoplastic state(9). It is not known whether the low susceptibility of C57BL mice to viral oncogenesis has an immunologic basis. Since mice thymectomized in the neonatal period are incapable of responding to foreign cellular antigens(10), the influence of thymectomy on the oncogenic activity of polyoma virus in C57BL mice was investigated.

Materials and methods. Mice. Inbred C57BL/KaLw mice of either sex were used. This strain was obtained from Dr. Henry Kaplan in 1951 and has been inbred here continuously since that time by brother to sister matings. Salivary gland tumors within the breeding colony have not been observed.

Virus. Dulbecco's large plaque polyoma virus strain(11) derived from LID-1, originally obtained from Dr. W. P. Rowe of Nat. Inst. of Health, was grown on C3H mouse embryo cells. The virus used for inoculation was a 40-fold diluted material, 0.05 ml being

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