Effect of 2,4-Dinitrophenol on Replication of Poliovirus in HeLa Cells.* (25215)

GEORGE E. GIFFORD AND BILLY R. BLAKEY†
Dept. of Microbiology, University of Florida College of Medicine, Gainesville

The presence of critical concentrations of 2,4-dinitrophenol (DNP) in aerobic oxidative phosphorylative systems decreases the ratio of high energy phosphate atoms formed to the atoms of oxygen consumed and stimulates rate of oxidation of substrate. Thus, oxidation is permitted to proceed without the concomitant fixation of phosphorous. Replication of influenza virus(1-3), Theiler's GD VII mouse encephalomyelitis virus(4), and pneumonitis virus(5) has been reported to be inhibited by concentrations of DNP which increased respiration and substrate utilization of host cells but which decreased incorporation of inorganic phosphorous. In addition, synthesis of these same viruses were inhibited when host cells were maintained anaerobically (4-7). It has been postulated that the energy required for viral synthesis is supplied by high energy phosphate bonds generated by aerobic oxidation of substrate(1,5,6). On the other hand, it has been reported that poliovirus replicated in HeLa cells maintained anaerobically(8). In those studies the latent period of virus synthesis in cells maintained anaerobically was considerably prolonged but the final virus yield/destroyed cell was essentially the same as under aerobic conditions. The increased latent period can be interpreted as interference with the energy producing mechanisms required for virus synthesis. Since DNP also interferes with energy production it was of interest to investigate its effect upon virus synthesis using the poliovirus-HeLa cell system. Presumably, DNP should not inhibit replication of poliovirus in this system but should only prolong the time taken for synthesis if its mechanism of action is to uncouple phosphorylation solely from aerobic oxidative processes.

Methods. Poliovirus, type 1 (Mahoney strain) was employed as a pool of supernatant fluid from HeLa cell cultures of the 73rd virus passage. Virus suspensions were stored in ampules at −50°C. HeLa cells were grown according to the method of Syverton and Scherer(9). In place of human serum, serum protein prepared from outdated human blood bank blood was employed and supplemented with 0.2% yeast extract (Difco "Yeastolate"). For virus assay by the plaque technic, cells were trypsinized, dispensed, and established as monolayers in a medium consisting of 20% calf serum (CaS_{20}) or chicken serum (ChS_{20}) and 80% maintenance solution (MS_{80})(10) containing 0.2% yeast extract. The same medium was employed in manometric studies except that the yeast extract was omitted and 0.01 M tris(hydroxymethyl) aminomethane (THAM) was added as a supplementary buffer. *Manometric method.* Oxygen uptake was measured by the direct method in the conventional Warburg respirometer as previously described (11). Duplicate flasks were employed for each analysis. Krebs' modification of Pardee's carbon dioxide buffer was employed in oxygen uptake studies to maintain an equilibrium concentration of 2% carbon dioxide in the gas phase. *Virus assay.* Virus assays were done by the plaque method using HeLa cell monolayers in 30 x 60 mm screw-capped square, soft glass bottles(8). *Glucose.* Glucose was measured by a modified procedure using Dreywood's anthrone reagent(12). Cooled anthrone reagent, 4.0 ml, was added slowly to the samples in the icebath and they were shaken without removal. Such treatment resulted in little or no color development. Tubes were then placed into a boiling water bath for exactly 8 minutes after which they were rapidly cooled to room temperature. Optical density was read in the Beckman DU Spectrophotometer at 625 mμ. Concentrations of glucose of from 1 to 40 μg could be accurately estimated by this procedure. *Phosphorous.* Phosphorous was determined by the

* Aided by grant from Nat. Inst. Health.

† NIH Medical Student Research Fellow.

TABLE I. Effect of 2,4-Dinitrophenol upon Glucose Utilization and Viral Replicating Capacity of HeLa Cells.

Conc. of 2,4-dinitrophenol ($\times 10^{-4}$ M)	Glucose utilized, μg		Yield of poliovirus. PFU ($\times 10^5$)*
	Infected	Control	
.0	102	112	12
1.0	135	162	16
2.5	133	202	19
10.0	0	0	0†

* Inoculum contained 300 PFU.
† No plaques on 10^{-4} dilution.

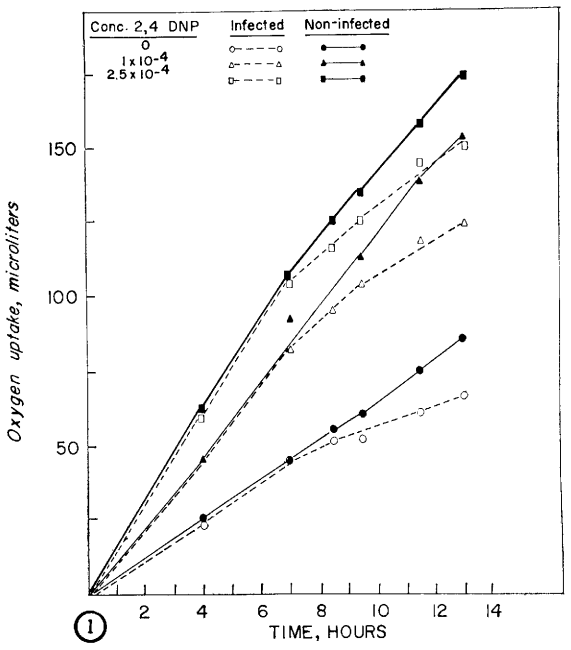
method of Fiske and Subbarow(13) or the modified procedure of Dryer, Tammes and Routh(14).

Results. The initial studies were performed with test tube cultures of HeLa cells. Concentrations of 2,4-dinitrophenol of 1 x 10^{-3} M, or greater, did not usually permit survival of HeLa cells. Concentrations of 1 and 2.5 x 10^{-4} M DNP permitted survival provided a well buffered medium was employed to prevent cell damage due to stimulation of glycolysis and concomitant acid production. Table I presents the results of an experiment in which approximately 300 PFU (plaque forming units) of poliovirus were added to replicate cultures of HeLa cells with various concentrations of DNP and incubated for 96 hours. Virus and DNP were added at the same time. Each HeLa cell culture contained approximately 80,000 cells. The results represent supernatant fluids from 5 replicate tubes and indicate that concentrations of 2,4-dinitrophenol which stimulated glycolysis did not inhibit replication of poliovirus. The data also show that the virus yield per cell was not adversely altered by these concentrations of DNP since the titers of virus given represent the total amount of virus in tubes after complete destruction of the cells.

The following procedure was done to test the possibility that the latent period of poliovirus replication in HeLa cells was altered by the presence of DNP. Warburg flask cultures containing approximately 1 x 10^6 freshly trypsinized HeLa cells were prepared and various concentrations of DNP added to flasks in quadruplicate. Two flasks of each concentration of DNP were inoculated with 1.5 x 10^6 PFU of poliovirus and the 2 remaining flasks inoculated with a similar amount of medium to serve as non-infected controls. This amount of virus would be sufficient to destroy a significant portion of the HeLa cells after one cycle of replication to be detectable manometrically as previously described(8). The time taken to destroy the virus synthesizing cells could be accurately measured by determining when these infected cell cultures consumed oxygen at a diminished rate. The results (Fig. 1), demonstrate that there was no significant alteration in the latent period of virus synthesis in the case of cells incubated in presence of the concentrations of DNP used. Table II includes the results of virus assays, rates of oxygen uptake and rates of glucose utilization in presence of 2 concentrations of DNP. Phosphorous determinations, total and inorganic, did not show significant deviation in any case from the uninoculated medium.

The same concentrations of 2,4 DNP were used in cultures of HeLa cells incubated anaerobically in Warburg respirometers. In this case, 1.6 x 10^7 PFU of poliovirus were employed with approximately 1 x 10^6 freshly trypsinized HeLa cells. The results are shown in Fig. 2. There was no apparent effect of these concentrations of DNP upon rate of acid production by HeLa cells under anaerobic conditions as indicated by rate of release of carbon dioxide from the bicarbonate buffer. Similarly, there was no alteration in the course of carbon dioxide evolution from infected cultures in the presence or absence of DNP. The delay in virus replication under anaerobic conditions, as previously determined by this procedure(8), was apparent and was not altered by the presence of DNP.

Discussion. Our results presented validate the hypothesis, based on results of anaerobic studies with HeLa cell and poliovirus(8), that poliovirus would be replicated in HeLa cells treated with critical concentrations of 2,4-dinitrophenol which markedly stimulated oxygen uptake and glucose utilization under aerobic conditions. Similar virus titers were obtained with concentrations of DNP which stimulated respiration as with the control cultures. Concentrations of DNP which inhibited glycolysis also inhibited virus synthesis; however, these concentrations of DNP did not



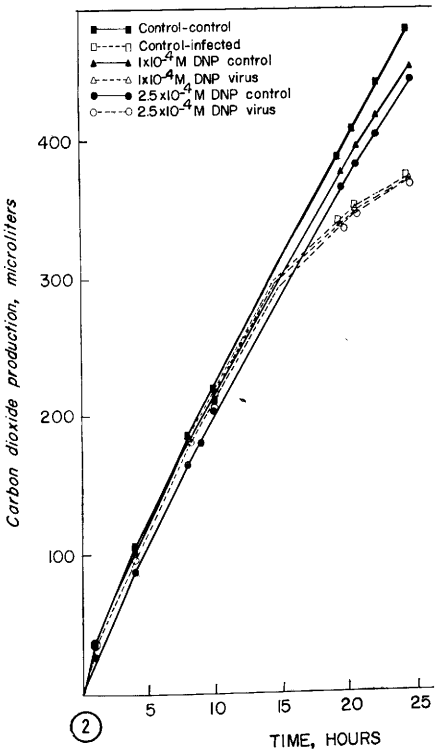


FIG. 1. Effect of 2,4-dinitrophenol upon respiration of infected and non-infected HeLa cells.
FIG. 2. Effect of 2,4-dinitrophenol upon release of carbon dioxide from bicarbonate buffer by HeLa cells under anaerobic conditions.

appear to be compatible with survival of the cells since the latter did not remain attached to the glass.

Unlike the anaerobic effect, 2,4-dinitrophenol did not alter the latent period of virus synthesis. The time required before a decrease in rate of respiration of infected cells became apparent was similar in the presence or absence of the agent. These results suggest that the energy obtained through anaerobic glycolytic pathways by HeLa cells was sufficient to permit replication of poliovirus and hence it is possible that the prolonged latent period found under anaerobic conditions (8) was not due to a lack of energy but rather to another unrecognized factor.

The failure to find any significant difference in inorganic or organic phosphorous in the medium before and after exposure of the cells to this medium demonstrates that the medium is a true maintenance medium since net growth should result in phosphate uptake. Since the inorganic and organic phosphorous concentrations of the medium remained constant in the presence of a marked increase in rate of oxygen uptake in the presence of DNP there was an uncoupling of oxidation and phosphorylation. Utilization of glucose was

TABLE II. Effect of 2,4-Dinitrophenol upon Oxygen Uptake, Glucose Utilization and Virus Yield over a 21 Hour Incubation Period.

Conc. of 2,4-dinitrophenol ($\times 10^{-4}$M)	O_2 uptake, μl/hr	Glucose utilized, μg/hr	O_2 uptake (moles) / Glucose used (moles)	Virus yield, PFU ($\times 10^7$)
.0	6.5	10.2	5.02	4.6
1.0	12.0	66.3	1.28	4.3
2.5	14.0	104.8	.95	1.7

more greatly stimulated than oxygen uptake by the presence of DNP as shown by the molar ratios of the 2 substances. Apparently the dissociating effect of DNP on aerobic phosphorylating systems in HeLa cells is compensated by greatly increased glycolysis and anaerobic phosphorylating systems. An increase of glucose uptake of 10-fold was found with 2.5 x 10^{-4} M DNP whereas uptake of oxygen was increased only 2-fold. These observations may explain the apparent discrepancies between the results presented here and those previously reported(1-7) since it is possible that the tissue cells they employed could not compensate as well with non-aerobic phosphorylating systems under conditions of anaerobiasis or in the presence of DNP. Furthermore, these previous reports on inhibition of virus synthesis by DNP have all used cells which were derived from normal animals whereas HeLa was derived from malignant tissue. Warburg(15) has stated that in cancer cells there is an increase in fermentation such that the failure of respiration is compensated for energetically.

Anaerobically maintained HeLa cells did not appear to be affected by the concentrations of DNP which markedly alter the metabolism of aerobically maintained cells. This is in accord with the generally accepted view that this substance interferes solely with aerobic processes.

Summary. Poliovirus was found to replicate in HeLa cells treated with critical concentrations of 2,4-dinitrophenol which markedly stimulated oxygen uptake and glucose utilization under aerobic conditions. The time required before a change in rate of respiration of infected cells became apparent was similar in absence or presence of DNP, indicating that this substance did not alter the latent period of virus synthesis. Apparently the uncoupling effect on aerobic phosphorylating systems is compensated for by increased glycolysis and the concomitant fixation of phosphorous by systems not effected by DNP.

1. Ackermann, W. W., Johnson, R. B, *J. Exp. Med.,* 1953, v97, 315.

2. Eaton, M. D., Perry, M. E., *J. Inf. Dis.,* 1953, v93, 269.

3. Ingraham, J. L., Roby, T. O., Peterson, J. H., *Arch. Biochem. and Biophysics,* 1953, v46, 215.

4. Pearson, H. E., *J. Immunol.,* 1950, v64, 447.

5. Moulder, J. W., McCormack, B. R. S., Itatani, M. K., *J. Inf. Dis.,* 1953, v93, 140.

6. Ackermann, W. W., *J. Biol. Chem.,* 1951, v189, 421.

7. Tamm, I., *J. Bact.,* 1956, v72, 42.

8. Gifford, G. E., Syverton, J. T., *Virology,* 1957, v4, 216.

9. Syverton, J. T., Scherer, W. F., *Ann. N. Y. Acad. Sci.,* 1954, v58, 1056.

10. Gifford, G. E., Robertson, H. E., Syverton, J. T., *J. Cell. and Comp. Physiol.,* 1957, v49, 367.

11. ———, PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 515.

12. Morris, D. L., *Science,* 1948, v107, 254.

13. Fiske, C. H., Subbarow, Y., *J. Biol. Chem.,* 1925, v66, 375.

14. Dryer, R. L., Tammes, A. R., Routh, J. I., *Eastman Chem. Abst.* No. 432.

15. Warburg, O., *Science,* 1956, v123, 309.

Received July 31, 1959. P.S.E.B.M., 1959, v102.