THE LEVEL OF LACTIC DEHYDROGENASE ACTIVITY AS AN INDICATOR OF THE GROWTH OF INFLUENZA VIRUS IN THE EMBRYONATE EGG*

By RICHARD KELLY‡ and DONALD GREIFF,§ Sc.D.

(From the Department of Pathology, Marquette University School of Medicine, Milwaukee)

(Received for publication, August 29, 1960)

The agglutination of erythrocytes by viral agents has been used extensively to quantitate, characterize, or purify certain viruses. This phenomenon has been useful also as an indicator for the detection of viruses of the myxoma group when determining infectivity titers in embryonate eggs (4). Misleading results of the above have been obtained sporadically because of milky chorioallantoic fluids or the presence of abnormal amounts of hemagglutination inhibitor which obscure or prevent hemagglutination respectively. During certain seasons we have encountered difficulty also in obtaining satisfactory red blood cells.

During recent and continuing investigations of the "enzyme profiles" associated with the growth of viruses and rickettsiae in embryonate eggs, we observed significant alterations in the levels of activities of certain enzymes in chorioallantoic fluids or yolk sacs. The marked increase in the levels of activity of lactic dehydrogenase in fluids from eggs infected with the PR8 strain of influenza virus suggested a new approach for determining the presence of this virus.

Materials and Methods

The coenzyme substrate solution used in this study was prepared by dissolving 8 mg. of sodium pyruvate and 100 mg. of dihydrodiphosphopyridine nucleotide (DPNH), Sigma type 1 (Sigma Chemical Co., St. Louis) in 100 ml. of a phosphate buffer (pH 7.5). This solution was stored at −38° until used.

The stock solution of dinitrophenylhydrazine, color reagent, was prepared by dissolving 200 mg. of 2,4-dinitrophenylhydrazine in 85 ml. of concentrated hydrochloric acid and diluting up to 1 liter with distilled water. This solution remained stable for several months under refrigeration.

* This investigation was supported, in part, by a research grant, C-3935, from the National Cancer Institute of the National Institutes of Health, United States Public Health Service and in part by the Atomic Energy Commission, Contract No. AT (11-1)-596.

‡ Predoctoral Trainee, Pathology Training Grant, 2G-170, Division of General Medical Sciences, National Institutes of Health, United States Public Health Service.

§ Senior Research Fellowship (SF-89) from the United States Public Health Service.

Quantitative Method.—The levels of lactic dehydrogenase were determined quantitatively by the colorimetric method developed by Berger and Broida (1). Each Berger-Broida unit of activity was equivalent to that amount of enzyme which caused a decrease of optical density, measured at 340 mu, of 0.001/minute in a reaction mixture of 3 ml.

Qualitative Method.—Lactic dehydrogenase was determined qualitatively by a modification of the colorimetric method of Berger and Broida (1). Samples of the chorioallantoic fluids to be tested (0.1 ml.) were placed in the depressions of a disposo-tray (Linbro Chemical Company, New Haven) and 0.3 ml. of coenzyme-substrate solution was added to each fluid. The reacting systems were incubated for 1 hour at 37°. Following incubation, 0.3 ml. of dinitrophenylhydrazine solution was added to each reactant. After 15 minutes at room temperature, 0.5 to 1.0 ml. of 1 N sodium hydroxide was added. Chorioallantoic fluids with minimal lactic dehydrogenase activities showed a dark brown color after several minutes; fluids possessing increased enzyme content did not change color.

To minimize false values caused by the release of lactic dehydrogenase by contaminating red blood cells, all eggs were chilled prior to the removal of chorioallantoic fluids.

RESULTS

The chorioallantoic sacs of a group of 11 day old chick embryos were inoculated with 0.1 ml. of a 10^{-6} dilution of a stock suspension of the PR8 strain of influenza virus ($ID_{50} = 10^{-8.5}$). Twenty-four hours after inoculation of virus, and at 12 hour intervals thereafter, chorioallantoic fluids were removed from each of 10 non-infected and 10 infected eggs. The levels of lactic dehydrogenase were determined quantitatively. The means of the values obtained are shown in Fig. 1.

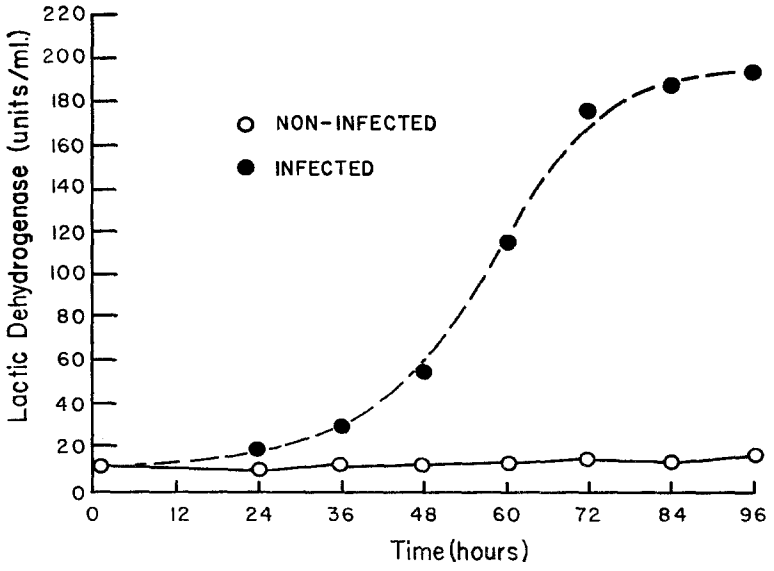

FIG. 1. The relation between time and the levels of lactic dehydrogenase activity of chorioallantoic fluids from non-infected and infected (influenza virus) embryonate eggs.

Twenty-four hours post inoculation the mean lactic dehydrogenase activity of the fluids from infected eggs was 3 times greater than that of fluids from non-infected eggs. The mean values followed a sigmoid curve with the greatest rates of change occurring between 48 and 72 hours after inoculation of virus. The mean values of the fluids from the infected eggs at the 72nd hour or later were approximately 18 times greater than those of the non-infected control.

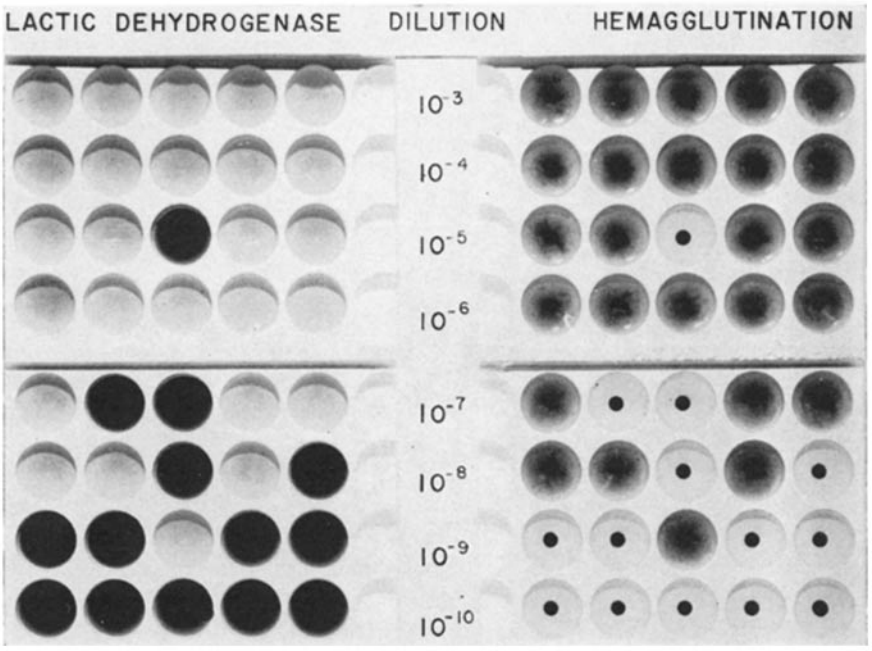

FIG. 2. Comparison of infectivity titers as determined by the qualitative color test for lactic dehydrogenase and by hemagglutination. The colorless chorioallantoic fluids are positive for lactic dehydrogenase, and therefore, positive for infection; the fluids showing the typical diffuse pattern of red blood cell agglutination are positive for influenza virus.

The mean values for lactic dehydrogenase of the fluids from non-infected eggs did not vary significantly during the course of the experiment.

To determine the correspondence between the detection of virus by hemagglutination and by qualitative changes in lactic dehydrogenase content, a group of embryonate eggs incubated for 11 days was divided into several series of 10 eggs each. One series was used as a control. The remaining series were inoculated with 0.1 ml. of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} dilutions of stock virus. Seventy-two hours after inoculation, all eggs were tested simultaneously for the presence of virus by the ability of their chorioallantoic fluids to agglutinate chicken red blood cells and by qualitative changes in lactic dehydrogenase content of the fluids. A 1:1 correspondence was observed.

In another series of experiments, using the observations above, infectivity titers of stock suspensions of virus were determined by making tenfold dilutions of virus in physiological saline and injecting 0.1 ml. of each dilution into the chorioallantoic sacs of each of 5 eggs (2). Fluids were tested for infection 72 hours later by the presence of hemagglutinins and by qualitative changes in lactic dehydrogenase content. In these experiments identical infectivity titers were found (Fig. 2). During the last several months routine infectivity titers in our laboratories have been determined by both hemagglutination and by the enzyme test; identical titers have been obtained in all tests.

DISCUSSION

The reaction catalyzed by the enzyme lactic dehydrogenase is as follows:

$$\text{pyruvic acid} + \text{DPNH} + \text{H}^+ \xrightarrow{\text{(lactic dehydrogenase)}} \text{lactic acid} + \text{DPN}^+$$

In the presence of an excess of pyruvic acid and DPNH the speed or rate of the reaction above is proportional to the amount of lactic dehydrogenase in the system to be tested. Because pyruvic acid reacts with 2,4-dinitrophenylhydrazine to form an intensely colored hydrazone we can accurately determine that part of the pyruvic acid that is not converted to lactic acid by lactic dehydrogenase.

By varying the amounts of chorioallantoic fluids and the times of incubation, parameters of a reacting system were established in which the concentrations of lactic dehydrogenase present in the fluids of non-infected embryonate eggs were sufficient to convert only minimal amounts of pyruvic acid to lactic acid. Under similar conditions, the high levels of lactic dehydrogenase present in fluids from infected eggs reduced completely the pyruvic acid to lactic acid. Thus the presence or absence of color in the reactants divides accurately non-infected (color present) from infected (color absent) embryonate eggs.

Several enzymes, including lactic dehydrogenase, have been reported to increase in human body fluids concurrently with infarction, neoplasia, hemolysis, and tissue injury (6, 7). In general, these increases have been attributed to the liberation of intracellular enzymes from necrotic tissues. Cytopathology resulting from the synthesis of virus in the extra-embryonic membranes of the embryo or the action of toxic agents liberated during the growth of influenza virus in embryonate eggs (3) may be responsible for the increased amounts of lactic dehydrogenase in the chorioallantoic fluids.

An increased turnover of the pentose cycle with the simultaneous synthesis of lactic dehydrogenase functioning as a TPNH-oxidizing enzyme has been reported to occur in virus-infected chorioallantoic membranes (5). Simple diffusion or release from injured cells may account for the high levels of lactic dehydrogenase activity in chorioallantoic fluids of infected eggs.

SUMMARY

The relation between time and the levels of lactic dehydrogenase activity of chorioallantoic fluids from embryonate eggs infected with the PR8 strain of influenza virus were determined quantitatively. The mean values, based on 10 determinations for each time interval, followed a sigmoid curve, with the greatest rates of change occurring between 48 and 72 hours after the inoculation of virus. The activities of the fluids from infected eggs at the 72nd hour or later were approximately 18 times higher than those from non-infected eggs. Based on the data above, a qualitative test for the presence of infection with influenza virus was developed.

BIBLIOGRAPHY

1. Berger, L., and Broida, D., The colorimetric determination of lactic dehydrogenase, *Sigma Techn. Bull.*, 1959, No. 505.
2. Blumenthal, H. T., Greiff, D., Pinkerton, H., and DeWitt, R., Influenza. I. The hemagglutination and infectivity titer curves of PR8 influenza cultured in embryonate eggs at different temperatures, *J. Exp. Med.*, 1950, **91,** 321.
3. Greiff, D., Blumenthal, H. T., and Pinkerton, H., Influenza. III. Rapid alterations in the respiratory rate of embryonate eggs apparently caused by influenza virus toxin, *J. Exp. Med.*, 1950, **91,** 335.
4. Hirst, G. K., The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus, *Science*, 1941, **94,** 22.
5. Kun, E., Ayling, J. E., and Siegel, B. N., Enzymic mechanism of increased utilization of glucose during virus multiplication in the chorioallantoic membrane of the chick embryo, *Science*, 1960, **131,** 1318.
6. Magill, G. B., Wroblewski, F., and LaDue, J. S., Serum lactic dehydrogenase and serum transaminase in human leukemia, *Blood*, 1959, **14,** 870.
7. Wroblewski, F., Clinical significance of alterations in lactic dehydrogenase activity of body fluids, *Am. J. Med. Sc.*, 1957, **234,** 301.