

SOME ENERGY RELATIONS IN A HOST-VIRUS SYSTEM*

BY W. WILBUR ACKERMANN, PH.D., AND R. BERNAL JOHNSON, PH.D.

WITH THE TECHNICAL ASSISTANCE OF H. KURTZ AND L. E. KEITH

(From the Department of Epidemiology and Virus Laboratory, School of Public Health
University of Michigan, Ann Arbor)

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Under special conditions in the presence of tissues surviving *in vitro*, influenza virus will propagate at a rate and to an extent approaching that obtained in the intact animal (1, 2). If, however, the respiration of the tissue is restricted by the use of metabolic inhibitors which act upon the citric acid cycle, it is observed that the yield of virus is directly proportional to the residual oxygen consumption (1). It has been postulated that the energy required for viral synthesis derives from the cellular aerobic metabolism. It would follow that the synthesis of virus must depend upon the maintenance of high energy phosphate bonds in the host tissue.

In the present report several experiments are described from which the correctness of the inference is clearly established.

Materials and Methods

Virus and Tissue.—The PR8 strain of Type A influenza virus used in these studies has undergone 7 passages in ferrets, 593 passages in mice, and 131 passages in eggs. The host tissue was obtained from the chorioallantoic membrane of 14 day embryonate eggs.

Warburg Flask Culture.—The host-virus system was maintained in a Warburg apparatus using a modified Simms solution. Each flask contained 200 mg. of chorioallantoic membrane which was used without mincing. As routine the cultures were incubated with shaking at 37°C. A description of this method has been reported elsewhere (1, 2), and details of the individual experiments are found in the corresponding figures. The inoculum was prepared by making suitable dilutions of infected allantoic fluid in Simm's solution. In all instances 0.3 ml. of inoculum was used per flask, and the final volume of fluid and tissue was 3.0 ml.

Virus Titrations.—The amount of virus was estimated by determining the infectious titer for eggs. For this purpose tenfold serial dilutions were prepared in broth and 4 eggs were inoculated with 0.1 ml. of each dilution. After 3 days of incubation at 37°C., samples of allantoic fluid were removed from each egg and tested for virus by the addition of red blood cells. The 50 per cent infectious titer was calculated using the method of Reed and Muench (3).

Oxygen Uptake.—The respiration of cultures was measured manometrically in the conventional manner with the Warburg apparatus.

Phosphorus.—The flask contents were deproteinized with trichloroacetic acid, and inorganic phosphate was determined by the method of Lowry and Lopez (4).

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RESULTS

Reversibility of the Effect of 2,4-Dinitrophenol (DNP) on a Host-Virus System.—Recent advances in the understanding of the mode of action of DNP have increased its usefulness in the study of phosphorylative processes (5). However, prior to employing it to study the effect of induced metabolic altera-

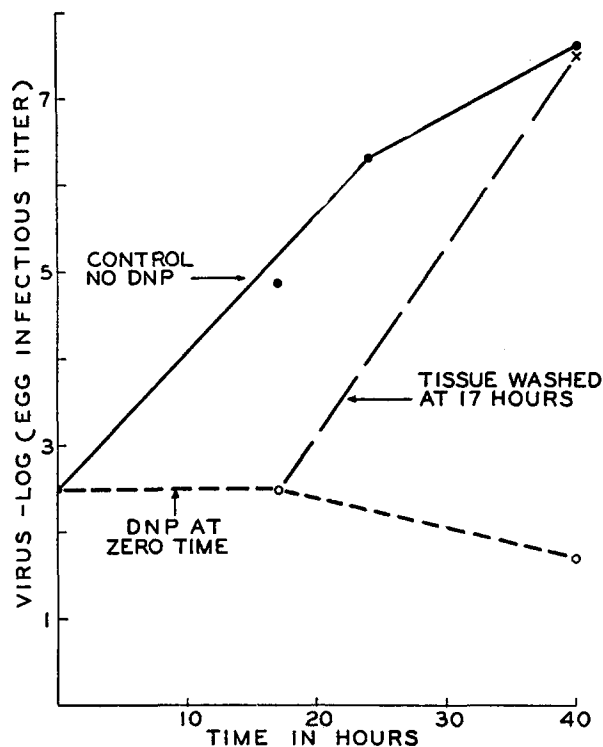


FIG. 1. Reversibility of DNP effect on host-virus system. The concentration of DNP employed in each flask was 27×10^{-5} M. Each flask contained 200 mg. of tissue, and each value is the average of 2 determinations.

tions in a host-virus system, it was desirable to determine the permanence of the changes wrought.

Two series of Warburg flasks were prepared containing virus, tissue, and medium. One series also contained DNP at a concentration of 27×10^{-5} M. Other details of this experiment are given in Fig. 1. Flasks were removed from each series at 18, 24, and 40 hours. On titration in eggs, the control flasks showed an increase of titer from $10^{-2.5}$ to $10^{-7.5}$ during the 40 hour interval. The flasks which contained DNP showed no increase in titer in 18 hours and by 40 hours their titer had decreased to $10^{-1.5}$. Tissues from two

flasks containing DNP were removed at 18 hours, washed in saline, and placed into fresh medium containing no additional virus or DNP. After 22 hours, the contents of these flasks were found to have a titer of $10^{-7.5}$. These data demonstrate that 27×10^{-5} M DNP, which is the highest concentration employed in any experiment reported here, will inhibit completely the propagation of influenza virus in chorioallantoic membrane. However, incubation of the tissue for 18 hours with this inhibitor does not permanently alter those metabolic processes required for the synthesis of virus. Further, in presence of the tissue there is no virucidal action of the DNP on the virus. Indeed, direct incubation of virus in allantoic fluid with this concentration of DNP at 37°C. for 20 hours was found to have no destructive effect upon the infectivity or hemagglutinating properties of the virus.

Activation of the Adenosinetriphosphatase (ATPase) of Chorioallantoic Membrane by DNP.—Since DNP acts to decrease the phosphorus to oxygen ratio measured in oxidative phosphorylative systems and to stimulate the rate of oxidation of the substrate thus permitting oxidation to proceed without concomitant fixation of phosphate, this substance has been termed an “uncoupling agent” (6, 7). However, these effects of DNP might also be explained in part as a result of the activation of the ATPase of the system (5, 8). Experimentally, it has been demonstrated that the “latent” ATPase of certain preparations of liver mitochondria can be markedly stimulated by this reagent (5). The action of DNP in modifying the metabolism of chorioallantoic membrane is similar as will be demonstrated here.

The ATPase activity of this tissue was determined essentially under those conditions which were employed in other experiments for the measurement of oxidative phosphorylation (9). However, the substrate and supplements of hexokinase were not added. Thus, no attempt was made to estimate the total ATPase activity, but rather, the effect of DNP on the “latent” ATPase was observed. To increase the permeability of the tissue to the ATP which was added from an exogenous source, the tissue was minced with curved scissors. Appropriate dilutions of this minced tissue were made in sucrose and added in the reaction vessel to an essentially non-ionic medium. The composition of the medium, duration of the experiment, amounts of tissue employed, and temperature of incubation are given in Fig. 2. To these reaction vessels graded concentrations of DNP were added, and the rates of reaction were followed by measuring the liberation of inorganic phosphate.

Tissue treated in this manner showed a considerable ATPase activity (18 μ M of phosphate released by 88 mg. of tissue in 20 minutes). This activity may be a reflection of the condition of the ATPase in the intact membrane or may be a result of injury to the tissue produced by mincing (10). However, the addition of varying concentrations of DNP produced a considerable further activation of the ATPase. The addition of 27×10^{-5} M DNP produced

nearly 100 per cent stimulation (Fig. 2). This experiment demonstrates that the destruction of high energy phosphate bonds which occurs in minces of chorioallantoic membrane is enhanced by the addition of DNP.

Effect of DNP on the Rate of Viral Synthesis, the Oxygen Consumption, and the Release of Phosphate by Intact Cells.—Virus will not propagate in the presence of disrupted cells, nor has it proved feasible to demonstrate oxidative phosphorylation directly in intact cells. Hence, indirect methods must be employed to correlate the viral synthetic rate of intact cells with their phos-

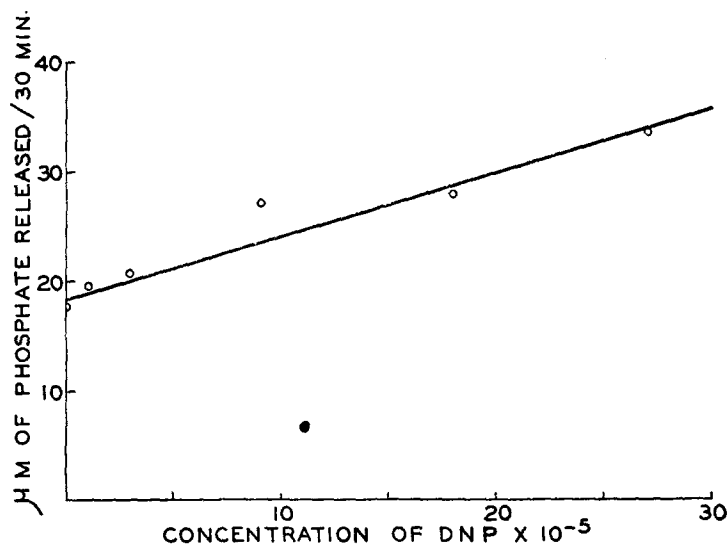


FIG. 2. Effect of DNP on the ATPase of chorioallantoic membrane. Duration of the incubation was 30 minutes at 31°C. Each flask contained 88 mg. of minced chorioallantoic membrane. Each point on the curve is the average of 4 determinations. Medium contained: 1.54×10^{-2} M glucose; 1.23×10^{-6} M cytochrome C; 0.040 M ATP; and enough 0.25 M sucrose to give a volume of 0.65 ml.

phorylative efficiency. From observations with cell-free systems, it is known that concentrations of DNP which do reduce the P/O ratio also stimulate oxygen consumption and release inorganic phosphate (7, 11). Thus, changes in these latter metabolites have been used as indicators of the efficiency of the oxidative phosphorylative process in intact cells. In this way, the effects of graded concentrations of DNP on the viral synthetic rate and on the oxidative phosphorylation have been compared.

One series of Warburg flasks was prepared as for the cultivation of virus. However, no inoculum of virus was added and twice the normal amount of chorioallantoic membrane was used (Fig. 3). Graded concentrations of DNP were introduced, and the flasks were incubated for a 3 hour interval while

the oxygen consumed and the inorganic phosphate released were measured. Into a second similar series of flasks also containing DNP, virus was introduced, and at the end of a 20 hour incubation period the yield of virus in each flask was determined.

Concentrations of DNP in the range of 1 to 18×10^{-5} M produced a proportional increase in respiration with the maximum stimulation being nearly

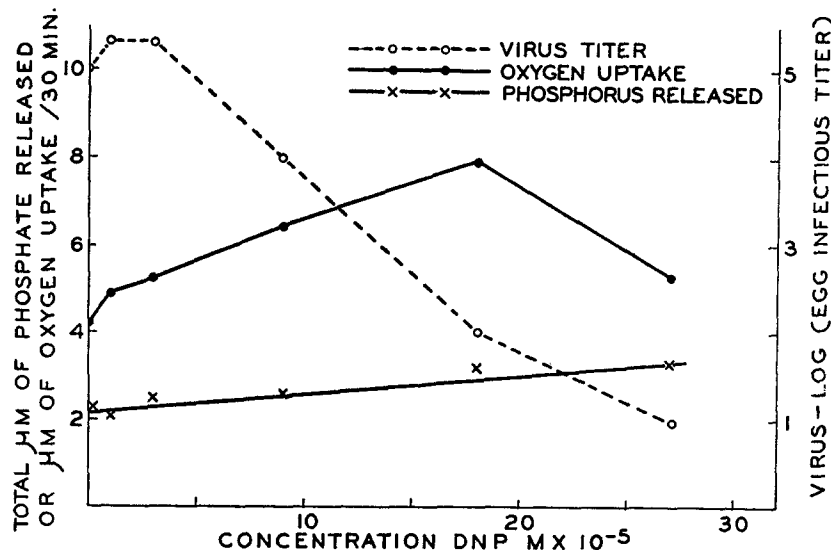


FIG. 3. Uncoupling effect of DNP on viral propagation. For oxygen and phosphate determinations an incubation period of 3 hours was used at a temperature of 30°C . The center well contained KOH, and 400 mg. of intact membrane was used. The medium is the same as that described for the growth of virus (1). Each point is the average of triplicate determinations. For the data obtained with the virus 200 mg. of tissue was used, and the center well contained Pardee's solution. The duration of the experiment was 18 hours, and the temperature of incubation was 37°C . Each point is the average of duplicate determinations.

100 per cent. DNP in amounts exceeding 18×10^{-5} M again depressed the oxygen uptake toward the normal control value (Fig. 3).

From the control tissue surviving under these conditions, there was a significant release of inorganic phosphate into the medium. However, all additions of DNP produced an additional proportionate stimulation. The maximum concentration (27×10^{-5} M) of DNP employed resulted in a 60 per cent increase in the release of phosphate.

From the data of Fig. 3, the effect of graded concentrations of DNP on the yield of virus can be compared with the effect of this reagent on the oxygen consumption and phosphate release of the host tissue. The yield of virus was essentially unaffected by 3×10^{-5} M DNP. Higher concentrations of the

phenol produced marked inhibition of viral propagation, and at 18×10^{-5} M DNP almost complete inhibition was observed. It will be noted that under these conditions the respiration of the tissue was twice the normal rate and the release of phosphate was increased by 30 per cent.

From these data, it is concluded that DNP at 18×10^{-5} M concentration is producing in the intact cell that "uncoupling" effect which is familiar in cell-free oxidative phosphorylating systems. Thus, oxidation is proceeding without a concomitant net increase of high energy phosphate bonds. Further, it is by virtue of this "uncoupling" effect that DNP inhibits the viral synthetic mechanism.

DISCUSSION

In isolated mitochondria, and probably in whole cells, the first rate-limiting factor in oxidative phosphorylation is the availability of phosphate acceptors (ADP) (12, 9). In cell-free systems the addition of cellular fractions containing ATPase, the addition of hexokinase and glucose, or the activation of "latent" ATPase with DNP all stimulate the conversion of ATP to ADP (12, 8, 5). Each of these components stimulates respiration.

In the case of DNP this stimulation is accompanied by a decreased rate of phosphorylation and an accelerated liberation of inorganic phosphate from intact cells. The latter effect is indicative of the breakdown of high energy forms of phosphate to which the cell membrane is normally impermeable. The effect of DNP on the stability of bound forms of phosphate may result from a stimulation of the catabolic activity of the cell in a manner similar to that observed directly in minces of the chorioallantoic membrane (Fig. 2), or may result from a reduced net yield of new high energy phosphate bonds in the presence of a constant catabolic activity.

The state of activation of ATPase is undoubtedly of importance in determining the new yield of high energy bonds in some phosphorylation systems. However, it should be noted that the "uncoupling" phenomenon produced by DNP cannot be explained completely by its influence on ATPase. The ATPase of spleen is not stimulated by DNP, and yet this reagent can completely inhibit the fixation of inorganic phosphate while respiration is maintained.¹ However, the respiratory rate and the release of inorganic phosphate are both indicators as to whether DNP is producing its uncoupling effect.

One salient characteristic of this host-virus system is the dependence of the viral synthetic mechanism upon energy which must be supplied by the host tissue. The importance of "actively metabolizing" tissue in the production of virus has long been recognized in a general way (13). However, it will be noted that active metabolism in the sense of a high rate of oxygen uptake is not

¹ These data obtained as a personal communication from Dr. Richard L. Potter, University of Michigan, Ann Arbor.

the essential requirement. When the oxidative rate of the tissue is restricted by the application of metabolic inhibitors, the yield of virus as measured by hemagglutination is directly proportional to the amount of oxygen consumed (1). However, the respiration of a tissue may be doubled with little or no yield of virus if concomitant phosphorylation does not take place. By the use of DNP it is possible to double the oxidative rate and demonstrate that it is the energy-producing capacity of the oxidative metabolism which is required for viral synthesis.

These findings do not exclude the possibility that the oxidation *per se* of particular intermediates of the Krebs cycle may have a specific relationship to viral propagation. The blockade of citrate oxidation with fluoroacetate *in vivo* permits much of the Krebs cycle to continue and to produce energy. The inhibition of viral propagation by that inhibitor probably results from the diverting of viral precursors into citrate formation (14-16).

Under some experimental conditions, the rate of viral synthesis is limited by the availability of precursors (2), while under others the yield is determined by the availability of energy (1). It would appear from the present data that virus does not derive from the transformation of large preformed units of the cell—a process which might require little or no energy—but rather arises from small elements which are organized after infection is initiated and in the presence of an energy-yielding mechanism.

SUMMARY

It was found that DNP (2,4-dinitrophenol) will inhibit completely the propagation of influenza virus in chorioallantoic membrane. This reagent did not permanently alter those metabolic processes required for the synthesis of virus and at the concentrations employed demonstrated no virucidal effects.

In minced preparations of chorioallantoic membrane DNP was shown to have a pronounced stimulatory effect upon ATPase (adenosinetriphosphatase).

When DNP was used with intact tissues, an excellent correlation was found between the inhibition of viral propagation and the stimulation of respiration and release of phosphate.

Concentrations of DNP which permitted a twofold increase in the endogenous respiration of intact membranes allowed little or no viral synthesis.

It is concluded that the energy required for viral synthesis derives from the oxidative phosphorylative activity of the host tissue.

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