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THE EFFECTS OF MEDIA CONCENTRATION ON METAL TOLERANCE IN *TETRAHYMENA FURGASONI* W

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ABSTRACT

Growth of axenic cultures of *Tetrahymena furgasoni* W is known to be inhibited by the heavy metal ions of cadmium, chromium and copper. This study was conducted to determine whether the concentration of organic matter in the growth medium could significantly alter the toxicity of these metals. The media used herein were enriched proteose peptone (Dobra *et al.*, 1980) and proteose peptone in a phosphate buffer (Bergquist & Bovee 1976). Each medium was used both full strength and diluted 1:10. Results obtained indicate that for each metal tested, inhibition of growth by the metal is greatest in the most dilute media and the least in the most concentrated media. Populations that had reduced population growth parameters also had an increased mean number of contractile vacuole pores.

INTRODUCTION

It is generally accepted that heavy metal toxicity in axenic cultures of ciliate protozoans is caused by the presence of the free ions in the supporting medium (Bovee *et al.*, 1979). However, Ramamoorthy and Kushner (1975) established that certain toxic, heavy-metal ions (*e.g.*, Cd^{+2}, Cu^{+2}) bind to specific microbiological medium components, including those containing proteose peptone and yeast extract. Although there are numerous reports in the literature of the toxic effects of metal ions on proto-

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zoans (Nyberg, 1974; Nyberg & Bishop 1983; Nyberg & Bogar 1986; Tingle *et al.*, 1973; Hutner, 1964; Dunlop & Chapman, 1981; Persoone & Dive 1978), little effort has been expended to date in determining the effect of the growth medium on the availability of these ions. As a consequence, the relative toxicity of bound versus free ions is still debated.

In addition, wide ranges in the concentrations of organic nutrients in media are noted in the literature. Some studies used strongly fortified nutrient media (Bergquist, 1974; Bovee, 1975), whereas others have used only distilled water as the agent delivering the dose (Carter & Cameron, 1973). Thus, to compare the toxicity data among the studies that have been done in these various media will require more knowledge of the effects of the organic materials on heavy metal toxicity.

This study was undertaken to determine the effects of the ions of the heavy metals cadmium, copper, and chromium (administered as chromate) on the ciliate protozoan, *Tetrahymena furgasoni* W when these toxic heavy metal ions were administered in varying concentrations of the supporting proteose peptone-based media that are commonly used with *Tetrahymena*.

Because the number and position of the contractile vacuole pores (CVPs) are important taxonomic characters, (Nanney 1967; Frankel 1972), it is useful to know if the number of CVPs remains stable under environmental stress. This investigation also includes some preliminary information on the use of the number of cortical contractile vacuole pores (CVPs) as a possible cytological indicator of the metal-induced cytotoxic stress.

MATERIALS AND METHODS

Tetrahymena furgasoni W (formerly *T. pyriformis* W) was obtained from C.F. Ehret at Argonne National Laboratory, Argonne, Il. The nutrient medium selected for growing the control culture was enriched proteose peptone (EPP) (Dobra *et al.*, 1980). For toxicity testing, EPP was prepared and used either full strength or as a 1:10 dilution (0.2% EPP). All other media containing proteose peptone (PP) were based on the medium of Sims (Bergquist & Bovee, 1976). These latter media contained 0.1% dibasic sodium phosphate and 0.1% sodium citrate. Proteose peptone was added to make final medium concentrations of 2%, 1%, 0.2% and 0.1% PP. All dissolved media were autoclaved at 121° C for 15 minutes at 15 psi in 100 ml aliquots in 250 ml Erlenmeyer flasks. The trace metals that were tested were supplied as cadmium chloride ($CdCl_2$), copper chloride ($CuCl_2$) and potassium chromate (K_2CrO_4). Solutions were prepared to produce stock concentrations of 1000, 500, and 100 mg/L cadmium; 10,000, 1000 and 100 mg/L chromium, and 100,000, 10,000 and 1000 mg/L copper. Two to three drops of concentrated HCl were added to the most concentrated copper solution to facilitate dissolution. This had no significant effect on the pH of the final solution. These stocks were autoclaved (15 psi for 15 min, 121° C), and the same sterile solutions were used for the duration of the experiments. The volumetric addition of one mL of metal stock solution to 100 mL of EPP yielded final concentrations of 1, 5 and 10 mg/L cadmium, 1, 10 and 100 mg/L chromium and 10, 100 and 1000 mg/L copper.

After the metal additions were completed, each flask containing 100 mL of broth was inoculated with a logarithmically growing population of *T. furgasoni* W to an initial cell concentration of approximately 1600 cells/mL. These were incubated in

duplicate flasks at 25° C in a Labline Instruments Co. Environ-Shaker. A rotation speed of 70 rpm and a 12:12 LD (light-dark) cycle continued throughout the testing period. Cells were harvested by withdrawing 1.0 mL by sterile pipette at six hour intervals, and fixed in 0.1 N perchloric acid. These samples were used for counting with a Coulter model F particle counter. The mean cell density per mL of the cultures at each sampling point was calculated by averaging six cell counts. The estimates of the initial cell concentrations were made by counting an aliquot of the inoculating cultures.

For each combination of media and metal ion concentration, growth rates were compared by linear least squares fits of time against the logarithm of cell number. In addition, Student's one-tailed t-test was used to compare cell concentration after 30 hours of growth among the metal-ion treatments within each medium concentration. A significance level of $p = 0.05$ was used in each analysis.

CVPs were enumerated from cells grown in 2% EPP at 30 and 60 hours. Samples were taken from flasks containing the control, 5 mg/L Cd^{+2}, 10 mg/L Cr^{6} and 100 mg/L Cu^{+2}. Ten mL of these cultures were centrifuged, fixed in 4% OsO_4, and then washed and post-fixed with Dafano's solution. The fixed cells were stained by the Frankel and Heckmann (1968) modification of the Chatton-Lwoff silver impregnation technique. Cells were scored for number of CVPs at 1000X magnification, using a light microscope.

One way analysis of variance (ANOVA) was performed on 250 cells from each treatment. When the F value for the ANOVA test ($p = 0.05$) exceeded the given value for the degrees of freedom of the data, the Student-Neuman-Keuls (SNK) test was employed to determine which metal treatments were significantly different from the control (Zar, 1974).

RESULTS

The results of the typical experiment are summarized in Table 1. Figures 1 through 3 are selected growth curves with the lines of best fit superimposed.

Figure 1 depicts the growth of *T. furgasoni* W in EPP and 0.2% EPP, with and without added cadmium. This heavy metal ion, as seen in Table 1, strongly inhibits growth in all media at a level of $\geq$ 10 mg/L. A maximum population and growth rate which did not differ significantly from the control were obtained at 5 mg/L only in the concentrated EPP solution. Although growth was not inhibited significantly at 1 mg/mL Cd^{+2}, in any of the concentrated media, *i.e*, EPP, 2% PP and 1% PP, all of the diluted media showed a pronounced inhibition of cell growth.

Figure 2 shows some of the effects of the chromate ion on the maxiumu population size obtained by *Tetrahymena* grown in 2% PP and 0.2% PP media. Table 1 indicates that growth was strongly inhibited in all media containing 100 mg/L CrO_4^{-2}. Inhibition in the 10 mg/L solution was significant in all cases, but had its minimal effect in EPP. In this medium, a high cell density was still attained. A level of 1 mg/L CrO_4^{-2} in 0.2% PP and 0.1% PP exhibited an inhibition of the population's growth rate.

Figure 3 presents growth data for copper-containing 1% and 0.1% PP. Inhibition of the growth rate and population density by copper (also see Table 1) were shown in all media at 1000 mg/L and 100 mg/L Cu^{2}. Growth rate and peak population concentration were reduced by 10 mg/L Cu^{+2} in 0.2% PP and 0.1% PP.

The statistical analyses of the number of CVPs are presented in Table 2. The ANOVAs of both the 30 hour and the 60 hour samplings showed significant differences among the treatments. The SNK test indicated that at 30 hours the only statistically significant different among the treatments was between the control and those containing copper, which had an increased mean number of CVPs. At 60 hours, the controls and cells treated with Cd^{+2} had no detectable change in the variance in the number of CVPs. There were significant ($p \leq 0.05$) increases in the number of cells with supernumerary CVPs (*i.e.*, more than the 2 CVPs that are typical for this species) between the control population and the cells treated with CrO_4^{-2} and Cu^{+2}.

DISCUSSION

The information obtained from these studies showed that the inhibition of growth of *Tetrahymena furgasoni* W by exposure to metal was affected by the amount of organic compounds in the medium. In all media concentrations tested, the growth of the controls displayed no statistically significant differences. For all metals used, the growth was inhibited at the highest metal concentrations in all media. Generally, higher metal concentrations were less toxic in the higher concentrations of PP, as judged by the growth of the culture.

The literature has numerous studies on the toxicity of heavy metals to protozoa (Persoone & Dive, 1978, Slabbert et al., 1983; Smith-Sonneborn 1983). *Tetrahymena* and *Paramecium* have received special attention in such toxicological research. Although this has generated a wealth of new information, the use of many different media has caused confusion in comparing the results of these studies.

Methods that measure toxicity by describing changes in a culture over several generations have been developed to estimate the toxic response of ciliates to metals. These include cell size characters (Nemeth & Csik, 1963), feeding behavior based upon comparative prey density (Bringmann & Kuhn, 1959), clone viability (Heaf & Lee, 1971; West *et al.*, 1962) and comparative growth curves (Apostal, 1973). Smith-Sonneborn (1983) used the autogamous species *Paramecium tetraurelia* to test the rate of genetic damage caused by toxic and carcinogenic agents.

Nyberg and Bishop (1983) used bacterized Cerophyl to test 48-h mean tolerance limit (TL-50) to Cd^{+2}, CrO^{-2} and Cu^{+2}. Carter and Cameron (1973) used a bioassay procedure with distilled water over a 24-h interval to ascertain a TL-50 of 1.67 mg/L for Cd^{2}. Yamaguchi (*et al.*, 1973) employed a 0.2% PP medium to determine that 0.44 mg/L Cd^{+2} was inhibitory to growth. Bergquist (1974) used 2% PP to measure growth inhibition using Cd^{-2}. Bovee (1975) did his studies in a 1% PP similar to the above four growth inhibition studies for Cd^{2}, Cu^{-2} and Cr^{-6}. Because these investigations and widely different toxicity testing procedures and media concentrations, the results were noncompatable or contradictory.

It is clear that there should be some standardization of media and testing procedures accompanying toxicological studies of the ciliates. For bioassay work, Schultz *et al.* (1980) advocate the use of a medium similar to the EPP used here. As this study demonstrates, additional organic components in the media decrease the toxic effect of the metal ions examined. For *Tetrahymena*, we recommend that the effects of a toxin on growth rate and maximum population levels in 0.1% PP be reported with other data as a comparative measure because we observed a marked protection of cells from the toxic effects of heavy metals as the concentration of peptone increased.

This recommended concentration of proteose peptone has the following advantages: 1) it supports a population equivalent to that seen in 1% proteose peptone. 2) it is buffered (pH is known to alter toxicity <Wakatsuki *et al.*, 1984>). 3) it is easier to prepare than defined media and 4) growth inhibition by metal ions is more clearly visible here than in higher concentrations of peptone. Cerophyl (0.25%) bacterized with *Klebsiella aerogenes* (Nyberg & Bishop, 1983) is recommended for *Paramecium* and other hymenostomes that are not routinely grown axenically. Growth rate and population density are recommended as common points of comparison.

In many of the cultures in which the metal concentration was strongly inhibitory to growth, an initial doubling of the cell density was noted. Differences in cell number among the treatments became manifest between 12 and 18 hours. Using 0.1% PP in distilled water, Ramamoorthy and Kushner (1975) found that additions of 20 mg/L copper resulted in an 86% binding of the metal to the medium after 30 minutes, as measured by a selective free ion probe. Similarly, the addition of cadmium resulted in 57% binding. A solution of 0.1% yeast extract bound 98% of the added copper, but only 11% of the cadmium ions. Therefore, the differences in growth rates that were detected between 12 and 18 hours, as indicated by the regression analysis, were not due to further reductions in free metal ions.

Saar and Weber (1979) found that cadmium bound to fulvic acids. Babich and Stotzky (1977) were able to relate the reduced toxicity of cadmium toward microorganisms directly to the cation capacity of clays added to growth media. These lines of evidence indicate that the interaction of organic matter and metals in the growth medium in protozoans may be of concern to the environmentalist as well as the laboratory toxicologist. Again, care must be exercised in the comparison of toxicity data from studies conducted in different media.

Few morphological effects are known to be caused by heavy metal toxicity. Food vacuole formation shows intense stimulation in both number and increased size in response to copper ions (Nilsson 1981). Cells increase the number of refractile granules in the cytoplasm (Nilsson 1981) and the normal trajectory followed by cell volume changes through the modes of growth can be altered by toxins (Nemeth & Csik 1963).

In *Tetrahymena*, the complex cell surface, the cortex, ranks among the most intensly studied features of the cell. Due to its stable structure cortical characters are important as a taxonomic indicator (Frankel, 1972, 1974). Among the better taxonomic features in *Tetrahymena* are the number and position of the contractile vacuole pores (CVPs), which are the external opening to the contractile vacuole (Nanney, 1967). Factors that alter the mean number of position of the CVPs are therefore of interest to systematists.

Bergquist (1974) reported that cells treated with Cd^{+2} developed enlarged CVPs. Loefer et al., (1966); indicated that with unstressed *T. furgasoni* W, approximately two-thirds of the cells contained 2 CVPs, and one-third had 3 CVPs. A few cells had a single CVP and they did not report any strain with four or more.

We found supernumerary CVPs in association with two of the metal solutions tested. These were not believed to be due to the osmotic or ionic stress caused by the addition of ions to the medium. The changes in the osmotic and ionic strength were minute compared to the effect of the medium concentration. The increased incidence of supernumerary CVPs was seen only in cultures which exhibited a significant reduction in both growth rate and maximum cell count. Thus, the mean number of CVPs may represent a potential measure of physiological stress.

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Table 1. Effect of media on growth of 30 hours with heavy metal.

Metal Concentration (mg/l)	Cell Count Mean ± S.E.*	Probability (P <)	Cell Count Mean ± S.E.	Probability (P <)	Cell Count Mean ± S.E.	Probability (P <)
CONCENTRATED MEDIA	EPP		2% PP		1% PP	
Initial	1,158 ± 31		854 ± 24		1,206 ± 73	
Control	216,300 ± 621		73,223 ± 1,355		69,833 ± 5,675	
Cd 1	320,417 ± 49,504		56,950 ± 1,747	0.05	20,490 ± 1,249	
Cd 5	227,270 ± 6,981		2,583 ± 131	0.005	2,630 ± 212	0.05
Cd 10	3,450 ± 245	0.005	1,647 ± 220	0.005	3,227 ± 41	0.05
Cr 1	212,690 ± 8,663		58,843 ± 2,139		50,570 ± 3,013	
Cr 10	125,197 ± 224	0.005	20,733 ± 57	0.005	19,247 ± 2,090	0.05
Cr 100	3,783 ± 0	0.005	2,290 ± 33	0.005	2,252 ± 367	0.05
Cu 10	219,113 ± 10,949		74,690 ± 33		51,523 ± 1,315	
Cu 100	110,513 ± 759	0.005	5,880 ± 82	0.005	2,583 ± 367	0.05
Cu 1000	5,190 ± 465	0.005	1,807 ± 8	0.005	2,693 ± 188	0.05
DILUTED MEDIA	0.1 EPP		0.2% PP		0.1% PP	
Control	97,293 ± 600		49,963 ± 547		34,530 ± 114	
Cd 1	2,463 ± 106	0.05	1,537 ± 220	0.005	817 ± 139	0.05
Cd 5	2,683 ± 171	0.05	1,057 ± 82	0.005	2,850 ± 1,078	0.05
Cd 10	2,083 ± 139	0.05	957 ± 73	0.005	1,270 ± 16	0.05
Cr 1	363,997 ± 24,258		37,873 ± 751	0.05	2,980 ± 1,560	
Cr 10	207,507 ± 12,235	0.05	2,750 ± 122	0.005	2,603 ± 106	0.05
Cr 100	12,383 ± 572	0.005	1,050 ± 49	0.005	1,337 ± 24	0.05
Cu 10	64,213 ± 2,490		4,107 ± 371	0.005	2,783 ± 278	0.05
Cu 100	3,693 ± 384	0.05	1,037 ± 57	0.005	1,983 ± 122	0.05
Cu 1000	2,423 ± 49	0.05	1,093 ± 57	0.005	1,313 ± 41	0.05

*S.E. = Standard Error

Table 2. Statistical analysis of contractile vacuole pore number data from cells grown in EPP for 30 h and 60 h.

30 hour counts

Treatment	Number of CVPs Per Cell					Mean CVP Number	Stand. Error
	1	2	3	4	5		
Control	6	152	83	7	0	2.37	0.037
Cadmium, 5ppm	1	139	97	12	1	2.49	0.035
Chromium, 10ppm	3	156	84	7	0	2.36	0.038
Copper, 100ppm	7	102	132	8	1	2.58	0.040

One way ANOVA among metal Treatments

Source	DF	Sum	Mean	f
Treatments	3	8.14	2.71	7.65
Error	996	353.26	0.35	
Total	999	361.40		
probability ≤ 0.01				

Comparison of Treatment Means By the Student-Neuman-Keuls (S-K-N) Test

Control vs cadmium- NS
Control vs chromium- NS
Control vs copper- $p \leq 0.05$
Cadmium vs chromium- NS
Cadmium vs copper- NS
Chromium vs copper- NS

60 hour counts

Treatment	Number of CVPs Per Cell					Mean CVP Number	Stand. Error
	1	2	3	4	5		
Control	39	165	42	4		2.04	0.039
Cadmium, 5ppm	20	164	64	0	2	2.20	0.039
Chromium, 10ppm	20	143	88	10	1	2.42	0.041
Copper, 100ppm	6	115	115	14	0	2.55	0.041

One way ANOVA among metal Treatments

Source	DF	Sum	Mean	f
Treatments	3	38.12	12.70	31.87
Error	996	397.08	0.40	
Total	999	435.19		
probability ≤ 0.01				

Comparison of Treatment Means By the Student-Neuman-Keuls (S-K-N) Test

Control vs cadmium- NS
Control vs chromium- $p \leq 0.05$
Control vs copper- $p \leq 0.01$
Cadmium vs chromium- $p \leq 0.05$
Cadmium vs copper- $p \leq 0.05$
Chromium vs copper- NS

NS = not significant

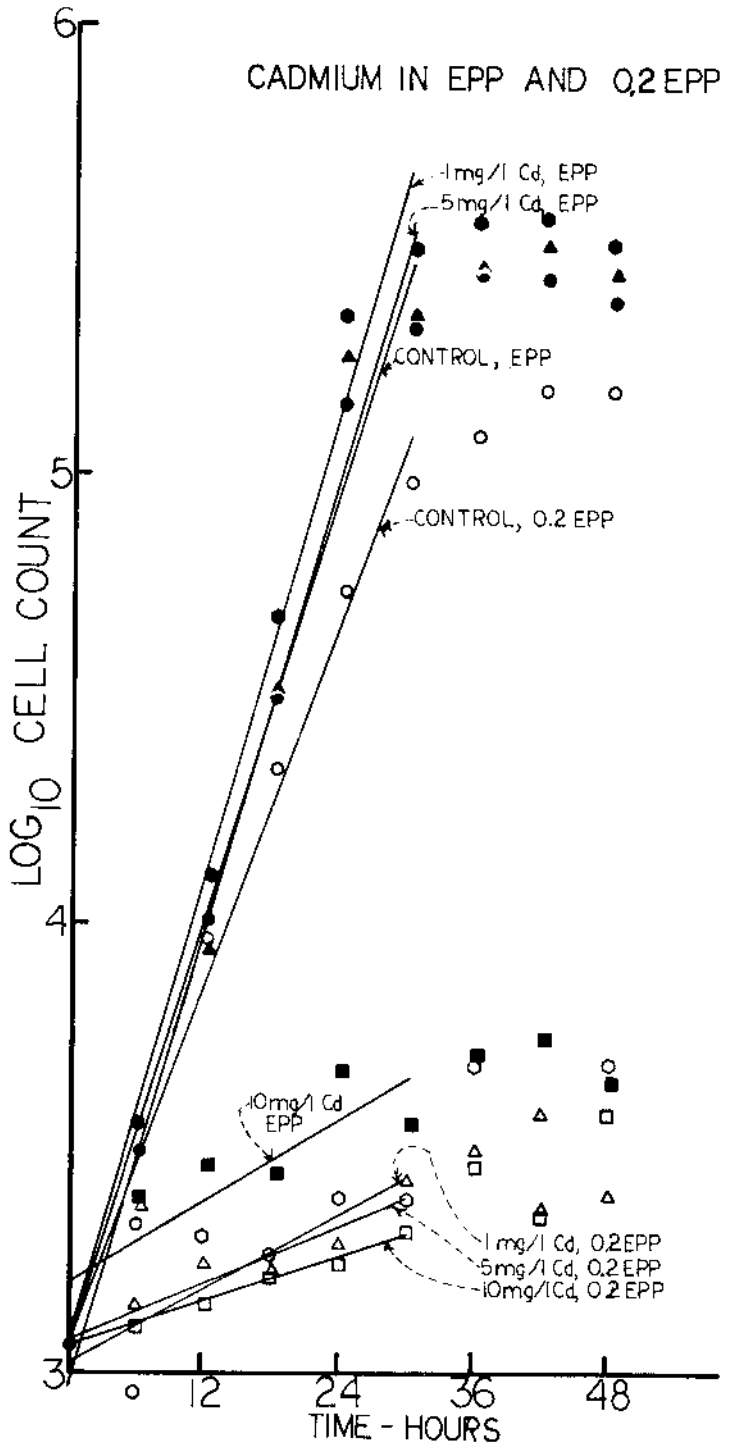

Fig. 1. The growth of *T. furgasoni* in full strength enriched proteose peptone (EPP) (2.0%) and in diluted EPP either alone or with 1-10 mg/L cadmium added.

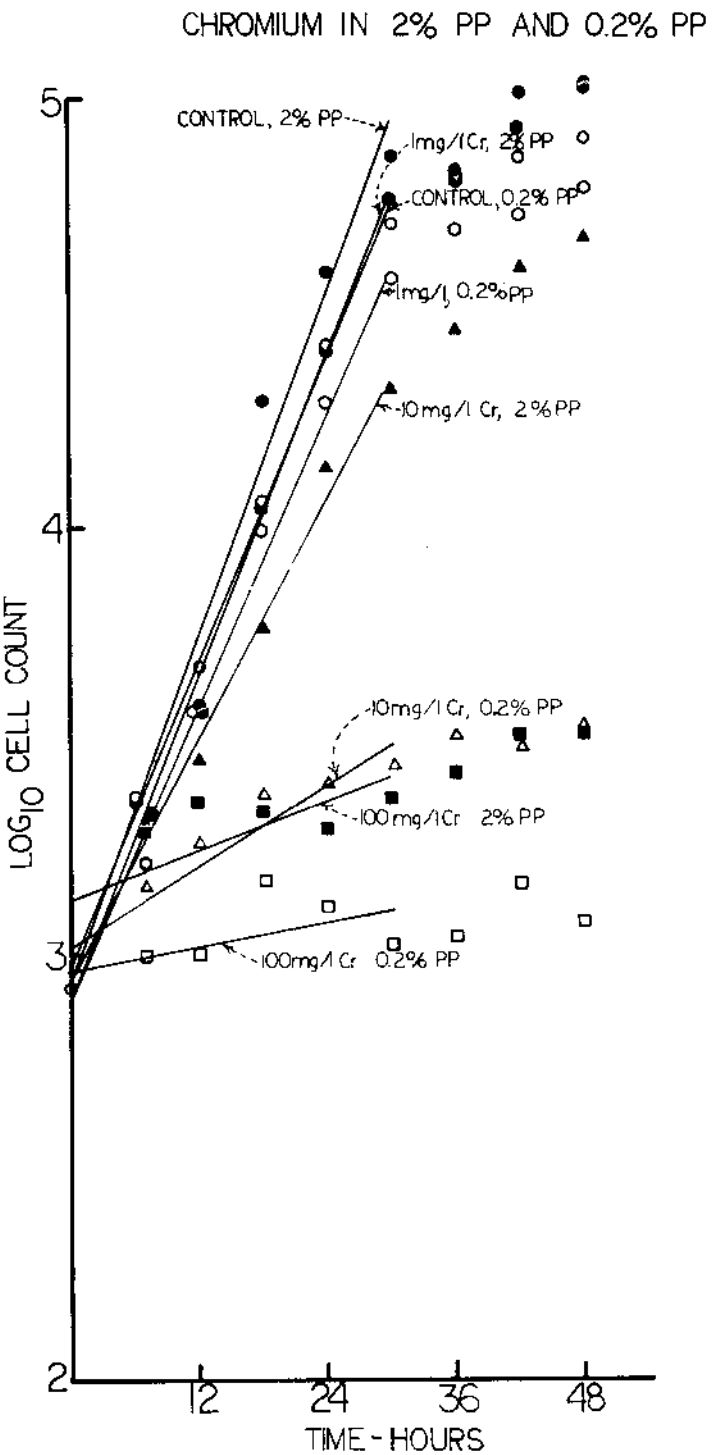

Fig. 2. The growth of *T. furgasoni* in similar media dilutions to the above, but with the addition of from 1 to 100 mg/L chromate.

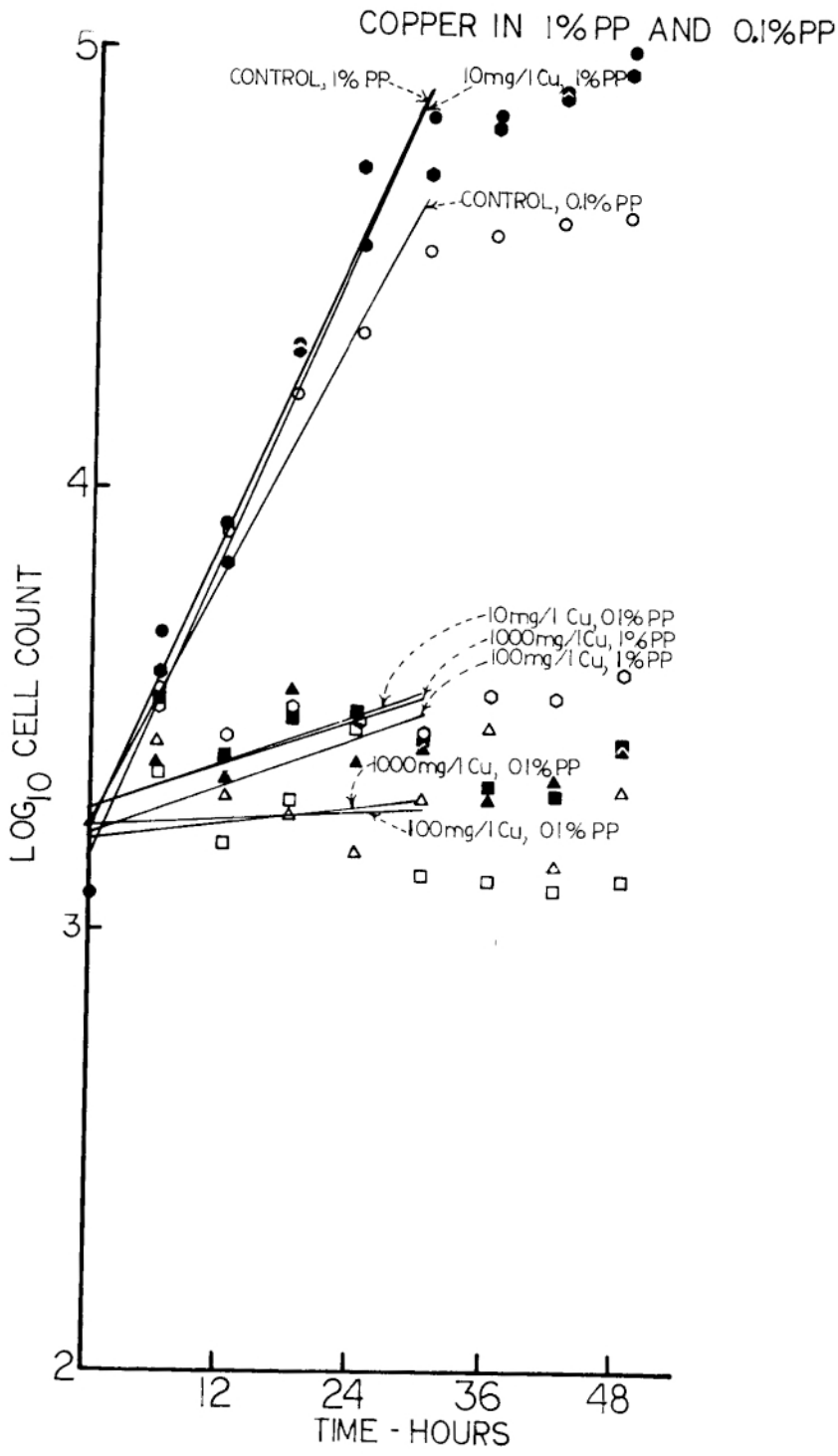

Fig. 3. The growth of *T. furgasoni* in 1 % proteose peptone medium and in diluted medium containing 10-100 mg/L copper.