IMMUNOLOGICAL AND CHEMICAL INVESTIGATIONS OF VACCINE VIRUS

V. Metabolic Studies of Elementary Bodies of Vaccinia

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The lack of knowledge of the exact nature of viruses has led to a great deal of discussion; certain workers believe that they are inanimate substances, while others look upon them as minute living organisms. According to the first view, a virus is capable of reproduction by a process of autocatalysis; this idea has received considerable support from the work of Stanley (1), who claims to have isolated a crystalline protein having the properties of tobacco mosaic. According to the second conception, viruses are autonomous living agents, which, being incapable of independent existence, are obligate intracellular parasites (2–4). If this conception be correct, it is not unreasonable to suppose that under proper conditions viruses might evidence independent metabolism even though they are capable of multiplication only in the presence of living host cells. With the object of demonstrating such metabolism, several attempts have been made in the past to measure the rate of respiration of viruses.

In attempts to measure the respiration of certain viruses, Bronfenbrenner (5) used a microspirometer, capable of detecting very small amounts of carbon dioxide. He failed to find elaboration of carbon dioxide by bacteriophage. Later, with Reichert (6), he attempted to measure the amount of oxygen consumed and carbon dioxide produced by the viruses of rabies and herpes simplex. Lacking cell-free preparations of these viruses, the authors resorted to the use of emulsions of brain tissue which contained the infectious agents; emulsions of normal brain tissue were employed as controls. They reported that the values obtained for the virus-containing emulsions did not differ significantly from those found for normal tissue. They also found that a certain amount of oxygen was consumed even after the tissues had been stored in the ice box for 6 months, by which time the tissues had lost the property of forming carbon dioxide; the consumption of oxygen was not related to the activity of the virus, continuing even after the virus had been

inactivated with phenol. More recently, Breinl and Glowazky (7) reported that they were able to demonstrate respiration of vaccine virus which had been freed from cells by means of differential centrifugation. Assuming an active metabolism of the virus, it is difficult, however, for one to understand how the figures they give could have been obtained by the methods described. In a brief communication, Kempner (8) has reported that the serum of birds containing the virus of fowl plague absorbs oxygen while the serum from normal fowls does not. This absorption of oxygen is not inhibited by octyl alcohol which quickly stops the absorption of oxygen by red blood cells.

In the past it has been difficult or impossible to obtain appreciable amounts of active virus in a state suitable for metabolic studies. In the case of vaccine virus, however, this difficulty has been overcome, because, by the application of recently developed technics, it is possible to prepare suspensions of elementary bodies of vaccinia which are almost entirely free from other particulate material, to wash the elementary bodies, and to concentrate a suspension of them to any desired degree, (9, 10). Furthermore, although the original preparations contain a few viable bacteria these may be killed with ether without materially harming the virus. Such suspensions of washed elementary bodies which are highly infectious have been used by us for the study of consumption of oxygen and production of acid by vaccine virus.

Methods and Materials

Preparations of Suspensions of Elementary Bodies of Vaccinia.—The manner of preparing suspensions of elementary bodies of vaccinia has already been described in detail (10). The elementary bodies in such suspensions have been subjected to repeated washing in a markedly hypotonic (0.004 M, citric acid-sodium phosphate buffer) solution by means of differential centrifugation, and were free from cells in so far as could be determined by the examination of numerous stained smears. Bacteria and any viable tissue cells that might have remained despite the repeated washings were killed by the addition of an excess of ether to the suspensions which had been reduced to a volume of 15 cc.; the mixture was stored at 4°C. until poured agar plates seeded with 0.1 cc. samples failed to reveal bacterial growth. For use in experiments in which the consumption of oxygen was to be measured the elementary bodies were suspended in 0.066 M phosphate buffer solution of pH 7.2; in experiments in which the production of acid was determined they were suspended in distilled water or Ringer's solution; sodium bicarbonate solution was added at the beginning of the experiment. In either case, before being used they were washed twice with the appropriate solution and finally suspended in 1.4 to 2.0 cc. of it, due precautions being taken to avoid

the introduction of bacteria. For each experiment the elementary bodies obtained from 10 rabbits were used. After the amounts of oxygen consumed and acid produced were estimated, the infectious titers of the preparations were determined by intradermal inoculation in rabbits of serial tenfold dilutions of the suspensions; most of them were active in a dilution of 10^{-9}. At the conclusion of each experiment the elementary bodies were washed 3 times in distilled water, dried *in vacuo* over calcium chloride, and weighed.

Warburg Apparatus.—Oxygen consumed and total acid (carbon dioxide plus fixed acid) produced, as measured by liberation of carbon dioxide from a buffer solution containing sodium bicarbonate and carbon dioxide, were measured in a Warburg apparatus of the usual type (11). Appropriate controls containing all the solutions used were included in each experiment and the figures recorded have been corrected for any pressure change shown by the control.

Tissue Extract Containing a Respiratory Supplement.—As a source of respiratory supplement an extract of normal kidney prepared in the following manner was used. A normal rabbit was killed by the intravenous injection of air, and the kidneys were removed aseptically. These were minced with scissors and ground with alundum and twice their weight of isotonic buffer solution, pH 7.2. The emulsion was spun for 30 minutes in an angle centrifuge, and the supernatant fluid following centrifugation was passed through a Berkefeld V filter. It was used within a short time after preparation.

Rabbit Erythrocytes.—To secure a suspension of red blood cells, a normal rabbit was bled from the heart under ether anesthesia; the blood was defibrinated, and the cells were washed twice with isotonic buffer solution or Ringer's solution. The top layer of cells was taken off after each washing in order to remove as many leucocytes as possible.

EXPERIMENTAL

In order to study the metabolism of a virus, it is necessary to have appreciable quantities of the active agent, free from living bacteria and viable tissue cells, suspended in small volumes of liquids. A suspension of the elementary bodies of vaccinia, prepared in the manner described, satisfies these requirements. In the following experiments we studied the consumption of oxygen and production of acid by washed elementary bodies of vaccinia under certain controlled conditions. Then, the effect of addition of freshly prepared tissue extracts containing a "respiratory supplement" to the suspension of elementary bodies was observed. Finally, the uptake of oxygen and production of acid by a mixture of elementary bodies and rabbit erythrocytes, under aerobic and anaerobic conditions, were measured.

In the first experiment the oxygen consumed by the elementary bodies was determined both before and after the addition of glucose, glucose monophosphate, and methylene blue, respectively.

Experiment 1.—A suspension containing 24 mg., dry weight, of elementary bodies free from living bacteria was prepared in isotonic buffer solution; the volume was then reduced to 1.6 cc. The elementary bodies were placed in a Warburg vessel; 0.1 cc. of 0.55 M glucose solution was put in one side arm and 0.1 cc. of glucose monophosphate solution in the other. Normal sodium hydroxide solution was placed in the inset vessel to absorb carbon dioxide. Both before and after addition of substrate, the amount of oxygen taken up during certain intervals of time was measured. Methylene blue was also added to the system and its effect noted. The results of the experiment are set forth in column 2 of Table I. In certain subsequent experiments similar observations on the amount of oxygen consumed by elementary bodies under the same conditions were made and the results of these experiments have been included in Table I for ease of comparison.

From the results shown in Table I it is obvious that there was a very small uptake of oxygen by the elementary bodies alone, most of which occurred in the first hour of observation; very little absorption followed the addition of glucose or glucose monophosphate; there was almost no effect from the addition of methylene blue to the system. These results differ markedly from those obtained with bacteria (12). The quantity of oxygen absorbed per milligram nitrogen, in the elementary bodies is much less (3 c.mm. compared with 15 to 340 c.mm.) than that taken up by resting bacteria (13). While the amount of oxygen consumed by bacteria is greatly increased by addition of glucose, this had no effect on the uptake of oxygen by elementary bodies. Furthermore, in the case of the elementary bodies the reaction is soon complete, and after a period of an hour or so no further absorption occurs, a phenomenon not noted in the case of bacteria observed for comparable periods of time. That the cessation of consumption of oxygen was not due to loss of activity of the virus was shown by inoculation of the virus into animals which revealed that the elementary bodies were as infectious at the completion of the experiments as they were at the beginning. The results obtained with elementary bodies differ also from those secured with spores. Goddard (14) has shown that the consumption of oxygen by dormant[1]

[1] The ascospores of the fungus *Neurospora* are normally dormant and will germinate only after they have been heated. The heat treatment which overcomes the dormancy consists in heating the spores to a temperature of 50°C. or higher for a few minutes and then cooling to room temperature. The spores germinate 3 to 5 hours after returning to the lower temperature.

TABLE I

Consumption of Oxygen by Elementary Bodies of Vaccinia

		Contents of vessel		
		cc.	*cc.*	*cc.*
24 mg. elementary bodies in 1.60 cc. phosphate buffer..		1.60		
11.5 " " " " 0.75 " " " ..			0.75	
8.0 " " " " 0.80 " " " ..				0.80
0.55 M glucose solution (in side arm)		0.10	0.10	0.20
0.10 M " monophosphate, potassium salt (in side arm).....		0.10	0.10	0.20
0.001 M methylene blue (added after opening vessel) ...		0.10		
Substrate	Interval	Oxygen absorbed		
	min.	*c.mm.*	*c.mm.*	*c.mm.*
No substrate	8	3.6		
	21	7.2		
	42	8.2		
	60	9.2		
Glucose added from side arm	7			2.3
	10		2.6	
	40	1.0		
	45		4.1	
	60	0.0		
	63			2.9
	69		4.1	
	137			2.9
	180			2.9
Glucose monophosphate added from side arm	15		−0.5	
	22	−1.5		
	31		0.0	
	41	−0.5		
	60		1.0	
	65	0.5		
	105		0.0	
Methylene blue added after opening vessel	6	1.0		
	30	2.5		
	55	3.0		

Temperature, 37°C.; gas in vessel, air; NaOH in inset vessel.

The figures of column 3 are taken from column 2 of Table III, those of column 4 are taken from column 2 of Table IV.

spores of *Neurospora tetrasperma* is very slight. When the spores are activated, however, the amount of oxygen consumed is immediately greatly increased, and shows the same continuous character as that evidenced by bacteria.

Having found that the amount of oxygen absorbed by elementary bodies is very small, we next determined whether acid is produced by them under anaerobic conditions by measuring the amount of carbon dioxide released from a buffer solution containing sodium bicarbonate and carbon dioxide.

Experiment 2.—Elementary bodies (26.0 mg., dry weight) were washed several times in distilled water and finally suspended in 2.0 cc. of it. A dilute solution of sodium bicarbonate was added, and, after equilibrating with a definite partial pressure of carbon dioxide, measurements were made of the amounts of carbon dioxide liberated. After appropriate intervals of time glucose and later glucose monophosphate were added. The results of this experiment, and of other experiments done under similar conditions, are set forth in Table II.

The figures given in Table II show that a small amount of carbon dioxide was released, almost all of which was liberated in the first hour of observation. These results agree with those of the experiments in which absorption of oxygen was measured, and provide little or no evidence of a measurable amount of metabolism of elementary bodies under the conditions employed. In view of the obligate parasitism of viruses this result was not unexpected, since viruses evidently depend on host cells for certain essential factors. The suggestion was made that a fresh tissue extract might provide a more satisfactory substrate for the virus which would be suitable for the demonstration of its metabolism. It is known that extracts of many tissues contain a "respiratory supplement" which is capable of stimulating the consumption of oxygen of certain cells, such as erythrocytes (15). In order to learn whether the addition of tissue extracts containing such supplement to a suspension of elementary bodies would increase their metabolism to such an extent as to make it measurable, the following experiment was performed.

Experiment 3.—A quantity of elementary bodies was prepared, suspended in Ringer's solution, and divided into two equal portions. To one of these, 0.75 cc.

TABLE II

Production of Acid by Elementary Bodies of Vaccinia under Anaerobic Conditions

		Contents of vessel			
		cc.	*cc.*	*cc.*	*cc.*
26.0 mg. elementary bodies in 2.00 cc. distilled water		2.00			
18.0 " " " " 0.90 cc. modified Ringer's solution			0.90		
10.2 mg. elementary bodies in 0.90 cc. modified Ringer's solution				0.90	
7.6 mg. elementary bodies in 0.70 cc. modified Ringer's solution					0.70
Modified Ringer's solution			0.90	0.90	0.60
0.31 M sodium bicarbonate		0.16	0.40	0.40	0.30
0.55 M glucose (in side arm)		0.10	0.20	0.20	0.20
0.10 M glucose monophosphate, potassium salt		0.10			
Substrate	Interval	CO_2 released			
	min.	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
No substrate	20	1.6			
	60	8.2			
Glucose added from side arm	6	−0.7		−2.5	
	22				1.3
	30	1.3			
	45	1.3	1.1		
	49				1.3
	68			−1.2	
	72	2.2			
	82				2.6
	95			0.0	
	109		3.3		
	125			−1.2	2.6
	155			−1.8	
	160		5.5		
	203			−0.6	
	212		11.1		
Glucose monophosphate added from side arm	19	0.7			
	38	2.6			
	56	3.3			

Temperature, 37.0°C.; gas in vessel, 5 per cent CO_2, 95 per cent N_2; pH 7.2.
The figures of column 3 are taken from column 2 of Table V.

of a tissue extract, prepared in the manner described above, was added; to the other an equal volume of a buffer solution was added. To test for the presence of a respiratory supplement in the extract two further mixtures were made, *viz.*, red blood cells plus buffer solution and red blood cells plus tissue extract. An additional control of tissue extract alone was run. The amounts of oxygen consumed by these preparations were measured, and the results are summarized in Table III.

TABLE III

Consumption of Oxygen by a Mixture of Elementary Bodies of Vaccinia and Tissue Extract, and by a Mixture of Rabbit Erythrocytes and Tissue Extract

		Contents of vessel				
		cc.	*cc.*	*cc.*	*cc.*	*cc.*
Elementary bodies in phosphate buffer solution, 23 mg. in 1.5 cc		0.75	0.75			
30% kidney extract			0.75	0.75		0.75
50% suspension rabbit red cells					0.75	0.75
0.066 M phosphate buffer solution pH 7.2		0.75		0.75	0.75	
0.55 M glucose (in side arm)		0.10	0.10	0.10	0.10	0.10
0.15 M glucose monophosphate (in side arm)		0.10	0.10	0.10	0.10	0.10
Substrate	Interval	Oxygen absorbed				
	min.	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
Glucose added from side arm immediately before beginning readings	10	2.6	4.1	1.1	4.2	5.7
	45	4.1	7.1	2.1	17.9	25.8
	69	4.1	9.4	4.3	23.9	34.1
Glucose monophosphate added from side arm	15	−0.5	−0.6	−0.5	3.6	7.7
	31	0.0	1.2	1.1	9.5	16.5
	60	1.0	3.5	3.2	20.3	31.0
	105	0.0	5.9	5.4	37.1	55.0

Temperature, 37.0°C.; gas in vessel, air; NaOH in inset vessel.

In Table III it will be seen that the amount of oxygen consumed by the mixture of elementary bodies and tissue extract is almost exactly the sum of that consumed by the two components of the mixture determined separately. On the other hand, the rate of consumption of oxygen by the red blood cells was definitely increased in the presence of the tissue extract, thus indicating that it contained an adequate amount of active respiratory supplement.

Since from the results of the experiment just described it appeared

that the addition of a respiratory supplement was without effect on the rate of consumption of oxygen by the elementary bodies, we decided to determine whether the elementary bodies carried substances capable of accelerating the metabolism of rabbit red blood cells.

Experiment 4.—In order to detect the presence in elementary bodies of a respiratory supplement-like substance the amount of oxygen consumed by a mixture of elementary bodies and red blood cells was determined, and compared

TABLE IV

Consumption of Oxygen by a Mixture of Elementary Bodies of Vaccinia and Rabbit Erythrocytes

		Contents of vessel		
		cc.	*cc.*	*cc.*
16 mg. elementary bodies in 1.6 cc. phosphate buffer solution		0.80	0.80	
50% rabbit erythrocytes			0.80	0.80
0.066 M phosphate buffer solution		0.80		0.80
0.55 M glucose (in side arm)		0.20	0.20	0.20
Substrate	Interval	Oxygen absorbed		
	min.	*c.mm.*	*c.mm.*	*c.mm.*
Glucose added from side arm immediately before beginning readings	7	2.3	3.4	3.3
	63	2.9	5.7	4.9
	137	2.9	9.1	6.5
	180	2.9	10.8	8.7

Temperature, 37.0°C.; gas in vessel, air; NaOH in inset vessel.

with the amount of oxygen consumed by each separately in comparable periods of time. The elementary bodies and cells were suspended in 0.066 M phosphate buffer solution in the presence of a glucose substrate. The results are recorded in Table IV.

It will be seen from the results shown in Table IV that there was no greater consumption of oxygen by the mixture of elementary bodies and red blood cells than could be accounted for by the addition of the values obtained for identical quantities of each separately. Similar experiments were then performed under anaerobic conditions.

Experiments 5, 6, 7.—For the experiments in which the amount of acid produced by a mixture of red blood cells and elementary bodies under anaerobic

conditions was determined, the elementary bodies and red blood cells were suspended, after washing, in Ringer's solution which had been modified by the omission of sodium bicarbonate. The sodium bicarbonate was added at the beginning of the experiment. The results of one of the experiments are given in Table V.

In the first experiment in which the rate of production of acid by a mixture of elementary bodies and red blood cells was measured, the quantity of acid produced by the mixture was definitely greater than

TABLE V

Production of Acid by a Mixture of Elementary Bodies of Vaccinia and Rabbit Erythrocytes under Anaerobic Conditions

		Contents of vessel		
		cc.	*cc.*	*cc.*
36.0 mg. elementary bodies in 1.8 cc. modified Ringer's solution		0.90	0.90	
50% suspension rabbit erythrocytes			0.90	0.90
Modified Ringer's solution		0.90		0.90
0.31 M sodium bicarbonate solution		0.40	0.40	0.40
0.55 M glucose (in side arm)		0.20	0.20	0.20
Substrate	Interval	CO_2 released		
	min.	*c.mm.*	*c.mm.*	*c.mm.*
Glucose added from side arm immediately before beginning readings	45	1.1	37.8	41.1
	109	3.3	86.5	87.5
	160	5.5	121.4	122.4
	212	11.1	155.0	159.0

Temperature, 37.0°C.; gas in vessel, 5 per cent CO_2, 95 per cent N_2; pH 7.2.

that produced by either component measured separately. In order to confirm this finding, two additional experiments were performed. The results of these two agreed with each other; the data obtained in one of them are given in Table V, from which it will be seen that the rate of production of acid by the mixture was no greater than the sum of the rates of the two suspensions composing it. An acceptable explanation for the increased rate of production of acid manifested in the first of these three experiments is not apparent.

SUMMARY AND CONCLUSIONS

Previous investigations of the metabolism of viruses have been hindered by the difficulty or impossibility of securing adequate amounts of the active agents in a pure state. However, by the application of recently developed technics, it is possible to prepare large quantities of vaccine virus free from living host cells, and to concentrate the suspensions to any desired degree. Advantage has been taken of this in the present investigation. Large quantities of washed elementary bodies of vaccinia were prepared, and suspended in small volumes of liquid. The amounts of oxygen consumed aerobically and of acid produced anaerobically were measured, the latter as carbon dioxide released from a buffer solution containing sodium bicarbonate and carbon dioxide. Even when large amounts of virus were used (as much as 26 mg., dry weight, of elementary bodies) the quantities of oxygen consumed and of acid liberated were very small. Furthermore, the greater part of the gaseous exchange which occurred took place in the first hour of observation; during the succeeding periods no absorption of oxygen or liberation of carbon dioxide was demonstrated. No increased absorption followed the addition of glucose, glucose monophosphate, or methylene blue. At the conclusion of the experiments the virus was shown to be fully active. Such findings are in sharp contrast to the results to be expected if true respiration were taking place, as for example in resting bacteria, in which case the quantities of oxygen consumed are much greater and are relatively constant during the period of observation.

It was considered that the failure of elementary bodies to consume oxygen might be due to lack of a proper substrate, or of respiratory supplements. In an effort to supply these essentials, a tissue extract was prepared which was shown to contain respiratory supplements, and this was added to the suspension of elementary bodies. It had, however, no effect on the rate of utilization of oxygen by the elementary bodies.

Since elementary bodies alone, and in the presence of simple and complex substrates, showed no evidence of continued respiration, it was decided to ascertain whether they contained substances capable of stimulating the metabolism of other cells. Rabbit erythrocytes were

used for this purpose; and the amounts of oxygen consumed under aerobic conditions and of acid produced under anaerobic conditions, respectively, by the red blood cells were determined. In neither case was any consistent stimulation of metabolism demonstrated.

In the interpretation of the results of our experiments it must be borne in mind that the conditions under which they were performed are highly artificial, and while they are compatible with the survival of virus, there is no reason to suppose that they would permit its growth (3, 4). It may be said, however, that under the conditions which have been described above, no evidence was secured that vaccine virus, in considerable amounts, freed from viable host cells and bacteria, is capable of continued utilization of measurable quantities of oxygen, or of continued release of appreciable amounts of acid.

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