Effect of 2, 4-Dinitrophenol and Sodium Azide on Host Cell Metabolism and Influenza A Virus[1]

J. L. Ingraham, T. O. Roby[2] and J. H. Peterson

From the Grasselli Chemicals, Stine Laboratory, E. I. du Pont de Nemours and Company, Inc., Newark, Delaware, and the Chemical Department,[3] Experimental Station, E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware

Received April 28, 1953

Introduction

From recent studies (1, 2) on the effect of various metabolic inhibitors on the growth of influenza A virus, Ackermann concluded that the normal functioning of the tricarboxylic acid cycle of the host cell is essential to virus propagation.

This apparent dependence of virus synthesis on the normal functioning of the tricarboxylic acid cycle might be explained in three ways: (*a*) Intermediates in the tricarboxylic acid cycle may serve as essential building blocks for virus synthesis. (*b*) The high-energy phosphate bonds formed during the operation of the tricarboxylic acid cycle may be essential to virus synthesis. (*c*) Tricarboxylic acid cycle building blocks and high-energy phosphate-bond formation may both be required. The aim of the present investigation is to determine if influenza A virus is synthesized in an environment where host cell respiration is not inhibited but phosphate uptake is depressed. Under such conditions, it may be assumed that the tricarboxylic acid cycle is operating but not forming normal amounts of high-energy phosphate bonds.

Loomis and Lipmann (3) have shown that 2,4-dinitrophenol (DNP) at sufficiently low concentrations inhibits phosphate uptake without affecting respiration. Hence, DNP apparently allows oxidative reactions

[1] It is a pleasure to acknowledge the technical assistance of Mrs. M. L. Cochrane and Mrs. L. T. Ewing.

[2] Present address: Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C.

[3] Contribution No. 327.

(including the tricarboxylic acid cycle) to continue, but inhibits the formation of high-energy phosphate bonds. Such a conclusion is supported by the findings that DNP prevents the normal functioning of energy-requiring systems such as adaptive enzyme formation (4) and oxidative and fermentative assimilation (5).

Sodium azide (NaN_3) is known to inhibit cytochrome oxidase (6) and various synthetic activities of the cell (5). Hotchkiss (7) has reported, however, that azide, at concentrations that do not inhibit respiration, still prevents a net increase in phosphate content of yeast cells metabolizing glucose aerobically.

It has been reported previously that DNP and sodium azide inhibit virus growth in cellular systems (8–11). Heagy (9) and also Eaton (12) assumed that virus inhibition is due to an interference with the cellular energy-transfer system. Rafelson, Pearson, and Winzler (13) reported an inhibition of virus growth by several other compounds (benzimidazole and lysine) which inhibit phosphate uptake. Eaton (12) reported a dependence of virus growth in the chorio-allantoic membrane upon the presence of glucose or pyruvate, which presumably served as energy sources. It was suggested that "the majority of the inhibitors [of virus growth] so far studied probably act through interference with synthesis or energy transfer within the cell."

The present studies were undertaken to investigate the effect of DNP and sodium azide on host-cell respiration, energy transfer, and virus synthesis in the chorio-allantoic membrane.

Subsequent to the completion of the present study, Ackermann and Johnson (14) published results that correlated the antiviral effect of DNP with its effect on stimulation of respiration and on phosphate release.

Methods

All experiments were run at 37°C. by conventional Warburg methods using Simms solution as a suspending medium and chorio-allantoic membrane from 14-day-old chick embryos. The CO_2 in the atmosphere was maintained at a constant level by adding Pardee solution (1) in the center well. Protocols are indicated in Table I.

In the virus-growth experiments, influenza A (PR8) virus, which was propagated via the allantoic sac of 9-day-old chick embryos, was added with Simms solution at a level of approximately 10 ID_{50} per Warburg flask. Eighteen hours after tipping the inhibitor, an aliquot was removed from each flask for infectivity titration. Serial decimal dilutions were prepared in nutrient broth, and 0.2 ml. of each dilution was injected into the allantoic sac of six 9-day-old chick embryos.

After 48 hr. incubation at 36°C., each embryo was tested for the presence of virus by mixing 0.5 ml. of the clear allantoic fluid with 0.5 ml. of 1% washed chicken erythrocytes. Hemagglutination after 1 hr. at 8°C. was interpreted as indicating the presence of virus; the titer was calculated as the 50% end point as described by Reed and Muench (15).

The P^{32}, in the form of phosphate, used in this investigation was supplied by Oak Ridge National Laboratory on authorization from the Isotopes Division, U. S. Atomic Energy Commission. Phosphate-uptake studies were performed in Simms solution containing approximately 15 microcuries (μc.) P^{32}/ml. These experiments were run at varying levels of DNP and NaN_3 without the addition of virus. After 20 hr. incubation, the chorio-allantoic membranes were removed, washed twice in 50-ml. amounts of saline, and digested with a mixture of 2.5 ml. of concentrated sulfuric acid and 1 ml. of 70% perchloric acid. The digests were diluted to 50 ml. Then 0.1-ml. aliquots were transferred to aluminum planchets, neutralized with an excess of ammonium hydroxide, and dried under an infrared lamp. The samples were counted with a gas-flow counter.

Experimental

The effect of DNP and NaN_3 on the respiration of chorio-allantoic membrane was measured by following oxygen uptake for the 2 hr. preceding and the 2 hr. following tipping of the inhibitor. Respiration was stimulated at all concentrations of DNP tested (5×10^{-5} M to 5×10^{-4} M), whereas sodium azide inhibited respiration at concentrations above 7×10^{-4} M. At this concentration and below, the inhibition was negligible (Table I).

The effect of DNP and azide on virus propagation and phosphate uptake was then determined. Dinitrophenol was found to inhibit phosphate uptake at concentrations greater than 2.0×10^{-5} M, and this inhibition became progressively greater, reaching a near maximal value at 4×10^{-4} M DNP. There is a similar inhibition of virus growth, reflected by the infectivity titer, at corresponding concentrations of DNP (Fig. 1). The same general effects were noted with sodium azide (Fig. 2). These data suggest a dependence of virus growth on the ability of the host cell to fix inorganic phosphate.

Since azide and DNP have been shown not to inhibit oxygen uptake at concentrations less than *ca.* 5×10^{-4} M (Table I), it is assumed that the oxidative reactions, including the tricarboxylic acid cycle, are functioning normally. The depression in virus synthesis and phosphate uptake below 5×10^{-5} M azide and DNP is thus probably attributable to a lack of high-energy phosphate-bond formation by the host cell. The possibility that the tricarboxylic acid cycle serves both as a source of

TABLE I

Effect of 2,4-Dinitrophenol and Sodium Azide on Oxygen Uptake of the Chorio-Allantoic Membrane

Per Warburg flask: 2.7 ml. Simms solution (1), 200 mg. chorio-allantoic membrane (14-day-old chick embryo). Center well, 0.3 ml. Pardee solution. Side arms, 0.3 ml. of neutralized DNP or NaN_3 as indicated, 36°C., readings at 15-min. intervals.

Inhibitor	Concentration $\times 10^{-4}$ *M*	Oxygen uptake of membrane (200 mg.) μl./30 min. (*a*) (Before addition of inhibitor)	(*b*) (After addition of inhibitor)	*b/a*
NaN_3	20	34.2	16.2	0.47
	13	34.2	20.1	0.59
	10	29.0	21.3	0.74
	7	30.0	27.3	0.91
	5	28.4	29.9	1.05
	4.2	24.6	26.0	1.05
2,4-DNP	5	35.7	56.7	1.59
	2.5	37.5	66.8	1.76
	1.6	27.2	42.6	1.56
	1.0	38.0	49.0	1.28
	0.82	32.1	43.7	1.36
	0.71	32.5	48.1	1.48
	0.63	32.9	42.2	1.28
	0.5	33.7	40.2	1.19

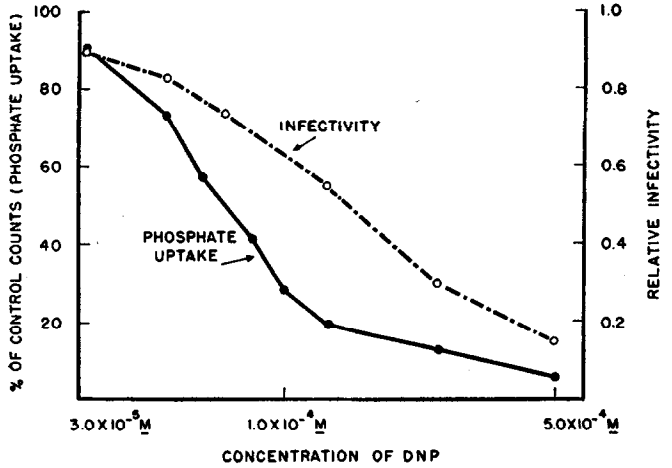

FIG. 1. Effect of DNP on phosphate uptake and virus growth. Relative infectivity = (log infectivity of flask with DNP)/(log infectivity of flask without DNP). Infectivity of flask without DNP was $10^{-8.2}$.

viral building blocks and essential high-energy phosphate bonds cannot be excluded by these results.

Since the virus titration was performed on the Simms solution, it is possible that DNP and azide may inactivate the virus after release into the medium. Tests of the infectivity of the suspending medium and of the chorio-allantoic membrane, after washing in saline and homogenizing, showed no essential difference. The seed virus upon incubation at 0.001 dilution for 16 hr. at 36°C. with DNP showed no greater decrease in

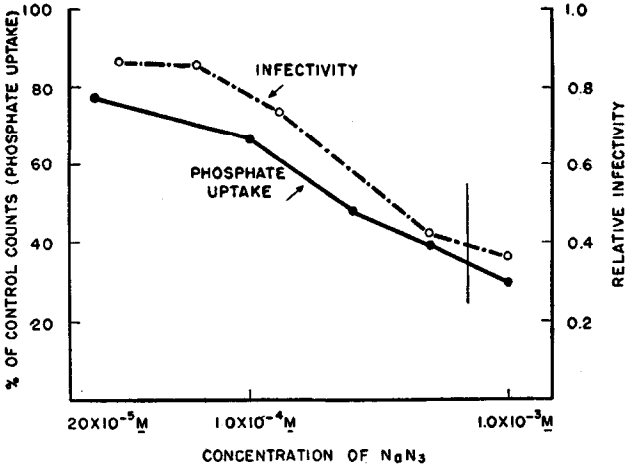

FIG. 2. Effect of NaN_3 on phosphate uptake and virus growth. Relative infectivity = (log infectivity of flask with NaN_3)/(log infectivity of flask without NaN_3). Infectivity of flask without NaN_3 was $10^{-8.2}$.

infectivity titer than the control. Similar incubation with NaN_3 gave only a tenfold decrease in infectivity titer over the control, indicating a possible slight virucidal effect of NaN_3. The lack of a direct virucidal effect of DNP has been previously reported (12, 16).

SUMMARY

2,4-Dinitrophenol and sodium azide, at concentrations that are not inhibitory to host cell respiration, have been shown to inhibit influenza A (PR8) virus synthesis and phosphate uptake in chick embryo chorio-allantoic membrane. Lack of virus development appears to be related to interference with the phosphate-bond formation.

References

1. Ackermann, W. W., *J. Biol. Chem.* **189,** 421 (1951).
2. Ackermann, W. W., *J. Exptl. Med.* **93,** 635 (1951).
3. Loomis, W. F., and Lipmann, F., *J. Biol. Chem.* **173,** 807 (1948).
4. Spiegelman, S., *J. Cellular Comp. Physiol.* **30,** 315 (1947).
5. Clifton, C. E., *Advances in Enzymol.* **6,** 269 (1946).
6. Keilin, D., *Proc. Roy. Soc.* (London) **B121,** 165 (1936).
7. Hotchkiss, R. P., *Advances in Enzymol.* **4,** 1953 (1944).
8. Spizizen, J., *J. Infectious Diseases* **73,** 212 (1943).
9. Heagy, F. C., *J. Bacteriol.* **59,** 367 (1950).
10. Thompson, R. L., *J. Immunol.* **55,** 345 (1947).
11. Price, W. H., *J. Gen. Physiol.* **31,** 135 (1947).
12. Eaton, M. D., Proc. 3rd Natl. Med. Chem. Symposium, Am. Chem. Soc., p. 46, June 1952.
13. Rafelson, M. E., Jr., Pearson, H. E., and Winzler, R. J., *Arch. Biochem.* **29,** 69 (1950).
14. Ackermann, W. W., and Johnson, R. B., *J. Exptl. Med.* **97,** 315 (1953).
15. Reed, L. J., and Muench, H., *Am. J. Hyg.* **27,** 493 (1938).
16. Burney, T. E., and Golub, O. J., *J. Immunol.* **60,** 213 (1948).