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Adenosine Triphosphate and Other Requirements for the Utilization of Glucose by Agents of the Psittacosis-Trachoma Group[1]

EMILIO WEISS

Department of Microbiology, Naval Medical Research Institute, Bethesda, Maryland

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ABSTRACT

WEISS, EMILIO (Naval Medical Research Institute, Bethesda, Md.). Adenosine triphosphate and other requirements for the utilization of glucose by agents of the psittacosis-trachoma group. J. Bacteriol. **90**:243–253. 1965.—The agent of meningopneumonitis cultivated in the allantoic cavity of chick embryos and purified by differential centrifugations was employed for most of the studies of the requirements for glucose utilization. The evolution of $C^{14}O_2$ from glucose-*1*-C^{14} was used as the criterion of metabolic activity in most experiments. The rate of glucose utilization increased somewhat during the first hour of incubation at 34.4 C and became approximately constant during the second hour. Changes in glucose concentration from 1 to 5 mM did not appreciably affect metabolic activity. More vigorous CO_2 production was obtained when the ratio of K^+-Na^+ was >1 and, under certain conditions, when the concentration of inorganic phosphate was relatively high (0.05 M). Glucose utilization was entirely dependent on added adenosine triphosphate (ATP) and Mg^{++}. The effect of ATP was greatly reduced when the microorganisms were partially disrupted with sonic energy. Adenosine diphosphate (ADP) could be substituted for ATP, but the activity was reduced to less than 20%. ATP was not required when glucose-6-phosphate was substituted for glucose. With ADP and glucose, glucose-6-phosphate was an effective competitor of glucose utilization. Nicotinamide adenine dinucleotide phosphate (NADP) enhanced CO_2 production from carbon 1, but not from other carbons, with glucose and, especially, glucose-6-phosphate as substrates. ATP and NADP produced the above-described effects only when their concentrations were comparable to those of the substrates. These concentrations always exceeded the amount of CO_2 produced (0.05 to 0.5 μmole/mg of agent protein). The concentration of NADP could be reduced when oxidized glutathione was added. Diphosphothiamine had no effect on CO_2 production. Qualitatively similar results were obtained with the agent of trachoma purified from yolk sac. These experiments furnish evidence that agents of the psittacosis-trachoma group, despite their enzymatic capabilities, require an exogenous source of energy.

It was previously shown (Weiss et al., 1964) that agents of the psittacosis-trachoma group produce CO_2 and pyruvate from glucose. For the demonstration of this metabolic activity, an arbitrary set of conditions was selected. The diluent contained a high K^+-Na^+ ratio and relatively high concentration of total ions and phosphate buffer (*p*H 7.0). The final reaction mixture also included Mg^{++} and Mn^{++}, bovine plasma albumin, adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide (NAD). The need for some of these conditions could be justified on the basis of previous experience with this group of agents or rickettsiae (*see* Moulder, 1962). The present investigation represents an effort to analyze these assumed requirements and extends to selected additional factors that were shown to influence glucose metabolism of the psittacosis-trachoma group. Most of the work was carried out with the agent of meningopneumonitis, because it can be cultivated in the allantoic cavity of the chick embryo (Francis and Gordon, 1945) and purified from allantoic fluid (Moulder, Novosel, and Tribby, 1963) more satisfactorily than from yolk sac. A few preliminary and confirmatory experiments were carried out with the agent of trachoma.

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MATERIALS AND METHODS

Microorganisms. The TE-55 strain of trachoma was grown in the yolk sac of chick embryos and purified as described previously (Weiss et al., 1964), except that the steps involving Celite were omitted, and in one experiment Pronase (Calbiochem) was substituted for trypsin.

The purification of the agent of meningopneumonitis (Cal-10 strain) from allantoic fluid was carried out as described by Moulder et al. (1963). J. W. Moulder kindly provided a detailed outline of this procedure. For each experiment, 150 to 250 embryos, 8 days old, were inoculated via the allantoic cavity with 0.2 ml of infected yolk-sac suspension diluted to 10^{-4} (approximately 10^4 LD_{50}) and incubated for 116 to 120 hr at 37 C. Embryos dying after the third day were carefully checked by smear for progress of infection and absence of bacterial contamination. The surviving embryos (50 to 80%) were refrigerated overnight; the allantoic fluids were harvested and maintained in ice water until the next day. A small sediment of red blood cells and of other relatively larger particles was discarded, and the fluid, heavily laden with elementary bodies, was subjected to three cycles of high-speed (30 min at 5,000 × *g*) and low-speed (15 min at 500 × *g*) centrifugation followed by a final centrifugation at 12,000 × *g* for 30 min. The pellets obtained from the four high-speed centrifugations were suspended in decreasing volumes of diluent, the first two in SP 25 (0.25 M sucrose, 0.02 M potassium phosphate, *p*H 7.0) and the second two in K 36 (0.1 M KCl, 0.015 M NaCl, 0.05 M potassium phosphate, *p*H 7.0). The purified preparations were usually used immediately, but in some cases samples were stored at 0 C and used within 2 days. The microorganisms did not lose metabolic activity during storage, provided they were not washed. The final preparations were checked for bacterial contamination and assayed for infectivity, total particle counts, and protein as described previously (Weiss et al., 1964).

Control preparations were prepared from the allantoic fluid of comparable uninfected embryos to which were added a sufficient number of chorioallantoic membranes (CAM) to approximate the protein concentration of infected material. The CAM were disrupted in a Sorvall Omnimixer, operated at 10,000 rev/min, for two cycles of 30 sec each. The suspensions were subjected to the above-described cycles of centrifugation.

Metabolic experiments. Chemical compounds of high purity were obtained from various commercial sources. With the exception of $MgCl_2$ and $MnCl_2$, they were dissolved in K 36 and the *p*H was adjusted with KOH to 6.8 to 7.0. They were used immediately or stored at −70 C. Glucose labeled with C^{14} in carbon positions 1, random, and 3, 4, and glucose-*1*-C^{14}-6-phosphate were also obtained from commercial sources (New England Nuclear Corp., Boston, Mass.; Calbiochem). They were diluted with their respective unlabeled compounds to the desired specific activity.

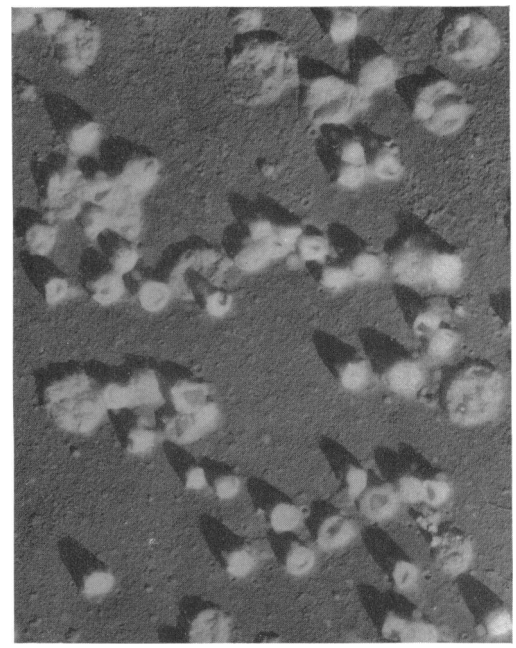

FIG. 1. *Agent of meningopneumonitis purified from infected allantoic fluid. Suspended in 0.33 M ammonium acetate, air-dried, and shadowed with chromium at an angle of 15° (× 11,000). Electron microscopy and total particle counts were done by Harry R. Dressler.*

Unless otherwise indicated, the microorganisms with added factors and substrate were placed in the main compartment of conventional Warburg vessels and incubated for 2 hr plus a 15- to 20-min allowance for thermal equilibration at 34.4 C in an atmosphere of air. The volume of the reaction mixture usually was 2.4 ml. CO_2 was trapped with 0.2 ml of Hyamine (Packard), placed in the central well. Its radioactivity was determined in a liquid scintillation counter as previously described (Weiss et al., 1964). All determinations were done in duplicate or triplicate. The results were expressed in terms of millimicromoles of CO_2 produced per milligram of agent protein, calculated from the disintegrations per minute (dpm, counts per minute divided by efficiency of counting), the specific activity of the substrates, and the number of carbon atoms labeled. Control preparations, consisting of the principal components of the reaction mixtures without microorganisms, were included in all experiments. The dpm obtained with these preparations were subtracted from the dpm of the test groups.

RESULTS

Characteristics of the purified preparations. Preparations derived from yolk sac were described in a previous publication (Weiss et al., 1964).

Figure 1 is an electron micrograph of the agent of meningopneumonitis purified from allantoic fluid. Most of the preparations appeared to contain little if any debris of host-cell material. The ratio of small agent particles to large agent particles was approximately 2.5:1. Among 18 preparations, the mean ratio of infectious to total particles was about 20, and the protein content per particle (± standard error) was $4.5 \pm 0.4 \times 10^{-11}$ mg. These values are comparable to those obtained by Moulder, Grisso, and Cho (1965*a*). The results of metabolic experiments presented below were corrected on the basis of protein, since this parameter was determined with least error.

Preliminary experiments. Previous studies (Weiss et al., 1964) were carried out with a glucose concentration of 0.33 mM and an incubation period of 2 hr at 37 or 34.4 C. Figure 2 illustrates the effect of glucose concentration on CO_2 production by the agent of trachoma. Unless otherwise stated, the experiments were carried out with glucose-*1*-C^{14} and only the CO_2 derived from carbon 1 was measured. Metabolic activity was enhanced somewhat by raising the concentration of glucose, but was relatively constant with glucose concentrations of 1 to 5 mM. Subsequent experiments were done with 0.8 to 1.6 mM glucose. The rate of CO_2 production by the agent of meningopneumonitis at 34.4 C is illustrated in

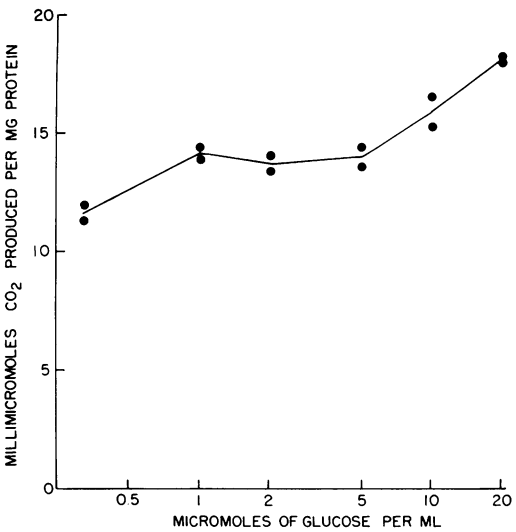

FIG. 2. *Influence of glucose concentration on CO_2 production by the agent of trachoma. Each flask contained, in a total volume of 3.0 ml: partially purified cells (6 mg of protein), K 41 diluent, ATP (15 μmoles), NAD (7.5 μmoles), Mg^{++} (6 μmoles), Mn^{++} (1.2 μmoles), bovine plasma albumin (Calbiochem, 7.5 mg), and glucose, in the amounts shown, containing 1 μc of glucose-1-C^{14}.*

TABLE 1. *Rate of carbon dioxide production from glucose by the agent of meningopneumonitis**

Time (hr)	CO_2 produced (mμmoles/mg of protein)
0.0	0.0; 0.0
0.5	6.6; 6.8; 6.6
1.0	18.9; 17.2; 16.6
1.5	30.4; 29.8; 29.3
2.0	39.1; 40.3

* To the central compartment of each flask were added 0.4 mg of agent protein suspended in K 36 plus the reagents listed in Fig. 2 in a total volume of 1.6 ml. Glucose-*1*-C^{14} [4 μmoles (2 μc) in 0.4 ml of K 36] was tipped in after thermal equilibration. The reaction was stopped at the time intervals indicated by tipping 0.3 ml of 1 M citric acid. CO_2 was collected after an additional 0.5 hr of incubation.

Table 1. The rate appeared to accelerate during the first hour—a finding confirmed in other experiments—and to become approximately uniform during the second hour. A 2-hr period of incubation plus time for thermal equilibration, therefore, appeared to be adequate, and was adopted for use in subsequent experiments. With the agent of meningopneumonitis and under conditions approximating those listed for Fig. 2, CO_2 production from carbon 1 of glucose per milligram of agent protein varied from 40 to 100 mμmoles. When these conditions were changed and metabolic activity was compared with that of the control group in each experiment, relatively small differences could be detected because variation among duplicate or triplicate flasks was usually quite small.

Diluent. In previous experiments (Weiss et al., 1964), the microorganisms were suspended in diluent K 41, containing relatively high concentrations of KCl and potassium phosphate. The ionic strength was later slightly reduced, and the diluent was designated K 36. The adequacy of this diluent for the sustenance of metabolic activity was studied in the four experiments presented in Table 2. The following conclusions can be drawn from these experiments. (i) Metabolic activity was greatly reduced when Na^+ was substituted for K^+ as the principal cation (experiments 18 and 25). Note that the concentrations of Na^+ and K^+ were not exactly reciprocal, because glucose and some of the added factors were dissolved in K 36. (ii) With KCl and potassium phosphate as the only major components of the diluent, metabolic activity was favored by a relatively high concentration of phosphate. In experiment 19, when the phosphate concentration was reduced from 0.049 to 0.017 M and KCl was increased to maintain osmolarity, CO_2 pro-

TABLE 2. *Influence of diluent on glucose utilization by the agent of meningopneumonitis*

Expt no.	Diluent designation	Principal components* (final concn, mM)					Osmols (calculated)	CO_2 production (mμmoles/mg of agent protein)
		K^+	Na^+	Cl^-	HPO_4^- $H_2PO_4^-$	Other		
18	K 36	185	14	110	49		0.36	51; 47; 46
	Na 36	39	160	110	49		0.36	12; 11; 11
	SP 25	65	2	18	25	Sucrose 200	0.32	55; 55; 53
	AA 8	39	8	18	9	Ammonium acetate 64	0.21	1; 1; 1
19	K 36	185	14	110	49		0.36	70; 66; 66
	K 36†	150	11	87	39		0.29	58; 60; 60
	LP 36	168	19	151	17		0.36	35; 36; 34
24	K 36	186	14	113	48		0.36	82; 83; 84
	K 36‡	170	12	95	48		0.33	85; 83; 89
	K 36‡	154	9	76	48		0.29	89; 94; 90
	K 36‡	136	6	55	48		0.25	88; 87; 88
	K 36‡	117	4	34	48		0.21	87; 91; 88
25	K 36	186	14	113	48		0.36	36; 40; 38
	SSC	59	140	137	13	Citrate 10	0.36	16; 16; 17
	KSC	163	36	137	13	10	0.36	53; 54; 53

* Each flask received the microorganisms in 2.0 ml of the indicated diluent plus 0.5 ml of a mixture of Mg^{++} (4 μmoles), Mn^{++} (0.8 μmole), ATP (10 μmoles), bovine plasma albumin (5 mg), and glucose-*1*-C^{14} (2 μmoles, 1 μc). The last three reagents were prepared in K 36, except for a minor difference in the ATP preparation used in experiments 18 and 19. In experiment 25, the cells were suspended in a small volume of K 36 to which were added the indicated diluents. The additions and changes of procedure are reflected in the final concentrations presented for the principal components. Agent protein in the four experiments ranged from 0.22 to 0.48 mg per flask.

† Diluted.

‡ Modified.

duction from glucose declined to approximately 50%. A slight reduction of activity was also obtained by simply diluting K 36 with distilled water to 80% strength. On the other hand, experiment 24 clearly showed that the concentration of KCl could be reduced without loss of activity, provided the phosphate level was maintained. Changes in *p*H could not be implicated in the above observations, because no changes were noted in the course of these experiments. (iii) Some compounds, such as sucrose (experiment 18) or citrate (experiment 25), could substitute, at least in part, for phosphate. On the other hand, ammonium acetate, which is an excellent stabilizer of the viability of the agent of meningopneumonitis (Moulder, 1964), was found to be almost totally inadequate for the support of metabolic activity. This latter effect could not be attributed to competition of ammonium acetate with glucose metabolism. In an experiment not shown in Table 2, the addition of 60 μmoles of ammonium acetate to microorganisms suspended in K 36 had little or no effect on CO_2 production from 4 μmoles of glucose. (iv) The osmotic pressure of the diluent, within the range studied, did not influence metabolic activity. With other requirements satisfied, almost identical results were obtained with osmolarity ranging from 0.36 to 0.21 osmol (experiment 24).

Added factors. Prior to the experiments described in Table 3, bovine plasma albumin, ATP, NAD, Mg^{++}, and Mn^{++} were added to all reaction mixtures. Although a function for bovine plasma albumin could not be clearly established in a preliminary experiment, it was added because of its demonstrated stabilizing effect on viability (Weiss and Dressler, 1962; Moulder, 1964). The effect of the other factors is shown in Table 3. It is quite obvious that these additions were essential for CO_2 production. The results also indicated that the effect was produced on whole cells and not on the enzymes liberated from disrupted cells. When approximately 50% of the cells were sonically disrupted, as demonstrated by electron

TABLE 3. *Influence of added factors on glucose utilization by the agent of meningopneumonitis**

Expt no.	Factors added	Cells sonically treated†	Incubation time (hr)	CO_2 production (mμmoles/mg of protein)
9	Yes	No	1	17.3; 16.7
			2	42.0; 42.2
	No	No	1	0.5; 0.3; 0.3
			2	0.6; 0.5; 0.3
11	Yes	No	1	13.1; 13.9
			2	32.5; 38.0
	Yes	Yes	1	1.7; 1.6; 1.5
			2	4.9; 5.4; 4.9

* Bovine plasma albumin was added to all groups. The added factors included ATP, NAD, Mg^{++}, and Mn^{++} in the concentrations given in the legend for Fig. 2. Agent protein contents were 0.64 and 0.4 mg per flask. The substrate was glucose-*1*-C^{14}, 4 μmoles (2 μc) per flask.

† In a Raytheon 10-kc sonic oscillator for 30 min. Electron microscopic examination indicated that approximately 50% of the cells were disrupted. They were used immediately without further washing.

microscopy, CO_2 production in the presence of the added factors was greatly reduced.

Table 4 presents a more detailed study of the effect of the added factors. It is quite obvious that ATP was an absolute requirement, whereas NAD had no effect. The divalent cations can be considered as another absolute requirement, if the small amount of activity obtained with ATP alone or the ATP and NAD mixture can be attributed to the presence of trace amounts of divalent cations prior to additions. In an experiment not presented in Table 4, it was shown that Mn^{++} or Ca^{++}, added to Mg^{++} and ATP, did not appreciably affect CO_2 production. Most surprising were the results, shown in Table 4, of a study of the relationship between ATP concentration and metabolic activity in the presence of excess Mg^{++}. CO_2 production remained negligible with as much as 0.12 μmole of ATP per flask. Very small amounts were produced with 0.6 μmole and relatively large amounts with 3 μmoles, but metabolic activity was further enhanced when the concentration was raised to 15 μmoles. In the group in which ATP was used at highest efficiency, 3 μmoles of ATP were required to utilize 0.012 μmole of glucose (by 0.38 mg of agent protein). The ratio of ATP present to glucose utilized was approximately 250:1.

Nicotinamide adenine dinucleotide phosphate (NADP). Previous experiments (Weiss et al., 1964) suggested that glucose was utilized by agents of the psittacosis-trachoma group via the pentose pathway. Moulder, Grisso, and Brubaker (1965*b*) demonstrated the presence of two enzymes of the pentose pathway, glucose-6-phosphate and 6-phosphogluconate dehydrogenases, in cell-free extracts of the agent of meningopneumonitis. Since the cofactor for these enzymes is NADP, its effect on CO_2 production from carbon 1 of glucose was investigated (Table 5). In the presence of adequate ATP and divalent cations, NADP effectively stimulated CO_2 production. Despite some variation among experiments, the effect appeared to increase with the concentration of NADP from 0.3 to 3.0 μmoles per flask. With 1.5 μmoles, CO_2 production was approximately doubled. In several of the experiments, sufficient hexokinase was added to convert most of the glucose to glucose-6-phosphate. Under these conditions, CO_2 production was also doubled (experiment 20A), indicating that glucose-6-phosphate was utilized more efficiently than glucose. NADP was particularly effective with glucose-6-phosphate as the substrate. The increase in CO_2 production with combined hexokinase and 1.5 μmoles of NADP was approximately fivefold.

The relatively large amount of NADP needed for enhancement suggested that the resulting reduced NADP ($NADPH_2$) was not readily reoxidized via the cytochrome system and atmos-

TABLE 4. *Influence of added factors on glucose utilization by the agent of meningopneumonitis**

Expt no.	Added factors (μmoles/flask)				CO_2 production (mμmoles/mg of protein)
	ATP	NAD	Mg^{++}	Mn^{++}	
10	15	7.5	6.0	1.2	45.4; 53.2; 48.7
	—	—	—	—	0.0; 0.0
	15	—	—	—	5.2; 5.1
	—	7.5	—	—	0.0; 0.0; 0.0
	—	—	6.0	1.2	0.0; 0.0
13	15	7.5	6.0	1.2	87.6; 92.9
	15	—	6.0	1.2	85.8; 85.2
	15	7.5	—	—	8.4; 8.5; 7.6
	—	7.5	6.0	1.2	1.6; 1.0; 0.0
24A	15	—	20.0	—	46.6; 52.8; 68.4
	3	—	20.0	—	31.6; 28.1; 31.6
	0.6	—	20.0	—	3.3; 2.8; 2.8
	0.12	—	20.0	—	0.6; 0.5; 0.2
	0.024	—	20.0	—	0.4; 0.4
	0.0	—	20.0	—	0.2; 0.3

* Agent protein contents in the three experiments were 1.0, 0.62, 0.38 mg per flask. The substrate was glucose-*1*-C^{14}, 4 μmoles (2 μc) per flask.

TABLE 5. *Influence of NADP on glucose utilization by the agent of meningopneumonitis**

Exp no.	Added factors (per flask)			CO_2 production (mμmoles/mg of protein)
	NADP (μmoles)	Hexokinase (units)	GSSG (μmoles)	
12	—	—	—	92; 100; 95
	0.3	—	—	150; 161; 146
13	—	—	—	86; 85
	0.75	—	—	176; 185; 174
20A	—	—	—	59; 61; 63
	1.5	—	—	127; 127
	—	10	—	131; 131
	1.5	10	—	315; 323; 322
22B	—	10	—	124; 128
	1.5	10	—	353; 357
	3.0	10	—	425; 437
23†	—	10	—	146; 149; 159
	0.3	10	—	171; 189
	1.5	10	—	382; 392; 406
	—	10	15	154; 154; 162
	0.3	10	15	278; 276
	1.5	10	15	396; 395; 390

* To each flask were added ATP, 15 μmoles; Mg^{++}, 6.0 μmoles; Mn^{++}, 1.2 μmoles; and glucose-*1*-C^{14}, 4 μmoles (2 μc). Agent protein in the five experiments ranged from 0.4 to 0.7 mg per flask.

† Incubation time was 2.3 instead of 2.0 hr.

pheric O_2. Moulder et al. (1965*b*) showed, however, that cell-free extracts could reoxidize $NADPH_2$ in the presence of oxidized glutathione (GSSG). The influence of GSSG on the above-described reactions in intact cells is shown in Table 5 (experiment 25). Although GSSG had no effect on CO_2 production by itself or with 1.5 μmoles of NADP, it enhanced the stimulation of 0.3 μmole of NADP. These results were confirmed in a second experiment not shown in Table 5.

In the two experiments shown in Table 6, glucose-6-phosphate was used, instead of a mixture of glucose, hexokinase, and ATP. Under these conditions, it was shown (experiment 14) that ATP was not required for CO_2 production. Without ATP the metabolic activity was about the same, or slightly higher, than that expected with ATP and glucose. NADP produced approximately a threefold enhancement. ATP added to glucose-6-phosphate alone or to the substrate plus NADP enhanced CO_2 production, in either case, by 20 to 30 mμmoles. NAD, too, appeared to have a moderately enhancing effect in the one instance tested, in combination with ATP. The activity of sonically disrupted cells was reduced when cofactors were not added. However, NADP enhanced the activity of disrupted cells to a greater degree than that of intact cells (experiment 25A). This was expected, in view of the results of Moulder et al. (1965*b*).

Adenosine diphosphate (ADP). The experiments

TABLE 6. *Utilization of glucose-6-phosphate by the agent of meningopneumonitis**

Expt no.	Added factors (μmoles/flask)			Cells sonically treated†	CO_2 production (mμmoles/mg of protein)
	ATP	NAD	NADP		
14	—	—	—	No	79; 80
	15	—	—	No	103; 110; 107
	15	7.5	—	No	132; 141; 133
	—	—	1.5	No	259; 271; 272
	15	—	1.5	No	290; 303; 303
25A	—	—	—	No	106; 113; 112
	—	—	—	Yes	57; 53; 54
	—	—	2.5	No	507; 472; 464
	—	—	2.5	Yes	768; 675; 818

* The substrate was glucose-*1*-C^{14}-6-phosphate, 0.47 μmole (1.14 μc) and 4 μmoles (0.4 μc) per flask in the two experiments. Agent protein amounts were 0.66 and 0.61 mg per flask. Mg^{++} and Mn^{++} were added to all flasks in these and all subsequent experiments.

† See Table 3.

TABLE 7. *Influence of ADP on glucose utilization by the agent of meningopneumonitis**

Expt no.	Added factors (μmoles/flask)				CO_2 production from glucose (mμmoles/mg of protein)
	ATP	ADP	NADP	G-6-P†	
17	15	—	—	—	68.3; 66.3; 66.6
	—	—	—	—	1.7; 0.0
	—	15	—	—	4.0; 3.4
	—	—	1.5	—	0.0; 0.0
	—	15	1.5	—	11.1; 10.6
28	15	—	—	—	71.2; 69.9; 67.3
	—	15	—	—	13.5; 11.9; 11.9
23B	—	4	—	—	5.8; 7.8
	—	4	—	4	0.0; 0.0
	—	4	1.5	—	17.0; 19.2
	—	4	1.5	4	0.2; 0.2

* Agent protein contents in the three experiments were 0.35, 0.86, and 0.40 mg per flask.

† Glucose-6-phosphate, unlabeled. Glucose-*1*-C^{14}, 4 μmoles (2 or 0.4 μc) per flask was the labeled substrate.

TABLE 8. *Carbon dioxide production from various carbon atoms of glucose and glucose-6-phosphate by the agent of meningopneumonitis**

Expt no.	Added factors (μmoles/flask)					CO_2 production (mμmoles/mg of protein) from carbon position			
	ATP	Hexokinase (units)	NAD	NADP	DPT	1	1–6	2–6 (mean calculated)	3,4
21	15	10	—	—	—	135; 134; 138	269; 253; 245	120	
	15	10	15	—	—	201; 200	325; 318	121	
	15	10	—	1.5	—	323; 303; 310	437; 415; 427	114	
25B	—	—	—	—	—				0; 0; 0
	15	—	—	—	—				70; 85; 89
	15	10	—	—	—				131; 130; 138
	15	10	15	—	—				143; 136; 143
	15	10	15	2.5	—				149; 144; 140
15			—	—	—	119; 126			
			—	1.5	—	438; 439; 423			
			—	—	1.5	125; 133; 133			
			—	1.5	1.5	431; 409			
21B	15	10	—	—	—		153; 148		
	15	10	—	1.5	—		410; 401		
	15	10	—	—	1.5		154; 133		
	15	10	—	1.5	1.5		374; 405		

* Agent protein amounts in the four experiments were 0.46, 0.39, 0.57, and 0.30 mg per flask. The following substrates were used: experiment 21, glucose-*1*-C^{14} and randomly labeled glucose, both 4 μmoles (2 μc) per flask; experiment 25B, glucose-*3,4*-C^{14}, 4 μmoles (0.13 μc) per flask; experiment 15, glucose-*1*-C^{14}-6-phosphate, 2 μmoles (0.31 μc) per flask; experiment 21B, randomly labeled glucose, 4 μmoles (2 μc) per flask.

shown in Table 7 represent attempts to determine whether the requirement for ATP could be met by adding ADP. As shown in experiment 17, a small amount of CO_2 (less than 10% of the amount produced with ATP) was produced with added ADP. NADP, which by itself had no effect on CO_2 production, enhanced metabolic activity when combined with ADP, as in previous experiments with ATP. Unfortunately, after experiments 17 and 23B were completed, an assay of the ADP preparation for the presence of ATP (done by the NADP reduction method in the presence of glucose, hexokinase, glucose-6-phosphate dehydrogenase, and NADP) indicated that contamination with ATP amounted to approximately 2%. Experiment 28, however, was done with a preparation of ADP containing no demonstrable ATP, and CO_2 evolution was approximately 18% of that obtained with ATP. In an experiment not shown in Table 7, CO_2 evolution in the presence of ADP was not reduced by the addition of 2,4-dinitrophenol. Two attempts to demonstrate ATP production in the presence of glucose-6-phosphate and ADP by enhanced evolution of CO_2 from labeled glucose were unsuccessful. Experiment 23B (Table 7) indicated that in the presence of ADP glucose-6-phosphate was a very effective competitor of glucose utilization.

CO_2 production from various carbon atoms of glucose. Experiments described thus far were conducted with glucose labeled in carbon position 1. Previous work (Weiss et al., 1964), however, indicated that CO_2 was produced from at least one other carbon, most likely 3 or 4. Attempts to extend to the other carbons the above-described observations were conducted either by comparing CO_2 production from glucose-*1*-C^{14} and randomly labeled glucose or by the use of glucose-*3,4*-C^{14}. The results (Table 8) indicate that CO_2 production from carbon atoms other than 1 occurred only in the presence of ATP and was stimulated by hexokinase. NAD and NADP had no effect. Furthermore, diphosphothiamine (DPT) by itself or in combination with NADP failed to stimulate CO_2 production from carbon 1 or other carbons of glucose-6-phosphate.

Agent of trachoma and control preparation. Except for the experiment illustrated in Fig. 1, all of the above studies were done with the agent of meningopneumonitis. An experiment embodying the principal features of these studies was carried out with the agent of trachoma, and is

TABLE 9. *Influence of added factors on glucose[a] metabolism of agent of trachoma and control preparations*

Prepn	Added factors (μmoles/flask)			CO_2 production[b] from carbon position		
	ATP	Hexokinase (units)	NADP	1	1–6	2–6 (mean calculated)
Agent[c]	—	—	—		0; 0	
	15	—	—	12; 13; 13	43; 42; 42	30
	15	10	—	17; 16; 18	50; 52	34
	15	10	2.5	184; 176	216; 209	33
Control[d]	—	—	—	0; 0	0.0; 0.4	0.2
	15	—	—	0; 0; 0	0.0; 0.2	0.1
	15	10	—	0; 0	0.2; 0.6; 1.4	0.7
	15	10	2.5	4.0; 4.2; 4.8		

[a] Glucose-*1*-C^{14}, 4 μmoles (0.4 μc) per flask; randomly labeled glucose, 4 μmoles (1.3 or 2.0 μc) per flask.
[b] Calculated on the basis of 1 mg of protein for the agent of trachoma and of 0.15 mg of protein for the control preparation.
[c] Agent of trachoma, 0.74 mg of protein per flask.
[d] Uninfected allantoic fluids and chorioallantoic membranes, 0.078 mg of protein per flask. The starting material consisted of 104 allantoic fluids plus 26 chorioallantoic membranes of uninfected chick embryos treated as described in Materials and Methods. The protein content of the starting material was comparable to that of infected allantoic fluids.

presented in Table 9. The results were qualitatively the same, but there were some quantitative differences. The total amount of CO_2 produced was smaller. ATP was essential as with the agent of meningopneumonitis, but the addition of hexokinase produced little enhancement. On the other hand, the effect of NADP on carbon 1 was much more pronounced. As in the case of meningopneumonitis agent, NADP had no effect on CO_2 production from carbons other than 1.

Previous experiments (Ormsbee and Weiss, 1963; Weiss et al., 1964) included numerous control preparations, and it was clearly established that glucose metabolism was a property of the agents and not of contaminating host particles. An additional control experiment with uninfected allantoic fluid and mechanically disrupted chorioallantoic membranes is presented in Table 9. Although an attempt was made to reproduce the equivalent of the crude agent preparation, the crude control preparation most likely contained somewhat larger amounts of connective-tissue cell debris and intact mitochondria and somewhat smaller numbers of particles of epithelial origin. When this preparation was subjected to steps of purification used for the agent of meningopneumonitis, the amount of protein recovered amounted to about 15% of that normally obtained with infected allantoic fluids. For purposes of comparison, CO_2 production was corrected on the basis of 0.15 mg of protein, instead of the usual 1.0 mg. As shown in Table 9, amounts of CO_2 produced were insignificant, with the exception of the group containing ATP, hexokinase, and NADP. Under these conditions, the amount of CO_2 produced was lower than the corresponding value for the agent of meningopneumonitis by two orders of magnitude. Thus, in this case also, the contribution of host-cell material can be considered to have been negligible.

DISCUSSION

The generally accepted view is that nucleotides and other phosphorylated compounds do not enter bacterial cells. This view is supported by a few carefully executed experiments. For example, Lichtenstein, Barner, and Cohen (1960) incubated *Escherichia coli* with deoxycitidylate labeled with C^{14} and P^{32} and showed that the C^{14} isotope was incorporated while P^{32} remained in the supernatant fluid. This finding was attributed to the presence of a phosphatase, presumably located at the cell surface, which slowly liberated inorganic phosphate from the nucleotide. These results confirmed previous indications that this happens with other nucleotides (Bolton, 1954; Balis, Lark, and Luzzatti, 1955; Lesley and Graham, 1956). Mitchell (1961) demonstrated the presence of a glucose-6-phosphatase on the cell membrane of *E. coli* protoplasts. Although glucose was rapidly utilized, the rate of glucose-6-phosphate utilization corresponded to that of the liberation of free glucose by the phosphatase. Abrams (1959) showed that the turbidity of *Streptococcus faecalis* protoplasts decreased when glucose was added. This phenomenon was attributed to the swelling of the protoplasts caused by the osmotic changes accompanying the entrance of glucose. The swelling disappeared when

the substrate was completely utilized. In contrast to glucose, glucose-6-phosphate, glucose-1-phosphate, or hexose diphosphate produced no changes in turbidity. Examples of phosphorylated intermediates having little or no effect on metabolism or growth, in contrast to their parent compounds, are too numerous to be quoted here.

There are obvious exceptions to this general rule, even if experiments dealing with interactions through impaired cell membranes or with incorporation of genetic material are excluded. Among these exceptions are the relatively few examples of requirements by bacteria for complete coenzymes. Best known is the requirement for NAD or NADP by *Haemophilus* elucidated by Lwoff and Lwoff (1937*a*, *b*). Gingrich and Schlenk (1944) confirmed the suggestion made by the Lwoffs that NAD, or $NADH_2$, was the preferred cofactor. Nicotinamide riboside supported minimal growth of the microorganisms, but heavy growth was not obtained with this compound. Griffin (1950, 1952) demonstrated a requirement for DPT by *H. piscium*. This requirement could also be met by a considerably larger amount of ATP, and to a less satisfactory degree by monophosphothiamine, ADP, or adenylic acid, but not by their unphosphorylated components. In these and other similar cases, the amounts of coenzymes required by the microorganisms were in the order of 10^{-8} M. Reports of ATP enhancing growth or a particular biochemical reaction have appeared from time to time, but in most cases the effects have been moderate or have not been clearly evaluated. More pertinent to this discussion are the results of Landman and Spiegelman (1955). They demonstrated that the induction of β-galactosidase synthesis by *Bacillus megaterium* protoplasts or similarly treated intact cells required hexose diphosphate as an energy-generating system. The concentration used was 0.015 M. This requirement was imposed by the high ionic strength of the diluent, needed for the osmotic protection of the protoplasts. When the concentration of phosphate buffer was reduced from 0.5 to 0.05 M, the requirement for hexose diphosphate by the intact cells disappeared. Recently, Fraenkel, Falcoz-Kelly, and Horecker (1964) presented excellent evidence that glucose-6-phosphate enters *E. coli* cells and is utilized without prior hydrolysis.

In contrast to most other bacteria, the requirements for phosphorylated compounds by rickettsiae have been well established. Bovarnick and Allen (1954, 1957*a*) showed that rickettsiae under certain conditions can slowly lose and regain NAD and, presumably, coenzyme A. The role of exogenously supplied ATP is quite complex (Bovarnick and Allen, 1957*b*; Bovarnick and Schneider, 1960*a*). In undamaged rickettsial cells, exogenous ATP can prevent the loss of endogenous ATP, but the cells must produce their own ATP through the utilization of a substrate such as glutamate. Damaged cells, on the other hand, in some cases may be supplied with exogenous ATP. For incorporation of amino acids, both externally added and internally generated ATP are required (Bovarnick and Schneider, 1960*b*). NAD, coenzyme A, and ribonucleotides stimulate incorporation, whereas free vitamins and nucleosides are without effect.

The most surprising finding of the experiments described in this paper is the complete dependence of the agent of meningopneumonitis, for the utilization of glucose, on large quantities of exogenously supplied ATP. Breakdown of appreciable amounts of ATP during the course of the experiments cannot be excluded, but is not a likely explanation for these exorbitant requirements. The site of action of ATP is not known, but there is some evidence, besides the amount required, that ATP enters the cell or is incorporated into the membrane at a slow rate. CO_2 evolution increases with time (Table 1), and glucose-6-phosphate is a remarkably successful competitor of glucose metabolism (Table 7). Despite its inefficiency, the absolute requirement for exogenous ATP by the agent of meningopneumonitis, to my knowledge, is without close parallel among bacteria or rickettsiae.

The weak metabolic activity engendered by ADP suggests that the microorganisms possess a myokinase-type enzyme that can convert ADP into adenylic acid and ATP. From the data presented, it appears less likely that the microorganisms can bring inorganic phosphate into organic linkage.

Of the effects produced by the inorganic ions, the apparent requirement for K^+ is not unexpected, because it is encountered among other obligate intracellular microorganisms, such as rickettsiae (Bovarnick and Allen, 1957*b*) or *Wolbachia persica* (Weiss et al., 1962). Of the several explanations that can be invoked for this phenomenon, the following two appear particularly attractive: (i) K^+ is a direct requirement of the enzymatic system involved, as appears to be true in some bacteria (see, for example, Abrams, 1959); (ii) ATP is required to maintain the uphill transport of K^+ and Na^+ across the cell membrane, as is the case in other biological systems (Hokin and Hokin, 1963). In a diluent in which Na^+ predominates, there is an added demand on ATP.

The obvious function of ATP is to produce glucose-6-phosphate. This strongly suggests that the microorganisms possess a hexokinase. The failure of Moulder et al. (1965*b*) to demonstrate

the enzyme thus far may be attributed to the instability of the enzyme or to an unusual requirement for activity. ATP most likely has other functions, but they have not yet been identified.

The role of glucose-6-phosphate as a substrate, in preference to glucose, is another phenomenon which is uncommon among bacteria.

NADP, also, has an effect only when supplied in substrate amounts, in contrast to the trace amounts required by *Haemophilus* (Lwoff and Lwoff, 1937*a*, *b*). This indicates that the microorganisms do not have an efficient mechanism for the reoxidation of reduced NADP. The entry of NADP into the cell might be compared with that of NAD in rickettsiae, although Bovarnick (1962) emphasized that NAD is lost and regained very slowly by rickettsiae. It might be interesting to compare the rates of acquisition of the two cofactors by the two types of microorganisms and to determine whether they truly represent different mechanisms of cofactor transport across cell membranes. In contrast to ATP, some agent NADP is retained during the course of separation from host cells and purification, since appreciable activity can be demonstrated without its addition. The enhancement of CO_2 evolution that it produces is in agreement with the postulation that glucose is utilized via the pentose pathway (Weiss et al., 1964) and with the isolation of glucose-6-phosphate and 6-phosphogluconate dehydrogenases by Moulder et al. (1965*b*).

It was shown in previous (Ormsbee and Weiss, 1963; Weiss et al., 1964) and present work that glucose metabolism leads to the production of pyruvate and evolution of CO_2 from carbon 3 or 4, as well as from carbon 1. It was also shown (Weiss, *unpublished data*) that the microorganisms can metabolize pyruvate with production of CO_2 from carbon 1, but not from carbons 2 and 3. These results are entirely compatible with the supposition that, with glucose as the substrate, the CO_2 not derived from carbon 1 is produced through the decarboxylation of pyruvate. The pathway by which pyruvate is produced has not been identified. Substituting glucose-6-phosphate for glucose enhances CO_2 production from carbon 3 or 4, but NADP or NAD are without effect (Table 8). Thus, it must be postulated that CO_2 evolution from carbon 3 or 4 is unaffected by the rate of pentose production from glucose, or that this CO_2 is not obtained via the pentose pathway. The recovery of labeled pyruvate after incubation of the microorganisms with glucose-*1*-C^{14} (Weiss et al., 1964) favors the latter postulation.

The experiment with the agent of trachoma presented in Table 9 suggests that there are broad similarities in the metabolism of agents of the psittacosis-trachoma group. The quantitative differences that were encountered can be due to genetic or physiological factors, since the two agents were grown in different cells of the chick embryo.

Moulder (1962, 1964), in his careful reviews of the agents of the psittacosis group, came to the conclusion that the most obvious deficiency in these microorganisms is an apparent lack of any sort of energy metabolism. This conclusion was seriously challenged by our finding (Ormsbee and Weiss, 1963; Weiss et al., 1964) that these microorganisms can metabolize glucose. The present work strengthens Moulder's view, because it has provided evidence that useful energy is not produced by these reactions. Furthermore, it offers two specific examples of the type of assistance that these agents may obtain in vivo from their host cells. One is preformed ATP or phosphorylated glucose; the other is NADP or reoxidation of the cofactor, after it has been reduced by the agent.

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