ENZYMIC MECHANISM OF INCREASED UTILIZATION OF GLUCOSE DURING VIRUS MULTIPLICATION IN THE CHORIOALLANTOIC MEMBRANE OF THE CHICK EMBRYO

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The chorioallantoic membrane of the chick embryo offers distinct advantages over other tissues for the study of multienzyme systems. Since it is composed of only three layers of cells, limiting factors of diffusion hardly interfere with measurements of the metabolism of intact cells in vitro. For these reasons we have undertaken the study of complex biochemical processes with the aid of excised chorioallantoic membranes. The influence of virus multiplication on the metabolism of the host tissue was one of the problems under investigation.

Earlier metabolic studies on the excised membrane revealed that during multiplication of myxoma, fibroma, herpes simplex, vaccinia, Rous sarcoma, swine influenza, and Newcastle viruses, the rate of lactic acid formation from glucose was markedly elevated. It was also shown that this increase in the accumulation of lactate was not due to inefficient transfer of electrons to O2, an effect which might be assumed to occur as a consequence of "pathological changes caused by viruses," but was the result of an increased amount of glycolytic enzymes in cells where the multiplication of viruses took place. Aldolase and the glycolytic enzymes, catalyzing the formation of 3-phosphoglycerate from fructose 1-6-diphosphate were earlier identified and the increase of their concentration in infected membranes determined. It was found, furthermore, that the chorioallantoic membrane contains glucose-6-phosphate dehydrogenase. This was believed to indicate that the dual
pathway of glucose metabolism (i.e., the "glycolytic" and "pentose cycle") may operate in this tissue. Analysis for both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase revealed that the concentration of these enzymes were identical in normal and infected membranes. These observations resulted in the further study of the biochemical significance of increased glycolysis. An increase in the rate of the conversion of glucose to pyruvate would yield a net increase in the rate of ATP formation, which could presumably be made available for biosynthetic processes. It is expected that during virus multiplication there would be an increased rate of nucleic acid synthesis. Since the pentose constituents of nucleic acids arise from the direct oxidative pathway of hexose monophosphates, this pathway should operate at a higher rate when a "physiological demand" for pentose exists. Because no increase in the hexose-phosphate dehydrogenase content of infected membranes occurred, it was assumed that some other mechanism must be responsible for the activation of the oxidation of hexose monophosphates. It is subsequently shown that this mechanism is an increased rate of the oxidation of TPNH, mainly by pyruvate, catalyzed by lactate dehydrogenase. This enzyme is also synthesized at a higher rate in cells where the multiplication of viruses occurs.

Experimental.—Techniques used in the present studies were similar to the ones reported earlier. 1 2 Chorioallantoic membranes of embryonated White Leghorn eggs, 12 days old, were infected with a suspension of a variant avian strain of canary pox virus. This virus was purified and identified by Siegel 4 and stored as a standard pool in ampules at -50°C throughout the experiments. The amount of virus used for each inoculum was 10⁴ infectious particles per egg, as standardized by the method of pock counts. 4 It was shown earlier that inoculation with heat-inactivated viruses or extracts of chorioallantoic membranes free of viruses, had no effect on the metabolism of this tissue. 2 In most experiments biochemical measurements were made 48 hr after infection. The infected and control eggs were kept in an egg incubator at 36°C. The infected areas of the membranes were excised, cut into pieces of about 1/4 cm² and suspended in a liquid medium, kept previously at 0°C, washed by transfer to fresh medium, and either used directly for manometric measurements or subjected to homogenization (in an all-glass homogenizer) and centrifugal cell fractionation 5 in 0.25 M sucrose, containing 9 mg cysteine hydrochloride per 10 ml sucrose (pH was adjusted to 7.4 with NaOH). The suspending medium in earlier studies was a buffered salt solution 1 2 but in the majority of recent experiments the centrifuged allantoic fluid or a salt solution 5 containing 400 mg bovine serum albumin per 100 ml was employed.

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>+ Glucose*</th>
<th>Infected</th>
<th>+ Glucose*</th>
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<tbody>
<tr>
<td><strong>Aerobic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QO₂</td>
<td>0.34</td>
<td>0.31</td>
<td>0.30</td>
<td>0.43</td>
</tr>
<tr>
<td>Qlact.</td>
<td>0</td>
<td>0</td>
<td>0.56</td>
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<tr>
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<td>0.42</td>
<td>0.76</td>
<td>1.12</td>
<td>2.4</td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Qlact.</td>
<td>0.70</td>
<td>1.00</td>
<td>1.90</td>
<td>4.70</td>
</tr>
</tbody>
</table>

* 5 mg glucose per flask. Q values = moles per 1 mg protein per 2 hr. Temperature = 30°C. Suspending fluid = 3 ml of allantoic fluid. Duration of experiment = 2 hr.
Manometric measurements were performed in conventional Warburg vessels at 30°C. Lactic acid was determined according to Barker and Summerson,6 glucose by the method of Nelson,6 and N by a modified Nessler procedure.7 Glucose-1-C14 was a gift of Drs. E. Z. Hassid and E. F. Neufeld (Department of Biochemistry, Berkeley). The sample was purified by paper chromatography by Dr. Neufeld, and had a specific activity of 7.5 microcuries/mg. Analyses for C14 were done directly on filter paper strips (containing K2C14O3) in a two-channel scintillation well counter by Dr. Chin-Tzu Peng in the Radioactivity Research Center of the School of Medicine. Crystalline heart muscle lactate dehydrogenase8 was a gift from Dr. J. B. Neilands (Department of Biochemistry, Berkeley). All reagents were recrystallized from reagent grade sources; coenzymes were chromatographically purified samples, obtained from Sigma Chemical Co.

Results.—Manometric experiments on intact membranes: Recent experiments differed from earlier ones1, 2 in 2 respects: (a) the suspending medium contained protein, and (b) the aerobic manometric measurements were done in 95% O2 + 5% CO2. Under these conditions pressure changes in the system are the result of both O2 uptake and CO2 evolution. Anaerobic production of CO2 was determined under the same conditions with 95% N2 + 5% CO2. It was found that under aerobic conditions the normal membrane, suspended in allantoid fluid con-

| TABLE 2 | GLUCOSE UPTAKE AND LACTATE FORMATION BY CHORIOALLANTOIC MEMBRANES |
|-----------------|-----------------|-----------------|
|                 | Normal          | Infected        |
| Aerobic         | QO2             | 0.72            | 0.62            |
|                 | Qglucose        | 0.34            | 0.70            |
|                 | Qlact-          | 0.39            | 0.87            |
|                 | QCO2            | 0.55            | 0.92            |
| Anaerobic       | Qglucose        | 0.60            | 1.20            |
|                 | Qlact-          | 2.3             | 3.46            |

Time of experiment = 5 hr. Temperature = 30°C. Suspending medium = buffered salt solution containing albumin. Q values = μmoles per 1 mg protein.

containing glucose, produces only negligible amounts or no lactic acid at all. On the other hand, 48 hr after virus inoculation, during a period of exponential viral multiplication,7 aerobic lactate accumulation does occur. Under anaerobic conditions the infected membranes evolve much larger amounts of lactate and CO2 than the normal ones. A representative experiment is shown in Table 1.

The rate of lactic acid formation exceeded the amount of CO2 evolved, suggesting that the mechanism by which lactate is produced does not involve a marked change in the total H+ concentration. It is of interest to note that the use of protein containing suspending medium, as suggested by Warburg18 abolishes aerobic lactate accumulation in normal membranes. This indicates the susceptibility of electron transferring enzymes in intact cells to denaturation by relatively mild agents, e.g., salts.

The question arises whether or not the increase of lactate accumulation occurs simultaneously with a net increase in glucose consumption. The experimental answer to this question is complicated by the fact that intact membranes as well as allantoid fluid contain variable amounts of endogenous carbohydrate. The interference of endogenous sources of glucose can be diminished by the use of albumin containing suspending medium, and by prolonged incubation, when larger dif-
ferences in glucose utilization become measurable by analyses for glucose in the suspending medium. Although the accurate determination of glucose utilization is not possible, the large increase in glucose disappearance is a fair indication that increased lactate accumulation occurs simultaneously with increased glucose consumption. This is shown in Table 2. There is aerobic lactic acid production by the normal membranes suggesting that prolonged incubation in the albumin containing salt solution interferes with the efficient aerobic utilization of pyruvate.

The pathway of glucose utilization was further studied with the aid of C\textsuperscript{14} labeled glucose in the 1 position. The conditions were the same as described for the experiment summarized in Figure 1, except that each flask contained 2 microcuries of glucose-1-C\textsuperscript{14} as a tracer. The gas phase was either 100\% O\textsubscript{2} or 100\% N\textsubscript{2}. The reaction was stopped by tipping into the main compartment 0.3 ml 2 N HCl, which lowered the pH below 2.0. The CO\textsubscript{2} (containing C\textsubscript{14}O\textsubscript{2}) was absorbed on a filter-paper strip saturated with 10 N KOH, placed into the center well. Results are summarized in Figure 1. The rate of C\textsubscript{14}O\textsubscript{2} evolution is much greater under aerobic conditions, when both anaerobic and aerobic metabolic pathways operate. Under these conditions C\textsubscript{14}O\textsubscript{2} derived from oxidative decarboxylation of 6-phosphogluconate is in part further metabolized by the tricarboxylic acid cycle. The increase in C\textsubscript{14}O\textsubscript{2} formation during virus multiplication was 47.5 per cent. The rate of C\textsubscript{14}O\textsubscript{2} evolution from C\textsubscript{14}-glucose under anaerobic conditions is more closely a measure of the rate of decarboxylation of 6-phosphogluconate since only the anaerobic CO\textsubscript{2} fixing enzymic reactions interfere with the detection of the formation of C\textsubscript{14}O\textsubscript{2}. The increase in C\textsubscript{14}O\textsubscript{2} evolution by membranes supporting virus multiplication under N\textsubscript{2} atmosphere was 120 per cent. It is probable that the increased rate of hexose-6-phosphate oxidation varies between these extreme values (50 to
120\% ) in all membranes where multiplication of viruses takes place. Metabolic measurements on intact membranes thus revealed that both glycolysis and the hexose monophosphate shunt operate at a higher rate during active virus reproduction.
The effects observed with intact membranes were reproduced in cell-free systems. It was found that the centrifugal supernatant of sucrose homogenates (24,000 × g for 30 min) contained the whole enzyme system necessary for the demonstration of the virus-induced increase in CO₂ and lactate production. Some component of the system was found to be sensitive to oxidation by atmospheric O₂ as manifested in a progressive diminution of the virus effect upon standing. This instability was counteracted by the use of cysteine in the homogenizing medium. The cell-free system contained 30 μmoles of glucose-6-phosphate (K-salt), 2.5 μmoles of TPN⁺, 10 μmoles of MgSO₄, 20 μmoles of Na-pyruvate and 80 μmoles of nicotinamide, 30 μmoles of PO₄, 30 μmoles of HCO₃⁻, and 9 mg protein (supernatant of a homogenate, prepared in 0.25 M sucrose, containing cysteine). The volume of the reaction mixture was made up to 3.1 ml with sucrose-cysteine solution, and the reaction measured for 30 min under 95% N₂ + 5% CO₂. Since under these conditions it is expected that both the direct oxidation of glucose-6-phosphate and, due to endogenous adenine nucleotides and traces of DPN⁺, its fermentation to lactate take place simultaneously, the effect of added DPN⁺ was also studied. It was found that addition of DPN⁺ (2.5 μmoles) markedly diminished lactate accumulation but not CO₂ production in the preparations obtained either from normal or infected membranes. This inhibitory effect of DPN⁺ on lactate accumulation argues against the assumption that the formation of lactate is solely due to the reaction

\[
\text{pyruvate} + \text{DPNH}^+ + H^+ \rightarrow \text{lactate} + \text{DPN}^+. 
\]

Attempts to eliminate the interference of endogenous nucleotides by adsorption techniques were only partially successful and the susceptibility of the system to oxidation during these procedures led us to abandon this effort. The effects of added DPN⁺, and of virus infection on the rate of CO₂ and lactate formation are shown in Figure 2a, b. The “protection” by cysteine indicated that a thiol system is involved in this multienzyme system. The effect of iodoacetate was therefore determined. As shown in Table 3, a concentration of iodoacetate which completely inhibits triose phosphate dehydrogenase only partially blocks these reactions, again suggesting that an additional mechanism besides glycolysis is in part responsible for the accumulation of lactate.

Analyses of the condition of these reactions revealed that the mechanism of “non-glycolytic” lactate formation is

\[
\text{TPNH} + H^+ + \text{pyruvate} \rightarrow \text{lactate} + \text{TPN}^+. 
\]

The enzyme responsible for this reaction was found to be a lactate dehydrogenase (LHD) of the chorioallantoic membrane. The following observations support this conclusion:
(a) no evidence was found for a direct transfer of H between DPN+ and TPN+ in the soluble extract of this tissue;
(b) there is a parallel increase of LDH concentration with increased CO₂ and lactate formation;
(c) this LDH catalyzes the reduction of pyruvate by both DPNH and TPNH;
(d) DPN+ in a concentration range of 10⁻⁴ to 10⁻⁶ M almost completely inhibits the TPNH-pyruvate reaction catalyzed by either crystalline heart muscle enzyme, or the chorioallantoid LDH;
(e) the chorioallantoic LDH is susceptible to inhibition by iodoacetate when it catalyzes the reaction TPNH+H+ + pyruvate → lactate+TPN+. The DPNH→ pyruvate reaction is not inhibited by iodoacetate.

A substrate mediated transhydrogenation of the type suggested by Holzer is quite improbable because of the inhibition of the TPNH-pyruvate reaction by DPN+. It should also be noted that Holzer used 0.4 mg of crystalline LDH and 0.48 mg glucose-6-phosphate dehydrogenase (as TPNH generator) to produce an estimated absorbancy change at 366 mµ (presumably due to TPNH) of 0.120 in 22 to 58 min. These conditions do not seem to support strongly the suggestion that LDH can catalyze a substrate mediated transhydrogenation to a significant extent.

### Table 4

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Normal</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>43</td>
<td>55</td>
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<td>24</td>
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<td>30</td>
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<td>78</td>
<td>32</td>
<td>77</td>
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</table>

The results are expressed in micromoles × 10^⁻³ of DPNH oxidized per 1 mg protein per 1 min. The optical system contained 30 µmoles of Na-pyruvate 0.4 to 0.6 µmoles of DPNH, 250 µmoles of phosphate (pH 7.4) in a volume of 3 ml. The amount of protein (supernatant fraction) varied between 5 and 12 gamma per cuvette. Absorbancy changes were read at 30 sec intervals for 4 min.

It is of interest that neither the DPNH-pyruvate nor TPNH-pyruvate reactions catalyzed by heart muscle LDH are inhibited by iodoacetate in concentrations employed here. A detailed kinetic study of these differences will be dealt with elsewhere.

The increase in LDH content of chorioallantoic membranes, supporting the multiplication of avian pox virus, is shown in Table 4. The effect of iodoacetate on the oxidation of DPNH and TPNH by pyruvate and LDH of the chorioallantoic membrane and of heart muscle are summarized in Table 5.

It was of interest to determine whether or not the amount of other pyridine nucleotide dehydrogenases is changed when the membrane supports virus multiplication. The following enzymes were tested in the supernatant fraction: malic enzyme, malic dehydrogenase, glutamic dehydrogenase, and glutathione reductase. Results are shown in Table 6. A marked increase in malic dehydrogenase and GSSG reductase occurred 48 hr after virus infection. The glutamic dehydrogenase content of this tissue is low and the significance of the increase of this enzyme is questionable. On the other hand, the oxidation of TPNH by either GSSG or oxalacetate may be of importance. Since there is at present no experimental evidence indicating an increased rate of oxalacetate formation after virus infection
while the observed increase in glycolysis yields a higher rate of pyruvate production, it seems likely that the oxidation of TPNH by pyruvate has priority in the mechanism of increased glucose utilization. The role of GSSG reductase is probably a significant one, but at this time difficult to explain.

Discussion.—According to earlier experiments and the ones reported here, the chorioallantoic membrane responds to virus infection with a marked increase in glycolytic enzymes. Similar observations were made recently with poliomyelitis virus on tissue cultures of epithelium cells. It is probable that the primary event in virus infection, in a metabolic sense, is a specific influence of a virus component on the enzyme-synthesizing system, which responds by a hitherto-unknown mechanism with an activation of synthesis of certain enzyme proteins. As shown here, the increase of the concentration of certain enzyme proteins can profoundly modify the metabolic pattern of the “infected” cell. It seems reasonable to think that this modification of metabolism will eventually result in the reshuffling of the constituents of the virus itself. We have so far only studied certain phases of carbohydrate metabolism and the example for the hypothesis offered above is restricted to the increased formation of the pentose constituent of virus nucleic acid.

Many challenging questions arise. Calculations based on cell counts of chorioallantoic membranes and titration of infective particles 48 hr after inoculation reveal that the number of cells containing infective viruses is only 10 to 20 per cent of the total cell population studied. An increase of 50 per cent in the average concentration of certain enzymes in the whole tissue may mean an increase of several hundred per cent in the infected cell or it may reflect an effect even in the cells which do not contribute to virus multiplication. There is no answer as yet to this question.

The virus-induced onset of aerobic lactate accumulation in this apparently “aerobic” tissue suggests, according to Warburg, that in appearance the membrane exhibits characteristics of a cancerous tissue. While a generalization is as yet not timely, this observation suggests to us a reinvestigation of certain aspects of glucose metabolism of cancer cells and a search for the type of mechanism of lactate accumulation as found in virus-infected membranes. It is obvious that the increase in concentration of certain enzymes does not have to be excessive in order to bring about profound metabolic changes. Again, the appearance of glycolytic enzymes in the serum of tumor-bearing animals, as found by Warburg and confirmed by Sibley and Lehninger, may be an indication of their increased biosynthesis.

The change in glucose utilization by the chorioallantoic membrane is readily

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**TABLE 5**

**The Effect of Iodoacetate on the Enzymic Oxidation of DPNH and TPNH**

<table>
<thead>
<tr>
<th></th>
<th>Normal DPNH</th>
<th>O Iodoac.</th>
<th>Infected DPNH</th>
<th>O Iodoac.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chorioallantoic enzyme</td>
<td>32</td>
<td>34</td>
<td>79</td>
<td>77</td>
</tr>
<tr>
<td>Heart muscle LDH (cryst.)</td>
<td>7,000</td>
<td>.</td>
<td>.</td>
<td>1,900</td>
</tr>
</tbody>
</table>

Results are expressed as in Table 4. The concentration of TPNH was 1 micromole per 3 ml iodoacetate concentration = $1.4 \times 10^{-3} M$.

The centrifugal supernatant (chorioallantoic enzyme) was tested 48 hr after inoculation with the virus. The amount of heart muscle LDH used in the optical test was 0.05 to 0.1 gamma protein.
explained by an increased concentration of glycolytic enzymes. Lactic acid dehydrogenase plays a special role since it effects an oxidation of TPNH by pyruvate and causes an increased turnover in the "pentose cycle." Our results on the catalytic role of LDH in TPNH oxidation are in close agreement with those of Navazio, Ernster and Ernster.17 The striking inhibition of the TPNH → pyruvate reaction by physiological concentrations of DPN⁺, also observed by these authors, may have a physiological significance. It is noteworthy that TPN⁺ has no inhibitory effect at all on the DPNH → pyruvate reaction. This implies that the oxidation of TPNH by pyruvate, catalyzed by LDH would only be possible when the amount of available DPN⁺ in the cell is very low. The actual concentration of DPN⁺ in, for example, normal rat liver is about 0.6–0.7 umoles per gm,18 sufficient to cause a marked inhibition. It is quite possible that a change in the intracellular distribution of DPN⁺, in addition to the rate of its reduction, constitutes regulatory mechanisms which contribute to the control of the rate of oxidation of TPNH by pyruvate.

It is of considerable interest that the isotope experiments of Wenner et al.19 showed that in Novikoff hepatoma and ascites tumor cells the major oxidizing agent of TPNH is pyruvate. It seems likely that the enzymic mechanism of TPNH oxi-

<p>| TABLE 6 |
| CONCENTRATION OF DEHYDROGENASES IN THE CHORIOALLANTOIS 48 HR AFTER VIRUS INOCULATION |</p>
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>DPNH</th>
<th>TPNH</th>
<th>DPNH</th>
<th>TPNH</th>
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</thead>
<tbody>
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<td>Malic enzyme</td>
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<td>...</td>
<td>0.8</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>29.0</td>
<td>4.3</td>
<td>53.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Glutamic dehydrogenase</td>
<td>0.2</td>
<td>...</td>
<td>0.3</td>
<td>...</td>
</tr>
<tr>
<td>GSSG reductase</td>
<td>...</td>
<td>3.9</td>
<td>...</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Results are expressed as in Tables 4 and 5. Malic enzyme and glutamic dehydrogenase were assayed according to standard procedures,13 malic dehydrogenase, as described elsewhere,18 and glutathione (GSSG) reductase by the oxidation of TPNH (1 umole) in the presence of 15 moles of GSSG at pH 7.4 in 0.1 M phosphate buffer in a volume of 3 ml.

dation in these tumor cells may be the same as in the virus-infected chorioallantoic membrane.

The LDH of the chorioallantoic membrane differs from enzymes of other sources in several respects. The most striking difference is the iodoacetate sensitivity of the TPNH → pyruvate reaction. None of the other reactions catalyzed by this LDH are inhibited by iodoacetate. The heart muscle enzyme is quite refractory to thiol reagents other than p-chloromercuribenzoate. Ox heart LDH contains 7 to 9 SH groups per mole, but only 3 to 4 of these react with p-chloromercuribenzoate in the native protein.20, 21 The SH groups of heart muscle LDH probably participate in the binding of coenzymes to the protein.21 The significance of the thiol sensitivity of the LDH of the chorioallantois, in the binding of coenzymes, and their analogues, may become better understood when kinetic studies similar to those reported by Kaplan et al.22 have been completed.

Studies dealing with the LDH and malic dehydrogenase content of the chick embryo during aging have been reported by Solomon.24 LDH activity on a protein basis in organs of the embryo remain constant except in the heart, where a small increase occurs on the 14th day. In our experiments, the analytical results of virus-infected membranes were in each instance compared with normal embryos
of identical age. Therefore, slight variations caused by aging were constantly com-
pensated for.

Summary.—(1) Avian pox virus-infected chorioallantoic membranes of chick embryos, suspended in allantoic fluid, produce lactic acid from glucose under aerobic conditions. Under identical conditions, non-infected membranes do not accumulate lactate.

(2) The rates of glucose consumption and-lactate and CO₂ production (the latter two under anaerobic conditions) is markedly increased as the result of virus infection.

(3) The rate of CO₂ formation from C14-labeled glucose is increased between 50 to 120 per cent 48 hours after inoculation with virus.

(4) The same effect of virus infection was reproduced with a soluble enzyme preparation (centrifugal supernatant) and it was shown that virus infection causes an increased synthesis of glycolytic enzymes resulting in an increased rate of pyruvate formation. LDH of the membrane catalyzes the oxidation of TPNH by pyruvate. The increase in LDH after virus infection accounts for the increased operation of the hexose monophosphate shunt in intact membranes.

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† Established Investigator of the American Heart Association, Inc.
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