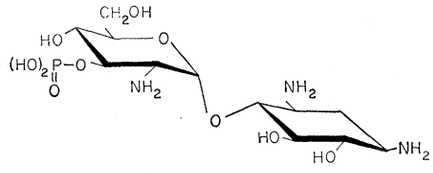
for $C_{12}H_{24}N_3O_7 \cdot PO(OH)_2 \cdot 2H_2O$, are C, 32.80; H, 6.88; N, 9.56; O, 43.70; P, 7.05. The crystalline inactivated paromamine consumed 1.6 moles of periodate at *p*H 4.05 in 24 hours. The hydrolysis of the periodate-oxidized inactivated paromamine gave D-glucosamine, but not deoxystreptamine. These data indicate that the 3-hydroxyl group of D-glucosamine moiety of paromamine is phosphorylated.



When the reaction mixture of inactivated kanamycin was treated by alkaline phosphatase, the recovery of kanamycin was not 100 percent; and the percentage recovery was higher when the concentrations of antibiotic and ATP in the reaction mixture were lower. When the reaction mixture containing 1 mmole of kanamycin and 8 mmole of ATP per liter was inactivated and the solution was diluted tenfold, treatment with alkaline phosphatase resulted in 60 percent recovery of kanamycin.

Adenosine triphosphate (ATP) was the absolute requirement for the inactivation reaction and addition of coenzymes (coenzyme A, nicotinamide-adenine dinucleotide, nicotinamide-adenine dinucleotide phosphate, flavin mononucleotide, flavin-adenine dinucleotide, folic acid), acetate, and amino acids did not show any enchancement of the inactivation reaction. Adenosine triphosphate could not be replaced by adenosine mono- or diphosphate, guanosine triphosphate, or uridine triphosphate.

Dihydrostreptomycin was inactivated by the kanamycin-inactivation system. Concentrations of dihydrostreptomycin and ATP in the reaction mixture were 100 μg/ml and 4 μmole/liter. After incubation at 37°C for 20 hours, the solution was heated to 90°C for 5 minutes and treated by alkaline phosphatase. This gave a 100 percent recovery of the activity. Thus, this strain of *E. coli* showed a phosphorylative inactivation of dihydrostreptomycin.

The relation between phosphorylation and resistance was shown by the fact that *E. coli* K12 and its resistant subcultures did not give an enzyme solution that inactivated kanamycin. *Escherichia coli* K12 was plated on a nutrient agar containing kanamycin, and a subculture which grew in a medium containing kanamycin (5 μg/ml) was obtained. Another resistant culture which grew in a medium containing kanamycin at 80 μg/ml was obtained by the successive transfers into a medium containing kanamycin. The enzyme solutions prepared from these resistant strains which had no R factor did not show inactivation of kanamycin.

Thus, *E. coli* that carries R factor produces an enzyme or enzymes which phosphorylate the 3-hydroxyl group of 2- or 6-amino-2- or 6-deoxy-D-glucose. Though it is not certain whether one enzyme alone catalyzes these reactions and whether the same enzyme catalyzes the same reaction on 2,6-diamino-2,6-dideoxy-D-glucose in neomycins, the isolation of the phosphorylated compounds and the dependence of the inactivation reaction on ATP indicates that phosphorylation is one of modes of inactivation of antibiotics by Gram-negative organisms carrying R factor.

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3. Obtained from *Crotalus adamanteus* venom Type II, purchased from Sigma Chemical Company.

27 June 1967

Antibody-Producing Cells in Division

Abstract. *Cells producing antibody were detected by a modified Jerne plaque technique. Of plaques initially containing a single central cell, 2.6 percent (45/1742) later had two. Mitosis of cells producing antibody was directly observed, with a mitotic time of not less than 40 minutes for the one mitosis timed at 37°C.*

A typical antibody response to a particulate antigen is characterized by the production of γM-globulin antibody followed shortly by γG-globulin. The Jerne hemolytic plaque technique (*1*) permits detection of antibody-producing cells during the γM phase of the response. The total number of these cells increases several thousandfold in the spleens of mice during a typical response. (We consistently observe that the number of antibody-producing cells doubles every 5 to 6 hours during the period 24 to 96 hours after cow red blood cells are given as antigen.) Cell division or cell recruitment, or both, could account for this increase (*2*). The experiments described here show that cells already known to be producing specific antibody do divide during the primary response, and they suggest that these cells continue to synthesize antibody after division.

We used a modified Jerne plaque technique which permits the direct observation of antibody-producing cells for many hours after they have formed hemolytic plaques (*3–7*). Plaques with central cells in good focus and with no nearby cells were selected for detailed study. In this way, it was ensured that hemolysis was the result of antibody made by central cells. Plaques, first observed after 30 minutes of incubation at 37°C, were numbered and inspected at intervals. The inspection part of the procedure was carried out at room temperature, since one experiment entirely at 37°C showed no qualitative or quantitative differences in the results at the two temperatures. Most observations were made with spleen cells taken from animals 72 hours after antigen administration, a time when the total number of antibody-producing-cells is still increasing logarithmically. Since observations made at other times (2¼ to 4 days) during the primary response do not differ importantly from these, all are described together.

When first observed, most of the plaques contained single central cells, but 4.8 percent (83/1742) already had two cells at this time. During the subsequent observation period (usually 4 to 5 hours), 2.6 percent (45/1742) of the single cells divided and gave two cells. All but one of the newly arising cell pairs remained in contact, although several of these pairs of cells showed vigorous amoeboid activity. In the instance when we did observe a

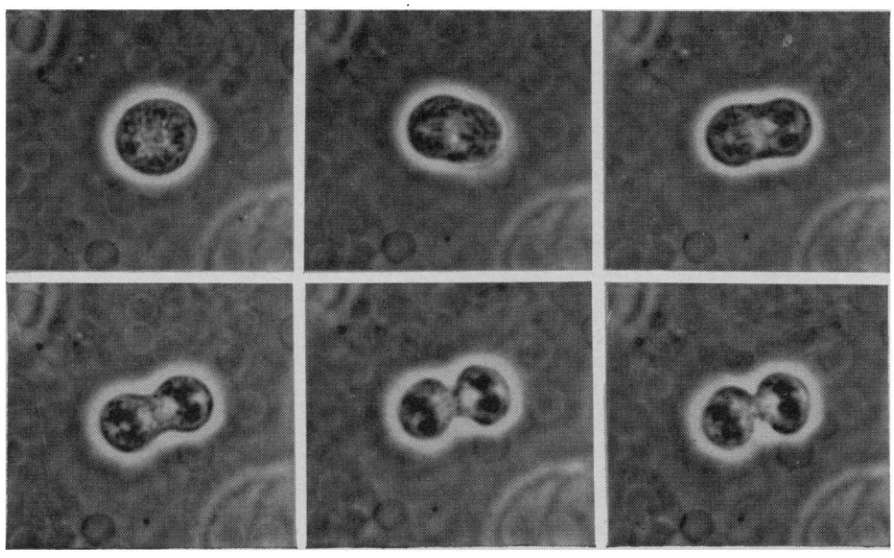

Fig. 1. Mitosis of a mouse spleen cell known to be producing specific antibody against cow red cells. Red cells ghosts approximately 6 μ in diameter are apparent in the background. The first and last pictures were taken about 25 minutes apart, at room temperature with a phase-contrast oil immersion lens (40 $\times$).

newly divided pair of cells migrate apart in the agar, the hemolytic plaque showed an asymmetric increase in size, indicating that the cells continue to release or synthesize antibody after dividing.

Occasional daughter cells showed obvious unequal enlargement 2 to 3 hours after mitosis, and two plaques initially scored as containing two cells were later observed to contain three cells. Two plaques initially containing a single cell later contained four cells; the time interval in one case was 20 hours; in the other, only 1.5 hours. The extremely rapid result was seen only once, at a time when the cells could not be observed adequately under high magnification. A binucleate cell undergoing quadripolar division could produce four cells in this short time.

In the course of counting the central cells in plaques, we learned to recognize cells likely to divide. These were relatively large cells, very often with condensed chromosomes. Fourteen cells were observed continuously and photographed while in mitosis (Fig. 1). Five of the resulting cell pairs fused again to give binucleate cells. Some plaques initially scored as containing two cells were also observed to have fused to give a single cell after several hours. Binucleate plasma cells have been described by Langevoort (*8*) in the spleens of mice during the first few days of a primary response to horse γ-globulin.

Twelve of the 14 closely watched cells which went through mitosis had condensed chromosomes when first observed; in the other two cases the chromosomes condensed and the cells went through mitosis during the period of observation. In one case, a cell with condensed chromosomes did not divide; the chromosomes seemed to disappear before mitosis occurred. It would appear that, in general, the cells which divide in this experimental system are already in mitosis when first observed.

Direct observation entirely at 37°C permitted the determination of a minimum time for mitosis in one case. Condensed metaphase chromosomes were present in this cell at first observation and division occurred after 40 minutes. This particular cell pair fused again and was clearly binucleate after 3 hours.

The importance of cell division in the immune response has been demonstrated repeatedly in the past (see *9*). Our experiments show that cells which are actually producing antibody can divide and suggest that the cells continue to produce antibody after dividing.

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3. Mice are injected intraperitoneally with 0.1 ml of 50 percent suspension of cow red blood cells washed in saline. Spleens are removed at various times later, placed in petri dishes containing cold dilute media (*4*), and teased apart with dissecting probes. Single-cell suspensions, obtained by rapid pipetting with a Pasteur pipette, are diluted to contain 1000 to 3000 plaque-forming cells per milliliter. One gram of Difco Special Agar-Noble is dissolved by boiling in 100 ml of distilled water. Two grams of dry DEAE-(diethylaminoethyl) cellulose is added in the form of hydrochloride (*5*). The DEAE-cellulose is filtered off after brief boiling, and the resulting agar solution is kept at 45°C. Pasteur pipettes, selected to give 20 drops per milliliter, are used to deliver 8 drops (0.4 ml) of agar solution to tubes (10 by 75 mm) in a 45°C water bath. Two drops (0.1 ml) of five-times-concentrated medium (*4*) and 4 drops (0.2 ml) of a 50-percent suspension of cow blood cells in saline are added and mixed by gentle shaking. Eight drops (0.4 ml) of guinea pig serum (*6*) and 2 drops (0.1 ml) of the spleen cell suspension are added, and the tube contents are mixed by pipetting. Approximately half of the mixture is placed on a lantern slide glass (8.2 by 10.2 cm) with No. 1 cover slips glued to each corner (*7*). The mixture is immediately covered with a cut-down glass slide (8.2 by 9.0 cm). (During experiments in which antibody-producing cells were observed at higher magnifications, similarly sized No. 3 cover slips were used in place of the top lantern slide glass plates.) The remaining mixture is placed between a second pair of slides as rapidly as possible. The edges of the smaller top slides are sealed with melted petroleum jelly to prevent drying during the incubation. The plates are incubated at 37°C for the required time (1 hour for counting plaques, ½ hour for observing cell divisions) and are then kept at room temperature. Plaques are counted at low magnification (10 to 20×).
4. Five times (5×) concentrated medium, *p*H 7.4 when diluted, was made as follows: 10 ml of saline-dextrose solution consisting of 136 g of NaCl, 8 g of KCl, 4 g of $MgCl_2 \cdot 6H_2O$, and 20 g of anhydrous dextrose, with water to 1000 ml; 8 ml of Eagle minimum medium (MEM) 50× concentrated in 0.2*N* HCl, obtained from Microbiological Associates, Inc., Bethesda, Md.; 10 ml of buffer solution, consisting of 42 g of $Na_3PO_4 \cdot 12H_2O$, 8 g of $NaHCO_3$, and water to 1000 ml; 7.7 ml of glutamine-phenol red solution, consisting of 7.6 g of L-glutamine, 0.2 g of phenol red, N_2OH to *p*H 7, and water to 1000 ml; 4 ml of 100× concentrated vitamin solution (obtained from Microbiological Associates, Inc., Bethesda, Md.); 0.1 ml of streptomycin sulfate solution (0.2 g per milliliter of water); and 0.2 ml of buffered penicillin G_1 solution (5×10^5 unit/ml). In the experiments for observing cells in plaques, 4 ml of this 5× concentrated medium was supplemented with 0.8 ml of NCTC 109 (Microbiological Associates, Inc.), and used as if still 5× concentrated. The resulting slight degree of hypotonicity in the final agar gave plates in which the red cells were more normal in shape than when the agar was theoretically isotonic; there was no apparent difference in cell divisions in the two agars.
5. Diethylaminoethyl-cellulsose (DEAE), obtained from Schleicher and Schuell Co., Keene, N.H., as standard grade Selectacel No. 70. is suspended in concentrated HCl diluted about tenfold. After thorough washing with distilled water, the product is sucked dry on a Büchner funnel and dried at 70°C.
6. Guinea Pig Serum Complement (Fresh Frozen), Hyland Lab., Los Angeles, Calif.
7. The adhesive used was "Clear Seal," General Electric Silicone Products Dept., Waterford, N.Y.
8. H. L. Langevoort, *Lab. Invest.* **12**, 106 (1963).
9. J. C. Kennedy, J. E. Till, L. Siminovich, E. A. McCulloch, *J. Immunol.* **94**, 716 (1965).
10. This investigation, paper No. 1133 from the Laboratory of Genetics, University of Wisconsin, was supported by NIH (GM08217) and NSF (GB4362).

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