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THE EFFECT OF COLD SHOCK ON THE METABOLISM OF *TRICHOMONAS GALLINAE*

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ABSTRACT

The influence of cold (4°C) used to prepare cells for metabolic investigations was examined with *Trichomonas gallinae* in this study. Cells washed with cold diluent in a refrigerated centrifuge were found to be less stimulated in their gas production at 37°C when exposed to glucose or maltose than cells washed at room temperature conditions. Such cold-prepared cells had higher initial glycogen contents after washing, faster endogenous glycogen degradation rates when incubated at 37°C, but lower glycogen synthesis in the presence of glucose or maltose when compared to cells not prepared in the cold. However, uptake of glucose and maltose at 37°C was not affected by pretreatment with cold. Washing with cold also reduced the total number of recoverable cells by an average of 20%. Cold washing of *T. gallinae* in three diluents (modified Ringers, Krebs Ringer phosphate, and 2% Trypticase) increased the recovery or lag time in STS medium when compared to use of the three washing diluents at room temperature.

INTRODUCTION

Cold shock is defined as injury or death to organisms caused by sudden chilling without freezing (MacLeod and Calcott, 1976). Cold shock is at times unintentionally caused in biological studies when there is such a chilling of living material to preserve metabolic stasis. In initial studies of carbohydrate utilization by *Trichomonas gallinae*, a pathogenic protozoan parasite of birds, it was noted that this organism responded poorly to exogenous carbohydrates in respirometry experiments. The cells had been prepared for experimentation by a washing and centrifugation procedure that was maintained at approximately 4°C until the cells were tested. It was suspected that this procedure may have been responsible for poor stimulation by sugar substrates. Therefore, cells from the same culture batch were divided into 2 groups with one receiving a cold-washing pretreatment as before and the other group of cells prepared under room temperature conditions. The two groups of cells were compared as to the effect of glucose and maltose on gas production, disappearance of these two substrates from suspension fluid, and changes in intracellular glycogen content. The effect of the two treatments on recovery in a minimal growth medium was also studied.

MATERIALS AND METHODS

The Jones' Barn strain of *Trichomonas gallinae* was used in this study and was maintained in culture using CPLM medium with 5% (final vol.) inactivated human serum. Cells for experiments were grown to a population density of $1-2 \times 10^5$ trichomonads/ml after approximately 24 hr incubation at 37°C. The medium, usually a one liter volume, was then divided into two equal portions, "A" and "B." Cells in "A" portion were centrifuged at 1000 g and washed three times with Krebs-Ringer Phosphate diluent (KRP) at room temperature. The KRP was 20 mM at pH 7.0, and prepared according to Umbreit et al. (1951). Cells in "B" portion were centrifuged at 2000 g in a refrigerated centrifuge (4°C) and washed with ice cold KRP diluent. International model CS (room temperature) and Lourdes Betafuge model A (cold) centrifuges were used. After the final washing, cells were resuspended in KRP and cell concentrations determined with a hemocytometer. Cell suspensions were then distributed to Warburg vessels and allowed to equilibrate to 37°C for 15-20 min.

Gas production was measured by the direct method of Warburg (Umbreit et al., 1951) using two flasks for determination of CO_2 and H_2. Since *T. gallinae* is a facultative anaerobe, the gas phase was 99.9% N_2; and this was obtained by flushing the flasks for 15 min. Endogenous flasks contained no carbohydrate whereas substrate flasks contained 0.2 ml of substrate in one side arm. When tipped into the cell suspension in the center compartment, final sugar concentration was 10 mM. Perchloric acid (70%) in the other sidearm was used to determine bound CO_2. The concentration of the organisms in the flasks was approximately 50 million cells/flask.

Substrate disappearance was measured on the supernatant of centrifuged Warburg flask contents by reducing sugar value (Nelson, 1944). Glycogen content of the centrifuged cell pellet was measured by the method of Good et al. (1933). Flask contents used for substrated uptake and glycogen content were not exposed to perchloric acid.

The recovery of cold-shocked cells in growth medium was studied using STS medium (Kupferberg et al., 1948) inoculated with cells from different treatment regimens which were as follows: Cells from 170 ml of a 48 hr old CPLM culture were centrifuged at low speed (500 g). The supernatant was decated, and the pellet resuspended in 10.0 ml of fresh CPLM medium. One ml of this mixture was added to 40 ml of six different sterile diluents. The six tubes of diluents were: 1) cold modified Ringers (Trussel and Johnson 1943), 2) cold KRP, 3) cold 2% Trypticase (BBL laboratories) and 4,5,6) room temperature tubes of these same three diluents. The cells in each tube were washed and centrifuged three times at their respective temperatures and samples from the final suspension inoculated into STS medium which was then incubated at 37°C. Growth was followed by hemocytometer counts of samples taken at predetermined intervals.

RESULTS

During a study of metabolic gas production by *T. gallinae*, the organisms were subjected to a pretreatment consisting of washings with cold KRP in a refrigerated centrifuge at 4°C. The purpose of this pretreatment was to maintain the organisms as close as possible to their original metabolic state when separated from the culture medium. Results were not in agreement with Read (1957), and it was noted that Read did not employ low temperatures in the preparation of his cells. Therefore a batch of organisms was divided into two parts with one receiving a cold treatment and the other room temperature treatment before experimentation (pretreatments). The effect of glucose and maltose on gas production of the two types of pretreatment can be seen in Table 1. Cold-treated cells showed less stimulation above the endogenous rate than room-temperature cells with both glucose or maltose as substrates. However, absolute values for en-

dogenous gas production (not noted in Table 1) were higher for cold-washed cells.

The ability of glucose and maltose to stimulate glycogen production was greater in the room temperature-washed cells (Table 2). The rate of endogenous glycogen depletion was greatest in the cold-treated cells. The percent change in glycogen content was calculated based on the endogenous rate of depletion for each treatment as 100%. In all four experiments maltose stimulated greater glycogen production in room temperature-treated cells than in cells prepared at 4°C. Glucose gave a major glycogen sparing advantage in two experiments for room temperature cells but no advantage in the two other experiments.

The disappearance of glucose and maltose from the supporting medium was also measured in paired experiments. The results of five experiments showed no significant differences in uptake of the sugars (per mole) between cold and room temperature treatments. Based on micromoles of substrate consumed/hour/10^8 cells (S.E.), 7.3 ± 1.4 mM glucose were taken up by cold-treated cells and 6.5 ± 2.3 mM by room temperature treated cells; 6.2 ± 2.1 and 8.0 ± 1.7 mM of maltose were taken up by cold- and room temperature-treated cells, respectively.

During the course of these experiments, it was noted that cold harvesting yielded fewer organisms than room temperature harvests. The data from paired experiments were analyzed for this effect. In each experiment cultures were pooled and divided into two equal portions. After respective pretreatment, the final pellet was brought to a convenient volume for dilution and cells counted. In each of six experiments there was a greater recovery of cells with room temperature treatment than with cold. The percent differences in population in each of these experiments were: 10% (1 experiment), 30% (1 experiment), and 19% (4 experiments). Use of the binomial expansion method shows that the probability of these differences occurring randomly is less than 2%. Cold pretreatment results in a destruction of organisms as well as their dimenished metabolic capability.
ity.

Utilizing the same method of dividing the cells for pretreatment, the glycogen content of the cells after the pretreatments was examined. In all five experiments glycogen content was higher in cells pretreated with cold (Table 3). Apparently some glycogen is degraded by *T. gallinae* at room temperature during washing and centrifugation but is spared with maintenance of cells at low temperature.

Table 1. The effect of pretreatment on metabolic gas production of *Trichomonas gallinae*. (Figures expressed as percent above endogenous.)

	10 mM Glucose[1]		10 mM Maltose	
Pretreatment	CO_2	H_2	CO_2	H_2
Room temperature	100	25	87	98
Cold temperature	3	8	60	12

[1]Average of three experiments.

In growth recovery experiments three diluents were used for the washing procedures: modified Ringers, Krebs-Ringer phosphate (KRP), and 2% Trypticase solutions. It was thought that certain diluents might have a protective effect against cold shock and that this might be detected in growth recovery. Modified Ringers is a minimal salt solution. The KRP and 2% Trypticase were at pH 7.0 and could act as buffers. The 2% Trypticase contains amino acids and peptides that might be lost from the cells as a result of washing. Also, it was expected that any changes in the growth curve would be seen in the lag and early log phases. Therefore a heavy inoculum of cells was used to insure accurate counts in the initial growth phases.

Table 2. The effect of pretreatment on the synthesis of glycogen from glucose and maltose by *Trichomonas gallinae*. Unclosed values represent mg change in glycogen content after 1 hr incubation. Closed values are percent glycogen change based on endogenous change as 100%.

Experiment	Mg of glycogen synthesized/10^8 cells/hr		
	Endogenous	Glucose	Maltose
1			
Cold	-0.77	-0.31 (159%)	+0.13 (217%)
Room Temperature	-0.33	-0.17 (148%)	+0.91 (375%)
2			
Cold	-0.97	-1.14 (72%)	-0.34 (160%)
Room Temperature	-0.86	-0.32 (162%)	+0.24 (227%)
3			
Cold	-1.20	-1.20 (100%)	-1.40 (83%)
Room Temperature	-0.50	+0.20 (240%)	0.00 (200%)
4			
Cold	-0.52	-0.32 (138%)	-0.47 (143%)
Room Temperature	-0.54	-0.37 (132%)	+0.26 (250%)

Table 3. Initial glycogen content of *Trichomonas gallinae* after pretreatment washing at room and cold temperature KRP (pH 7.0). Figures represent mg glycogen/10^8 cells.

Pretreatment	Experiments				
	1	2	3	4	5
Cold	3.06	4.82	5.26	7.20	4.47
Room Temperature	2.56	3.88	4.61	6.10	3.91

A typical experiment is seen in Figure 1 with modified Ringers used as the diluent. An increase in the lag phase is seen with the cold-treated cells and the population difference continues to 24 hr. Using KRP or 2% Trypticase did not change this effect to any marked degree since the curves obtained were quite similar to those seen in Figure 1. Using total cell counts as the criterion for population size, a slower recovery period was indicated after cold washing of cells.

DISCUSSION

Cold shock was first reported by Sherman and Albus (1923) in a bacterium, *Escherichia coli*. Most of what is known about cold shock has come from studies with bacteria, especially gram-negative organisms, but the phenomenon has been described from a variety of microbes and eucaryotic cells. The changes that occur with cold shock are varied and complex but are primarily associated with increases in membrance permeability and loss of substances from the shocked cells. A number of low molecular weight solutes such as amino acids, nucleotides, small proteins and low molecular weight peptides have been found to be released from shocked cells. Presumably, it is the loss of these compounds that causes death or reduced metabolic capabilities of the affected cells. Important variables are cell age, diluent (milieu), growth and recovery medium, presence of cations (Mg++, Ca++, Mn++), and rate of cooling, all of which may increase or decrease the effectiveness of cold shock. These aspects of cold shock in microorganisms have been summarily reviewed by MacLeod and Calcott (1976). Permeability changes in cold shock are believed due to a phase transition in the membrane lipids

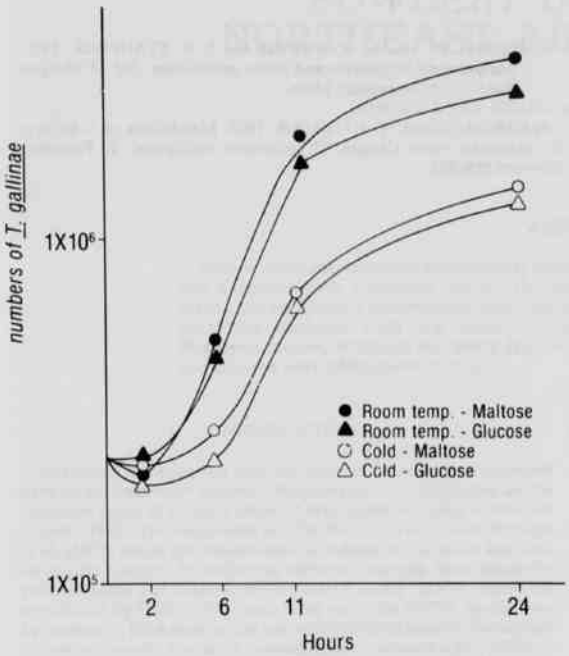

Figure 1. The effect of cold and room temperature pretreatment washings of *Trichomonas gallinae* with modified Ringers' solution on subsequent recovery in STS growth medium (pH 7.0). Sugar concentrations are 20 mM.

from a liquid to a solid state. Adam (1941) showed that macromolecular films of lipids will shrink upon cooling with a temperature range for each lipid. Salts of long-chain fatty acids will form microcrystals at a specific temperature known as the Kraft point (Dervichian, 1965). A sudden change in configuration of the lipids in the cell membrane should express itself as changes in permeability mechanisms. Steim et al. (1969) have suggested that such changes create hydrophilic channels allowing the escape of solutes from cells.

Cold shock effect on the viability of *Trichomonas gallinae* was demonstrated on logarithmic cells in experiments performed after the present study (Matthews and Daly, 1974). Using a colony count technique, cells exposed to slow cooling in modified Ringers showed a higher initial percent viability than cells exposed to sudden chilling. In allowing the cells to remain at 4°C, the death rate was greater with the slowly cooled cells. Little loss in viability was seen after the initial decrease with suddenly cooled cells. In the present study stationary phase cells were used which are usually more refractile (in bacteria) to cold shock (Meynell, 1958). There was an initial loss of these older cells in the cold-washing procedure indicating cold-sensitive cells had been present and had presumably disintegrated. Recovery in growth medium was slower with cold-washed cells with an increased lag phase. Unfortunately, no reliable viability test was available for these experiments at the time; therefore, it is possible that the increased lag seen may be due in great part to dead cells in the total cell count rather than slowly recovering cells. Three diluents were used to wash the cells for these growth experiments, since bacterial studies had shown the importance of the presence or absence of common cations and amino acids. No major differences in "recovery" of the cells were noted among the use of the three diluents.

In preliminary metabolic studies with *T. gallinae*, it was not suspected that the employment of a cold-washing procedure would adversely affect the condition of the cells. Microscopic examination of trichomonads kept at 4°C for up to 1½ hr showed no apparent decrease in motility when warmed to room temperature. Cold washing of parasitic protozoa in experimental preparation procedures is not generally done; therefore, these findings were somewhat fortuitous and unique. Warren and Kitzman (1963) noted that cold washing of *Schizotrypanum cruzi* resulted in a higher level of oxygen consumption but attributed this to cold preservation of available intracellular endogenous substrate. In the present study a greater glycogen pool was maintained in cold-washed cells, and subsequent endogenous gas production was higher than with room-temperature cells. However, stimulation of CO_2 and H_2 by exogenous glucose and maltose was greater with room temperature-treated cells. It may be argued that this is not evidence for a metabolic defect, since stimulation of cold-washed cells would appear to be lower only because the higher relative endogenous rate would result in a proportionally lower stimulation to the two sugars. The equimolar uptake of carbohydrate after both treatments would also appear as evidence against cold shock, especially since membrane permeability is a target of this effect. Glycogen metabolism, however, is noticeably changed since cells exposed to cold are less able to synthesize this polysaccharide. Endogenous depletion of glycogen at 37°C is higher for cold-washed cells. This may reflect an increased intracellular inorganic phosphate level due to permeability changes stimulating glycogen phosphorylase activity.

Observations of cold shock on the metabolism and recovery in growth medium of *T. gallinae* have been made in this study, but the mechanisms of this effect are not clear. However, it is obvious that cold treatment of this organism will result in greatly different physiological data than that from cells prepared under less drastic temperature changes. Cold shock should be avoided with trichomonads unless it is to be studied intrinsically or for examination of defective metabolism.

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