DEHYDROGENASE STUDIES OF CHORIOALLANTIOIC FLUIDS INFECTED WITH INFLUENZA A VIRUS (PR8).

by

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A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Master of Science.

Approved:

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University of Utah
1950

ACKNOWLEDGEMENT

The author wishes to express her appreciation to Dr. Louis P. Gebhardt, Dr. Paul S. Nicholes, Dr. Stanley Marcus, and Dr. Glen R. Leymaster, and to express her gratitude to the members of the Departments of Bacteriology and Biochemistry.

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INTRODUCTION

Comparatively few investigations concerning the metabolic behavior of virus had been reported before 1942. Since that time, there has been appearing a steady increase in the number of publications. Most of the studies have been concerned with isolated phases of this large problem. However, a widely accepted theory attempting to explain viral metabolism has been proposed. Upon entrance into a susceptible host cell, the virus particle shifts the normal metabolic activities of the host cell in such a manner as to result in the synthesis of more virus particles. The mechanism by which the virus particle accomplishes this is unknown.

The above theory in itself suggests a need for more extensive metabolic studies of viruses; for, if we are to know how the virus particle duplicates itself within its host, we must first gain a clearer and broader knowledge of virus metabolism. Also, the planning of a logical approach to a chemotherapeutic attack on virus diseases necessitates a better understanding of the biochemistry of virus infections.

This study was suggested by the work of Bauer (1947). Mouse brains infected with either of the viruses of yellow

LITERATURE REVIEW

Because of the minute size and apparent simplicity in composition of virus particles, it has been thought that the virus could not contain a full complement of metabolic enzymes to carry out its own metabolism and reproduction. So far there has been no report of an isolation of any metabolic enzyme from a virus particle. The fact that viruses cannot survive outside of a susceptible living cell suggests that the virus particle "steals" these necessary metabolic components from its host cell. Schultz (1948) has made the statement

> Viruses . . . must have certain of their functional needs supplied by the host cell, the demand is always for a special kind of protoplasmic substrate. . . . The offering by the cell might consist of something which could conceivably be modified by artificial means; it might, on the other hand, consist of some characteristic protoplasmic property which is ridgidly predetermined by the genetic constitution of the cell. . . . While there are reasons for believing that much of host-parasite relationships in the case of viruses is linked with genetically determined factors — factors which determine the suitablity of a given protoplasmic soil for a given virus — we have no knowledge of why certain kinds of cells are susceptible to a given virus.

Even in the early 1900's virologists working with plant viruses and the diseases caused by them noticed changes from the normal plant cell constituents when a cell was invaded by a virus. An increased oxidase activity was described in plants

fever, lymphocytic choriomeningitis or lymphogranuloma inguinale were studied. The virus-infected brains showed a significant increase in dehydrogenase activity when compared with that in normal brains. This increase was due, in part at least, to an increase in xanthine oxidase and pyruvic dehydrogenase activities. Bauer suggested that these two enzymes were in all probability essential for virus multiplication.

In the present investigation an attempt has been made to determine whether or not there is an increased dehydrogenase activity of chorioallantoic fluid of 13 day-old embryonated chicken eggs previously infected with influenza A virus (PR8).

suffering from the diseases of tobacco mosaic, potatoe leaf roll, and curly top of sugar beets. Tomato leaves infected with tomato spotted wilt virus contained oxidases that could not be detected in the leaves of healthy plants. An increased diastase content was noticed in sandal suffering from spike disease, and in tobacco plants infected with tobacco mosaic virus. Bawden (1943) gives a more complete account of these findings in his book.

Since these early observations, other such abnormalities upon viral infection have been reported. There appears to be fairly definite pH changes in the environment of the multiplying virus. Huang (1943) noted that in tissue cultures infected with western equinencephalomyelitis, St. Louis equinencephalomyelitis, or Jungeblut-Sanders mouse virus there was a slightly higher pH than in non-infected cultures. He attributed this to a decreased degree of glycolysis in the infected tissue. McLean, et al (1945) have reported that the chorioallantoic fluid of embryos inoculated with influenza cirus B maintained an alkaline reaction throughout the period of infection, whereas the pH of the fluid of normal embryos of a comparable age became progressively more acid in a similar period. These authors proposed that an infection with the virus lead to an alteration of the processes of acid metabolite excretion. Parodi, et al (1948) have also reported an

increase in the pH of the allantoic fluids of embryonated eggs infected with influenza A virus.

Changes in the oxidative and glycolytic metabolism of cells infected with virus have been noted. Huang (1943) reported a lower degree of glycolysis in tissue cultures infected with western equinencephalomyelitis virus. Parodi, et al (1948) noticed a decreased oxygen consumption and production of carbon dioxide when embryonated eggs were infected with influanza A virus. However, when these measurements were considered per gram of embryo there appeared to be no significant difference from the normal. Pearson and Winzler (1949) found that the propagation of Theiler's GD VII virus in minced 1 day-old mouse brain had no significant influence on the oxidative or glycolytic metabolism of the host tissue. Recently, Pinkerton, et al (1950, 1950a) reported that eggs infected with influenza A virus showed wide cyclic variations in the rates of increase in oxygen consumption which appeared to correspond quite accurately with the growth cycles of the virus. These variations were in contrast to the uniformity of increase noted in non-infected eggs. These workers felt that the variations of oxygen consumption were due to variations in the concentration of virus toxins.

A critizism was made by Utter, Reiner and Wood (1945) of

the early glycolytic studies of viral infected tissues. They were of the opinion that the experiments were not designed specifically enough to measure the desired factors, and thereby of doubtful value. The authors felt that changes occurring during viral infection would appear in (a) permeability or intracellular organization, (b) concentrations of coenzymes, substrates, or other essential components, and/or (c) concentrations or nature of enzymes (increases or decreases in normal enzymes, appearance of abnormal ones, or a disturbance of their relative proportions).

Just such a change in enzyme concentration, as postulated by Utter, Reiner and Wood, has been reported. Bauer (1947) made a study of mouse brains infected with the viruses of yellow fever, lymphocytic choriomeningitis and lymphogranuloma inguinale. Preliminary investigations showed an overall increase in dehydrogenase activity of infected tissue when compared with that of normal brain tissue. More specific experiments were designed in which the activities of xanthine oxidase, pyruvic dehydrogenase, α-glycerophosphate dehydrogenase, succinic dehydrogenase, lactic dehydrogenase, and malic dehydrogenase were measured by the use of the "Thunberg" technique. Significant increases in the activities of xanthine oxidase and pyruvic dehydrogenase were noted in the brains

infected with any of the three viruses studied. Bauer suggested from these findings that these two enzymes were essential for virus multiplication, and that they were required in greater amounts than the normal cell contained. It was postulated that the virus particles caused an assemblage of enzyme molecules from constituent parts of the host cells to create around it the biochemical environment necessary for its multiplication.

Isotope studies have revealed many changes occurring during a virus infection. Rafelson, Winzler and Pearson (1949), using labeled glucose, reported an increased extent of incorporation of glucose fragments into the "protein" fraction and a decreased incorporation into the lipid fraction of 1 day-old mouse brain tissue infected with Theiler's GD VII virus. This effect occurred during the period of maximal virus propagation. These workers interpreted this as being a redirection of carbon metabolism during an infection with the virus. Saenz and Taylor (1949) injected intra-allantoically into 11 day-old chick embryos radioactive phosphorous (P^{32}) and influenza viruses A or B. These workers noted an increased amount of P^{32} escaping from the allantoic fluid and a greater concentration of the isotope in the blood. These observations lead the authors to believe that the virus damages the chorioallantoic membrane so as to render it more permeable to the isotope. Rafelson, Winzler and Pearson (1949a) also utilized P^{32} in

studying minced 1 day-old mouse brain infected with Theiler's GD VII virus. During the period of maximal virus propagation, the uptake of P^{32} was markedly stimulated in the phospholipid and "total protein-bound" phosphate fractions. The increased turnover in the latter fraction was primarily due to an increased turnover of ribosenucleic acid (RNA) phosphorous. Desoxyribosenucleic acid (DNA) phosphorous was not affected. The virus infected tissue had an increased RNA phosphorous content and a decreased DNA content. From this last finding the authors ventured the suggestion that the increased turnover and amount of RNA in the virus-infected tissue might represent the contribution of the virus itself.

The differences in DNA and RNA turnover and content when cells are infected with virus has greatly interested many workers. Gratia, et al (1945) reported that silkworm jaundice virus, which has only DNA as its nucleic acid constituent, caused an increase in both the RNA and DNA content of infected cells.

Cohen (1948) has reported, in studying E. coli infected with T_2 bacteriophage, that only the nucleic acid found in T_2 (DNA) was synthesized, while normal cells synthesized about three times as much RNA as DNA. Also, in infected cells, essentially all the phosphorous going to form protein-bound constituents appeared as DNA. During the period of normal virus

multiplication, protein and DNA were synthesized at a constant rate, and this rate was constant regardless of the number of virus particles formed within a cell. It appeared then that the virus did not contain the enzymes for DNA or protein synthesis, and that new enzymes were not being formed, or if so, were not being used for this synthesis. Thus it was concluded that the metabolic constituents which synthesized the most complex virus components are the enzymes of the host cell, E. coli B, presumably according to the new models supplied by the infecting virus particles.

Cohen (1948a) studied the origin of the phosphorous found in the DNA of T_2 and T_4 bacteriophage of E. coli. With the host cells or the culture medium labeled with P^{32}, the DNA of the virus was found to be built mostly from the inorganic phosphorous of the medium. RNA was not a precursor in the synthesis of DNA, and was essentially inert in infected cells.

Price (1949) described the nucleic acid synthesis in Staphlococcus muscae cultures during phage formation. Nucleic acid synthesis per cell increased in infected cells during the whole course of infection, while it increased in normal cells during the lag phase and then remained fairly constant during the log growth. Also infected cells synthesized less RNA and more DNA than normal cell. This increased DNA content may actually be the DNA in the virus particles. Price purposed

that phage synthesis was closely related to normal cellular reactions of S. muscae and not an entirely independant series of reactions; that when the virus invaded the normal cellular reactions were shifted so as to bring about the formation of phage.

An interesting observation was made by Wildman, Cheo and Bonner (1949). Their evidence suggested that tobacco mosaic virus protein was synthesized in Turkish and Havana tobacco leaves at the expense of a normal nucleoprotein found in the leaf cell cytoplasm. Virus protein could be detected by electrophoretic methods 3 days after the inoculation of the tobacco leaves. It progressively increased in amount up to 12 days after which it appeared to have reached a static level. Coincidental with this virus protein increase there was a porportionate decrease in the normal nucleoprotein of the leaves. If these observations are correct, these workers wonder just how much of a change has occurred when the normal protein is transformed to virus protein. Is it an extensive breakdown, or a more or less direct transformation?

A number of investigations have been made to determine the influence of various substrates and compounds of metabolic significance on the multiplication of virus particles. Spizizen (1943) has reported that 3 compounds, glycine, glycine anhydride, and hippuric acid at certain low concentrations, supported the

multiplication of virus P_1 in the absence of any demonstrable bacterial growth of its host. The author felt that in all probability the glycine anhydride did not split to glycine. Spizizen (1943a) continued his studies on compounds influencing bacteriophage multiplication. Using bacterial cells suspended in concentrations of glycine anhydride that would inhibit bacterial growth but would still support the multiplication of phage, the following compounds were found to stimulate virus multiplication: nucleic acid, glycerophosphoric acid, ATP, coenzyme I, 4-C-dicarboxylic acids, and α-ketoglutaric acid. Compounds inhibiting phage reproduction were cyanide, iodoacetate, arsenite, 2,4-dinitrophenol, p-aminophenol and the sulfonamides. From these studies, Spizizen believes that virus reproduction requires certain fundamental cellular reactions, but not necessarily all the reactions leading to cell multiplication.

In investigations of the nutritional factors governing virus reproduction in an E. coli T_2 bacteriophage, Cohen and Fowler (1947) observed that indole-3-acetic acid (heteroauxin) stimulated virus multiplication on a limited media.

Price (1947a) made a study of bacteriophage formation without growth of the host bacteria, and the influence of some compounds on this formation. Iodoacetate, floride, azide, and gramicidin were found to prevent the formation of phage and to

inhibit the synthesis of ATP by S. muscae. These findings suggested that energy-rich phosphate was needed for the synthesis of phage. No differences, however, were found between normal bacteria and phage-infected bacteria in the inorganic phosphate, ATP, RNA and DNA contents of the cell. In further studies by Price (1947), it was seen that the addition of penicillin greatly increased the production of phage in bacterial suspensions. The addition of niacin also increased the formation of phage, but only without the addition of penicillin. The results indicated that niacin was necessary for the phage production and that the bacteria could not utilize niacin in the presence of penicillin. Phage multiplication required the presence of one or more factors besides niacin that were present in broth and yeast extracts which were not essential for bacterial growth. Penicillin did not prevent the utilization of these unknown substances. Continued studies (Price, (1948)) showed this unknown factor in yeast to be a ribonucleoprotein fraction that stimulated adaptive enzyme formation in the yeast. This fraction had no effect on the multiplication rate of normal S. muscae cells and was metabolized by them in the absence of virus to a form no longer available for virus production. The yeast extract stimulated the formation of S. muscae phage in cells in the log phase in synthetic medium. It appeared then that this substance played

a rather direct role in the synthesis of the virus. Since a similar fraction could be isolated from normal S. muscae cells, this fraction might be a limiting factor for virus synthesis.

Investigations utilizing the capacity of certain compounds to inhibit essential metabolic components have been made to determine the influence of the compounds on the formation of virus. Knight and Stanley (1944) studied the effect of some chemicals on purified influenza A virus (PR8) in phosphate buffer. Tests in chick embryos and mice showed that virus activity was destroyed by strong oxidizing agents such as iodine, salts of heavy metals, mercurochrome, formaldehyde and detergents. Reducing agents appeared to have little inactivating effect on the virus, with the exception of ascorbic acid.

Several acridine compounds were seen, by Fitzgerald and Lee (1946), to effectively inhibit bacteriophage multiplication in infected suspensions of E. coli. Ribose nucleic acid appeared to counteract the antiviral effect of the acridines, suggesting that some cellular mechanism involving nucleic acid or a related substance was essential for virus formation.

Rasmussen, et al (1947) reported the effect of acridine on the growth of influenza viruses A and B. Parallel infectivity and red cell agglutination titrations showed that nitroakridin 3582 (2,3-dimethyoxy-6-nitro-9-(diethyl-amino-oxypropyl)

aminoacridine dihydrochloride) inhibited the growth of Lee influenza virus in embryonated eggs. Administration of 0.5 to 1.0 mg of nitroakridin 3582 prior to the injection of 10 minimal infecting doses of virus resulted in varying degrees of suppression. In certain eggs the appearance of the virus was delayed 8 or more hours, but ultimately reached the concentration equal to that in untreated controls. Nitroakridin had a less pronounced effect on influenza A virus (PR8) infections in embryonated eggs. This compound was somewhat viricidal *in vitro*, but the amount was insufficient to account for the degree of inhibition observed *in vivo*.

A series of compounds were tested by Thompson (1947) in Maitland type of tissue cultures containing chick embryonic tissue in order to determine which substances possessed the capacity to increase or decrease the growth of the vaccinia virus. Substances found to inhibit virus proliferation were dinitrophenol (respiratory stimulant); cyanide, azide, atabrine, proflavin, iodoacetic acid, and other enzyme-inhibitors; substituted amino acids (methoxinine, amino methane sulfonic acid, ∝-amino isobutane sulfonic acid, and ∝-amino-phenylmethane sulfonic acid); benzimidazole (purine antagonist); and, ascorbic acid, 2-methyl naphthoquinone, benzoquinone, and hydroquinone. In general, it appeared that any agent which decreased the oxygen tension of the medium depressed the development of the

vaccinia virus.

Volkert and Horsfall (1947) presented evidence which indicated that pneumonia virus of mice (PVM) was affected adversely by concentrated lung tissue suspensions or by the presence of glutathione. Since iodoacetamide inhibited or eliminated the effects produced by these two substances, it was concluded that sulfhydryl groups caused PVM to lose its capacity to induce infection.

The effect of certain enzyme inhibitors on the in vitro activity and growth of psittacosis virus was studied by Burney and Golub (1948). Iodoacetamide, p-chloromercuribenzoate, hydroquinone, proflavin, atabrine, and mercuric chloride markedly reduced the activity of psittacosis virus as measured by egg titrations. Glutathione and cysteine were shown to antagonize the effect of p-chloromercuribenzoate with resumption of virus activity. This reactivation suggested that free sulfhydryl groups were involved in the inactivation, and that it was not due to an irreversible protein denaturization. Growth of the psittacosis virus in tissue culture was inhibited after previous exposure of the tissue to o-iodosobenzoate or hydroquinone. This inhibition suggested that it caused the suppression of certain enzymatic reactions in the host tissue requiste for normal reproduction of psittacosis virus.

Klein, et al (1948) reported that mercuric chloride, mer-

bromin, merodicein, metapher, and phenylmercuric nitrate effectively inactivated the influenza virus *in vitro*. Sodium thioglycolate and BAL reversed the inactivation of the virus by mercuric chloride. Merodicein instilled intranasally in mice possessed some prophylactic activity against the influenza virus. Intamuscular injection of BAL into mice effected the *in vivo* reactivation of the virus. Francis and Penttinen (1949) repeated the work of Klein, *et al* with merodicein, and found it to have no modifying action against influenza B virus (Lee) in mice, or influenza A virus (PR8) and influenza B virus (Lee) in embryonated eggs.

MATERIALS AND METHODS

Preparation of Stock Virus

The influenza A virus was chosen for these studies because, (a) the presence of the virus is easily detected by chick cell hemagglutination, (b) the virus can be stored in a carbon dioxide ice box for long periods of time, (c) the techniques used in the cultivation of this virus are comparatively simple, and (d) the egg-adapted virus is non-infective for man.

The influenza A virus[1] utilized in these investigations was a PR8 strain of the virus, and has a past history of an indeterminate number of ferret and mouse passages, and six egg passages. The egg infectivity titre (EID_{50}) of this virus, as calculated by the Reed-Muench method (1938), was approximately $1 \times 10^{-6.5}$. Its hemagglutinating titre by the method of Salk (1944) was 1:4096, and its titre by the method of Hirst (1942) was 1:128.

This virus material was subsequently passed in eggs to attain its maximum infectivity titre. The maximum titre of the virus was desired in order that the conditions under which

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[1]The virus was obtained from Dr. Glen R. Leymaster, Department of Public Health and Preventive Medicine, University of Utah Medical School, Salt Lake City, Utah.

the repeated enzyme studies of infected material were performed would be as similar as possible. The virus material (undiluted allantoic fluid of infected 13 day-old embryonated eggs) was diluted 1:100 with sterile beef heart infusion broth. One tenth cc of this material was injected into the allantoic sac of 11 day-old chick embryos. The eggs were incubated for 42 hours at 35° C. Following incubation, the eggs were kept at 10° C. for at least 4 hours. This lower temperature storage reduced bleeding of the embryos during the harvesting of the virus. The allantoic fluid of each egg was withdrawn (all bloody and albuminous fluid was disgarded), and the fluids pooled. Between egg passages the virus material was stored in a carbon dioxide ice box (-56°C.).

The original virus material was passed through 4 consecutive egg passages (from the 6th egg passage to the 10th). The material from the last egg passage was found to have an EID_{50} of $1 \times 10^{-6.8}$, as calculated by the Reed-Meunch method. It appeared that this was very nearly the maximum infectivity of this particular strain of virus.

A virus stock of the same egg passage was desired in such an amount that all enzymatic investigations could be made with a standard inoculum. The 11th egg passage of the virus was carried out using sufficient numbers of eggs to obtain such a quantity of virus material. One cc amounts of the

pooled allantoic fluids from the 11th passage were placed in vials and sealed. The material was quick frozen in a mixture of dry ice and alcohol, and the vials then stored in a carbon dioxide ice box (-56°C.). This virus material (PR8-E_{11}) had an EID_{50} of 1 x $10^{-6.7}$, and a Salk hemagglutination titre of 1:2560.

Turner and Fleming (1939) reported that influenza A virus (PR8) could be stored at -78°C. for 3 years with only a slight, if any, decrease in infectivity titre. After a period of one year's storage, the stock virus had an EID_{50} of 1 x $10^{-6.5}$. This value is slightly lower than that obtained at the beginning of the storage period, but it is within the range of experimental error for the determination, and in all probability no decrease in titre occurred.

Thunberg Studies

The presence and relative activities of the dehydrogenases in infected and normal allantoic fluids were measured by the use of the "Thunberg" technique. The procedure followed was that of Umbreit, Burris and Stauffer (1947), with a few minor modifications. One cc of 1/10,000 methylene blue, 2 cc of M/15 phosphate buffer (pH 7.4), and 2 cc of M/50 substrate were placed in a standard Thunberg tube. One cc of virus material was placed in the side arm cap. The tubes were sealed

by the use of anhydrous lanolin, and then evacuated with a water aspirator. The evacuation was checked with a mercury manometer. The tubes were next placed in a constant temperature water bath of 38°C. and allowed 10 minutes for temperature equilibration, inverted, and the methylene blue reduction followed visually.

In some experiments, the volumes of the reacting components in the Thunberg tubes were changed, according to the method followed by Bauer (1947). Five tenths cc of 1/20,000 methylene blue, 0.5 cc M/50 substrate, 1.0 cc of M/15 phosphate buffer (pH 7.4), and 1.0 cc of distilled water or enzyme activator were placed in a standard Thunberg tube. One cc of virus material was placed in the side arm cap. The tubes were then treated the same as those previously described.

Sterile conditions were maintained throughout the Thunberg studies, because of the lengthy reduction times of the materials tested.

The substrates utilized were sodium succinate, succinic acid, malic acid, fumaric acid, sodium lactate, and xanthine. These substrates were employed at a concentration of M/50, using distilled water as a diluent, and sterilized at 120°C. and 15 lbs. pressure. In the case of xanthine, the compound was not completely soluable at this concentration, and potassium hydroxide had to be added until all was dissolved.

TABLE I

pH OF SUBSTRATES

Substrate	pH of M/50 Substrate	pH of equal parts M/50 substrate and phosphate buffer.	pH of Thunberg tube mixture,* before reduction.	pH of Thunberg tube mixture,* after reduction.
Sodium succinate	7.2	7.4	7.35	7.45
Succinic acid	2.9	6.3	6.1	6.1
Malic acid	2.6	6.2	6.0	6.3
Fumaric acid	2.6	6.7	6.6	6.8
Sodium lactate	5.1	7.4	7.3	7.4
Xanthine	alk.	alk.	8.1	7.8

*Thunberg tube mixture consisted of substrate, buffer, methylene blue, and virus material.

The pH of the substrates under various conditions are given in Table I. The hydrogen ion concentrations were determined by a glass electrode pH meter (Beckman).

Because of the exceptionally long reduction times, an attempt was made to shorten them by increasing the concentrations

of the substrates to M/20. This, however, proved to be unsuccessful, so the original concentrations were employed.

The phosphate buffer used in these investigations was a potassium acid phosphate-sodium hydroxide mixture at a pH of 7.4 (Clark (1928)).

Two different preparations of virus material were utilized in the Thunberg studies.

(1) Untreated and Undiluted Infected Allantoic Fluid. This was obtained in the following manner. Eleven day-old embryonated eggs were injected intra-allantoically with 0.1 cc of a 1:100 dilution of the stock influenza A virus ($PR8\text{-}E_{11}$). These eggs were incubated for 42 hours at 35°C. The allantoic fluids were removed aseptically and pooled, with a minimum of 4 infected eggs in a pool. The fluid was used as soon as possible, never allowing more than 18 hours to elapse before use.

(2) Concentrated Infected Allantoic Fluid. This was obtained in the following manner. The initial procedure was the same as that presented in the above, (1). The freshly harvested pooled fluid (with 10 to 12 eggs per pool) was brought to a pH of 4.6 with 10% acetic acid. This material was centrifuged for 10 minutes, and the supernatant poured off. The precipitate was resuspended in M/15 phosphate buffer (pH 7.4) in an amount 1/10 the original volume of the

fluid. The virus material prepared as such retained its dehydrogenase activity for a week, but generally the concentrate was used for study within 24 hours after preparation. This method of preparing a concentrate of the allantoic fluid was suggested by the purifications of succinic dehydrogenase, lactic dehydrogenase, and malic dehydrogenase by Ogston and Green (1935), Green (1936), and Green and Brosteaux (1936).

To parallel the Thunberg studies testing the dehydrogenase activities of virus material, normal allantoic fluid (fluid from 13 day-old non-infected embryonated eggs) that had been treated the same as the virus preparations was submitted to the "Thunberg" technique.

Inhibition Studies

The procedure employed in the studies attempting to determine any decrease in the hemagglutinating titre of the virus upon treatment with "inhibiting" substances was as follows. One tenth cc of an "inhibiting" compound was injected intra-allantoically into 11 day-old embryonated eggs. A group of 6 eggs were used for the testing of each compound. Four hours following this injection, 0.1 cc of a 1:100 dilution of the stock virus ($PR8\text{-}E_{11}$) was injected intra-allantoically into these same eggs. The eggs were incubated for 42 hours at 35°C. After incubation, the eggs were kept at

10°C. for at least 4 hours. Following this time, the allantoic fluids were harvested, and the fluids from each group of 6 eggs were pooled. These fluids were stored at 10°C.

The hemagglutinating capacity of each pool of allantoic fluid was tested. Two-fold serial dilutions, using physiological saline as diluent, were made of each pool. An equal volume of 0.5% chicken red blood cells was added to each tube. The tubes were allowed to stand at room temperature until the red cells had settled and the typical hemagglutination patterns appeared. Along with the testing of the fluids from infected eggs that had been treated with an "inhibiting" substance, a hemagglutinating titre was carried out on fluid from untreated infected eggs.

The "inhibiting" substances used were 0.2M alloxan, 0.5 mg of acriflavin dye (a mixture of 2,8 diamino-10-methylacridinium chloride, and 2,8 diaminoacridine), 0.2M glutathione, 0.2M chlorohydroquinone, 0.2M pyruvic acid, 0.2M sodium pyrophosphate, 1.0M malonate, and 1/2,000 mercuric chloride.

EXPERIMENTAL RESULTS

Some Properties of Normal and Infected Allantoic Fluids

Allantoic fluid from uninfected 13 day-old embryonated eggs was a clear, colorless or slightly amber non-viscous fluid. The presence of ureates in this fluid often caused the formation of a dense, white precipitate. Allantoic fluid from infected 13 day-old embryonated eggs was a transluscent or opalescent, non-viscous fluid. Precipitated ureates were often also present in this fluid. Kilbourne and Horsfall (1949) reported an increased protein content in infected allantoic fluid. The property of transluscence or opalescence of infected fluid might be attributed to this increased protein content.

The treatment with acetic acid in the preparation of a concentrate of allantoic fluid caused a dense, yellow precipitate to form. In comparing the precipitates formed by the fluids from normal and infected eggs, there appeared to be a smaller amount formed by the infected eggs.

The pH of normal allantoic fluid from 13 day-old chick embryos was in the range of 7.4 to 7.9, while the pH of allantoic fluid from 13 day-old chick embryos that had been

previously infected with the influenza A virus (PR8) was in the range of 7.9 to 8.5. This confirms the findings of Parodi, et al (1948).

Thunberg Studies

Preliminary studies were made in which the dehydrogenase activities of untreated, undiluted infected allantoic fluid, and untreated, undiluted normal allantoic fluid were compared. The dehydrogenase activities tested by the "Thunberg" technique (using the procedure first described in the section Materials and Methods) were succinic dehydrogenase, xanthine oxidase, malic dehydrogenase, lactic dehydrogenase, and fumaric dehydrogenase. The results of these studies are given in Table II, and represented in Figure 1. Because of the extremely long methylene blue reduction times of these fluids, after 90 minutes incubation in a 38°C. constant temperature water bath the tubes were transfered to a 38°C. warm air incubator.

The reduction times obtained when xanthine was employed as the substrate were variable. It was learned that fats markedly accelerated the reduction of xanthine by xanthine oxidase (Booth (1938)), and the sealing grease that was being used in the sealing of the Thunberg tubes was anhydrous lanolin (a fatty extract of sheep wool). When white petroleum jelly

TABLE II

THE METHYLENE BLUE REDUCTION TIMES OF UNTREATED, UNDILUTED NORMAL AND INFECTED ALLANTOIC FLUIDS

Substrate	Number of Exper.	Reduction Times of Normal Fluid	Number of Exper.	Reduction Times of Infected Fluid
Xanthine	6	20-22 hours	4	19-22 hours
Succinic acid	9	19-21½ hours	9	3-6 hours
Na succinate	9	43-47 hours	9	1-3 hours
Malic acid	9	19-21½ hours	9	5-7 hours
Fumaric acid	9	19-21½ hours	9	18-22 hours
Na lactate	9	44-46 hours	9	½-3 hours

was used in the place of the lanolin, the reduction times obtained were reproducible.

The Methylene blue reduction times of the allantoic fluids from influenza infected eggs for the substrates succinic acid, sodium succinate, malic acid, and sodium lactate appeared to be significantly shorter than those reduction times of the allantoic fluids from normal eggs. The results would seem to indicate that an influenza A virus infection of embryonated eggs causes an increased content of succinic

dehydrogenase, malic dehydrogenase, and lactic dehydrogenase, over that normally present in the chorioallantoic fluid.

Succinic acid and sodium succinate were both used as the substrates for the testing of succinic dehydrogenase activity because of their differences in pH. The variations of the reduction times obtained with the two substrates might be explained by just such pH differences; one having a pH closer to the optimum pH for the dehydrogenase activity. However, there was an inconsistancy in that succinic acid appeared to have the pH nearer the optimum for normal allantoic fluid, while sodium succinate appeared to have the pH nearer the optimum for infected allantoic fluid.

Concentrates of the infected and normal allantoic fluids were made in hopes that by concentrating the enzymes present shorter and, therefore, more reliable reduction times would be obtained. The fluids were concentrated to one-tenth their original volume. The substrates employed were those showing differences in reduction times between the infected and normal allantoic fluids. The Thunberg procedure used was the second described in the section Materials and Methods. The results of the studies are presented in Table III, and represented in Figure 2. The Thunberg tubes were incubated in the 38°C. constant temperature water bath throughout the full length of these experiments.

FIGURE 1. THE RELATIVE ACTIVITY OF SOME DEHYDROGENASES IN UNDILUTED, UNTREATED INFECTED & NORMAL ALLANTOIC FLUIDS.

FIGURE 2. THE RELATIVE ACTIVITY OF SOME DEHYDROGENASES IN CONCENTRATED INFECTED & NORMAL ALLANTOIC FLUIDS.

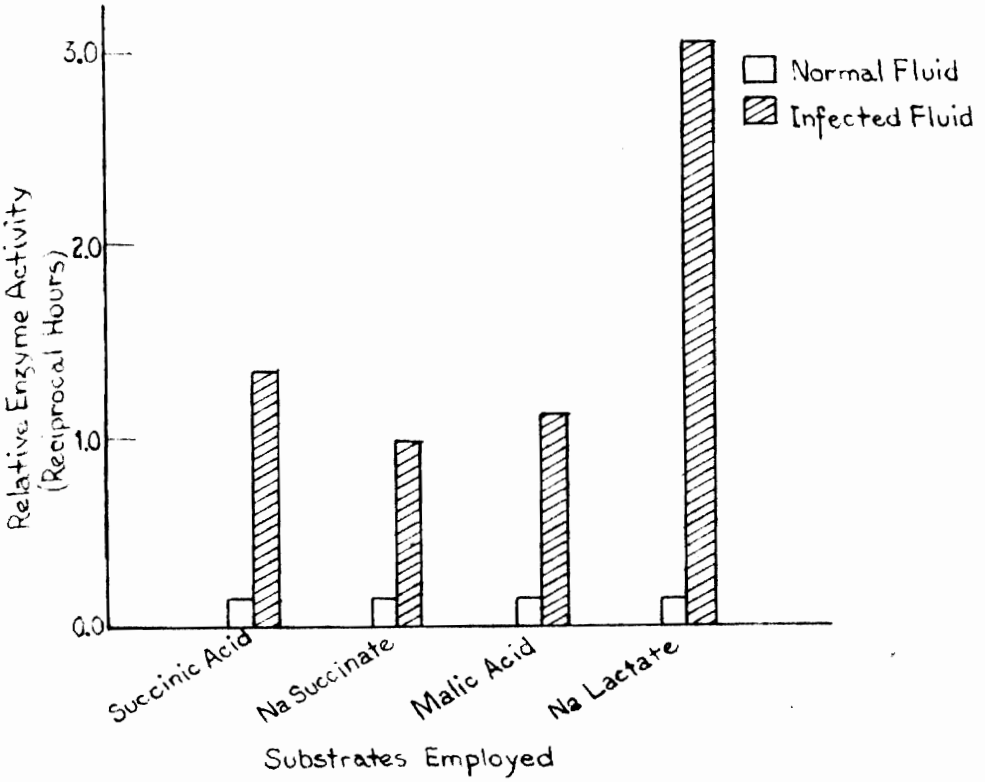

TABLE III

THE METHYLENE BLUE REDUCTION TIMES OF CONCENTRATED NORMAL AND INFECTED ALLANTOIC FLUIDS

Substrate	Number of Exper.	Reduction Times of Normal Fluid	Number of Exper.	Reduction Times of Infected Fluid
Succinic acid	1	longer than 8 hrs.	1	55 min.
	2	" " "	2	50 min.
	3	" " "	3	120 min.
	4	" " "	4	25 min.
	5	" " "	5	15 min.
Na succinate	1	longer than 8 hrs.	1	55 min.
	2	" " "	2	90 min.
	3	" " "	3	120 min.
	4	" " "	4	30 min.
	5	" " "	5	20 min.
Malic acid	1	longer than 8 hrs.	1	50 min.
	2	" " "	2	75 min.
	3	" " "	3	60 min.
Na lactate	1	longer than 8 hrs.	1	30 min.
	2	" " "	2	15 min.
	3	" " "	3	25 min.

When succinic acid, sodium succinate, malic acid and sodium lactate were used as the substrates, again the reduction times obtained were significantly shorter for the fluids infected with influenza virus than those reduction times obtained for normal fluids. The concentrated normal allantoic fluids

gave reduction times with these substrates that were longer than 8 hours, while the concentrated infected allantoic fluids gave times ranging from 15 minutes to 120 minutes.

Hoyle (1948) reported that influenza A virus grown in embryonated eggs reached its maximum titre after 24 hours growth, and then began a steady decline. It was thought possibly that at this point of maximum infectivity titre there would be even a more marked difference between the enzyme contents of the infected and normal fluids. Allantoic fluid in which the influenza A virus had been grown for 24 hours was concentrated and submitted to the Thunberg procedure. Normal allantoic fluid (from 12 day-old chick embryos) was run as a control. The results of this study showed very little difference in the enzyme content between the two fluids.

Inhibition Studies

The influence of enzyme-inhibitors and some chemicals on the growth of influenza A virus in embryonated eggs was investigated. The effect of specific inhibitors against the dehydrogenases that appeared to have increased in amount when the embryonated eggs were infected with influenza virus was studied. Succinic dehydrogenase has been reported as being inhibited by hydroquinone (Potter and DuBois (1943)), malonate (Sumner and Somers (1947)), and alloxan (Philips, et al (1947)).

Malic and lactic dehydrogenases have been reported as being inhibited by pyruvate (Green (1936)), and Green and Brosteaux (1936)). Sodium pyrophosphate has been reported as stimulating malic dehydrogenase activity (Green (1936)). Rasmussen, et al (1947) reported that acridines inhibited the growth of influenza A and B viruses in embryonated eggs. Mercuric chloride was reported by Klein, et al (1948) as inhibiting the growth of influenza A virus. Volkert and Horsfall (1947) reported that glutathione inhibited the growth of pneumonia virus of mice (PVM). The results of these studies are presented in Table IV.

The only compound which appeared to significantly effect the growth of the influenza A virus was mercuric chloride.

TABLE IV

THE EFFECT OF SOME COMPOUNDS ON THE GROWTH OF INFLUENZA A VIRUS IN EMBRYONATED EGGS

"Inhibiting" Compound	Hemagglutinating Titre of Allantoic Fluid
Acriflavin dye	1:1280
Pyruvic acid	1:1280
Glutathione	1:640
Alloxan	1:2560
Na pyrophosphate	1:640
Na malonate	1:1280
Mercuric chloride	<1:2
Untreated influenza virus	1:1280

DISCUSSION

It has been postulated that a virus particle on entering a susceptible host cell usurps the metabolic activities of this cell, and directs them into pathways more utilizable by the virus particle. Stanley (1931) is of the opinion that the influence of a virus might be very much like that of agents already present which direct normal metabolism, except that the virus exerts a dominating influence. He further suggests that the mechanism of virus synthesis within a diseased cell must be very similar to the synthesis of normal proteins and cellular constituents in the normal cell. Wright has concurred with Stanley, and additionally points out the similarity between genes and virus proteins, in regard to crystal growth and molecular mechanism.

This investigation reveals that the activity of dehydrogenases normally present in allantoic fluid is increased when an egg is infected with influenza A virus. This suggests that the virus directs the normal metabolic pathways of the host cell in such a manner that enzymes will be available for virus synthesis. It appears that the virus particle stimulates the normal cellular reactions, rather than changing the whole metabolism of the cell. It would seem unlikely that the enzymes which are normally present in the cell and appear to

increase in activity during a virus infection would be synthesized in the diseased cell by a different pathway than that in the normal cell. No attempt was made in this study to determine the appearance of abnormal enzynes in the infected host.

It was observed that allantoic fluid from infected eggs treated with acetic acid formed a smaller amount of yellow precipitate than treated fluid from normal eggs. This suggests the possibility that virus synthesis may be carried out at the expense of a normal protein in the allantoic fluid. Wildman, Cheo, and Bonner (1949) reported that tobacco mosaic virus grown in Havana and Turkish tobacco plants was formed at the expense of a normal plant protein constituent.

The failure of the specific inhibitors of the dehydrogenases to inhibit the growth of the virus might be explained. The mere introduction of a compound (especially pyruvic acid) into a host animal such as the embryonated egg gives no assurance that that compound will inhibit an enzyme. The compound may be immediately metabolized by the living host, giving it no chance to inhibit. The compound, on the other hand, might inhibit an enzyme, but this inhibition be of so short a duration that the virus multiplication is only temporarily delayed. The use of a more controlled system such as a tissue culture may reveal inhibition of virus growth by some

of the compounds tested. Burney and Golub (1948) found that compounds which inhibited the ~~vaccinia~~ psittacosis virus growing in Maitland type tissue culture had no effect on the virus growing in embryonated eggs.

SUMMARY

The results of this investigation suggests that an influenza A virus infection of embryonated eggs causes succinic dehydrogenase, malic dehydrogenase, and lactic dehydrogenase to increase in activity in the allantoic fluids of these infected eggs. An infection with influenza A virus also produced allantoic fluid which was slightly more alkaline than allantoic fluid from uninfected eggs.

Mercuric chloride was the only substance which appeared to significantly inhibit the growth of the influenza A virus in embryonated eggs.

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