Effects of sulfhydryl reagents on the fission number and mating ability of Paramecium multimicronucleatum

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Abstract

Lipoic acid and a range of sulfhydryl reagents were tested for their effects on the fission number and mating ability of Paramecium multimicronucleatum. Lipoic acid was given special attention because of its specific biological effects on many organisms.

It was found that the reagents affect fission in the following order of decreasing effectiveness: lipoic acid, mercaptoethanol, N-ethylmaleimide, reduced glutathione, cysteine, iodobenzoic acid and mercaptoethylgluconamide. The effects of these reagents on fission are immediately reversible after 24 hour treatments with each concentration used, except for 10^{-1} M mercaptoethylgluconamide and 10^{-2} M mercaptoethanol.

The same concentrations of mercaptoethylgluconamide and mercaptoethanol which have a long-lasting effect on fission inhibit conjugation. The inhibition of conjugation by lipoic acid increases with increasing concentrations of the substance, starting at 5×10^{-4} M. The other sulfhydryl reagents have no effect on conjugation.

EFFECTS OF SULFHYDRYL REAGENTS ON THE FISSION NUMBER AND MATING ABILITY OF PARAMECIUM MULTIMICRONUCLEATUM

by

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Introduction

Paramecium is a protozoan which is widely used in research because of the ease with which it can be cultured. In the present experimental work, we used the species Paramecium multimicronucleatum because of its large size which facilitates manual isolation of individual cells and observation.

Paramecium multimicronucleatum has a cigar-shaped body, round in cross section with a somewhat pointed posterior end. In our cultures, the body size was between 180 and 200 μ in length and 40 to 50 μ in width in its largest part. The nuclear apparatus of these paramecia is composed of one kidney-shaped macronucleus and three or four small, vesicular micronuclei. Two contractile vacuoles are one at each end of the body. The oral groove extends slightly beyond the middle of the body and is continued by the mouth and cytopharynx.

The body of the paramecium is covered by a semi-rigid pellicle which may serve as a barrier between the cortical cytoplasm and the medium. The pellicle is composed of three membranes: first, the cell-membrane proper which is continuous over the entire cell surface including cilia and all orifices, and then the outer and inner boundaries successively

of the balloon-like alveolus that lies close under the cell membrane (Pitelka, 1965). The pellicular alveoli form a surface pattern of hexagonal depressions. The center of each hexagonal area bears a cilium which has the typical ciliary structure with nine pairs of filaments surrounding two inner filaments.

Fission and Conjugation.

Paramecium multimicronucleatum like the other paramecium species has two modes of reproduction. Binary fission occurs more often and is an asexual process in which a fully grown specimen divides into two daughter cells. Division of the cell body is preceded by the division of the nuclei. The macronucleus stretches and divides amitotically while the micronuclei divide by mitosis. The fission rate is influenced by environmental conditions. It is optimum when the food supply is abundant and when the temperature and pH are respectively around 22° C and 7.00. A change in the above conditions will accordingly produce a lowering of the fission rate.

The other mode of reproduction is a sexual process, conjugation, which brings about a micronuclear exchange (Barnett, 1964). Conjugation usually occurs after a relatively long period of asexual reproduction and is followed by another period of asexual

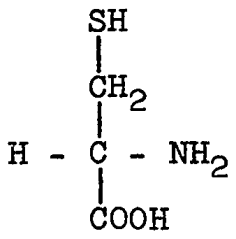
reproduction. When paramecia of complementary mating types are mixed together, conjugation takes place if certain conditions which we will describe later are fulfilled. A few seconds after the introduction of individuals of the opposite mating type, clumps of paramecia begin to form. These clumps increase in size during the following hour and can be comprised of several dozens of individuals. Then, the clumps break down gradually into smaller clumps about one and a half hours after mixing, and pairs begin to appear.

Mating is brought about by specific mating-type substances borne on the cilia. These substances seem to be proteins (Metz et al, 1954) whose interaction would involve a steric fitting or antigen-antibody type of union at the contact of two paramecia of complementary mating type (Cohen and Siegel, 1963). These substances appear with diurnal periodicity on the ventral surface of the paramecia, mainly around the oral cone (Cohen, 1965). The members of a pair usually adhere at their antero-ventral portion. A cytoplasmic bridge formed by breakage of the membrane of both cells at the site of adherance allows the micronuclear exchange to take place.

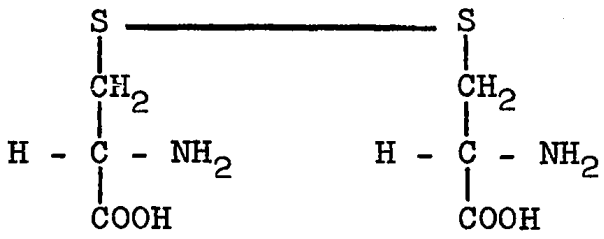
Biological effects of sulfhydryl reagents.

Numerous studies have been made on the effects

of sulfhydryl reagents on living cells and organisms since it became clear that proteins are an essential component of the structural framework of the cell and that the enzymes are proteins. Most if not all proteins contain cysteine and cystine, each of which contains respectively a sulfhydryl and a disulfide group.



Cysteine



Cystine

The tertiary configuration of proteins is stabilized to a large extent by disulfide bonds which occur between two cysteine residues. Also, the active site of a large number of enzymes contains a sulfhydryl group. For example, several oxidative enzymes like lipoyl dehydrogenase have a sulfhydryl group which is thought to participate in electron transfer.

Whether it is structural or enzymatic or both, a protein has a particular steric configuration which gives it its specific properties. A sulfhydryl reagent which oxidizes sulfhydryl groups, such as cystine, could affect the protein in several ways. Oxidation of otherwise free sulfhydryl groups could denature the

protein by binding different parts of the amino acid chain through formation of disulfide bonds between two cysteine residues. Oxidation of sulfhydryl groups with a primary role in the catalytic activity of an enzymatic protein could cause the loss of the catalytic activity of the enzyme. Oxidation of oxidizable metabolites such as ascorbic acid and glutathione might influence proteins indirectly.

Similarly, a sulfhydryl reducing agent such as cysteine could affect the protein. Breakage of protein disulfide bonds could denature the protein by changing its configuration. Reduction of catalytically active disulfide groups could cause a loss in the activity of the enzymatic proteins involved. Reduction of some cell metabolites could influence the proteins indirectly.

The role of disulfide linkages in the stability of the mitotic apparatus was demonstrated by Mazia and Dan (1952) who reported that reduction of disulfide linkages renders the spindle more soluble in detergent while oxidation stabilizes the spindle. They proposed that the formation of the mitotic apparatus might involve the polymerization of small protein molecules through a disulfide interchange reaction initiated by glutathione. According to this hypothesis, a change in the intracellular redox potential would be likely

to affect the mitotic apparatus. Indeed, the cleavage of fertilized eggs of Strongylocentrotus purpuratus may be blocked reversibly by mercaptoethanol, a non-toxic reducing sulfhydryl reagent. The resulting apparent disorganisation of the mitotic apparatus was interpreted as a loosening of the spindle structure (Mazia and Zimmerman, 1958). Mitosis in the blastomeres of the crustacean Artemia salina is also inhibited by mercaptoethanol and dithiodiglycol, the oxidized form of mercaptoethanol (Fautrez-Firlefyn, 1963). Homogenates of sea urchin eggs, ovaries, testes and gut, contain a cleavage-retarding factor whose delay may have a common basis with that induced by mercaptoethanol (Wolfson, 1959). Agents which would be expected to maintain the sulfhydryl groups of the extract in reduced form, such as reduced glutathione and cysteine increase the delay in cleavage, while agents which oxidize sulfhydryl groups such as oxidized glutathione, iodobenzoic acid and aeration, decrease the delay (Wolfson and Wilbur, 1960).

Cell division in the microorganisms Escherichia coli and Saccharomyces cerevisiae which are not likely to have a mitotic apparatus is also inhibited by mercaptoethanol and dithiodiglycol. It seems therefore, as suggested by Limbosch-Rolin (1963), that any

modification in the sulfhydryl-disulfide equilibrium has as a necessary consequence the inhibition of cell division and growth.

Aside from cell division and growth, sulfhydryl reagents affect the morphogenesis of developing embryos. Mercaptoethanol blocks gastrulation movements and nucleic acid synthesis in amphibian embryos. Both mercaptoethanol and dithiodiglycol inhibit closure of the medullary plate (Brachet and Delange-Cornil, 1959). Other examples of effects of sulfhydryl reagents on morphogenesis include observations on amphibian embryos treated with several oxidizing agents such as alloxan, oxidized glutathione and iodobenzoic acid. Only glutathione inhibited morphogenesis (Lallier, 1951). Monoiodoacetamide and chloropicrine which are sulfhydryl inhibitors, brought a loosening of the blastoporal lip and medullary plate of the amphibian embryos (Rapkine and Brachet, 1951).

Workers in the laboratory of Jean Brachet have studied the effects of mercaptoethanol, dithiodiglycol and mercaptoethylgluconamide, the latter of which does not penetrate into the cells, on the development of amphibian embryos, cap production in the alga Acetabularia mediterranea, regeneration of the tail in amphibian tadpoles and regeneration of the head in planarians.

In all cases, mercaptoethanol inhibits morphogenesis at concentrations which do not kill the organisms. Dithiodiglycol has slight stimulatory effects, especially in the case of Acetabularia, while mercaptoethylgluconamide causes a failure in lens formation of amphibian embryos and produces very abnormal caps in Acetabularia. Thus, a general conclusion is that morphogenesis in very different biological systems is dependent on a system involving the sulfhydryl-disulfide equilibrium (Brachet, 1959).

Lipoic acid.

Lipoic acid has specific biological effects such as a growth-promoting action on many microorganisms and an inhibitory action on the growth, morphogenesis or regeneration of other organisms. Some of these effects may be at least partly due to its role in the oxidative decarboxylation of α-keto acids. Lipoic acid also has liposoluble properties. The preceding characteristics led us to consider lipoic acid in greater detail than in the case of the other sulfhydryl reagents used.

Lipoic acid was discovered independently in four laboratories. Snell et al (1937) found that two unknown factors were necessary for luxuriant growth of Lactobacillus delbruckii and that an accessory factor

was essential for growth of fourteen species of lactic acid bacteria. This accessory factor was concentrated from Brewer's yeast by Guirard, Snell and Williams (1946), and was found to be a water-soluble substance which replaced acetate for its growth-promoting function in some lactic acid bacteria. Guirard et al called it the Lactobacillus bulgaricus factor (LBF), as it is essential for growth of this strain in particular. Kitay and Snell (1950) studied concentrates of the LBF factor and demonstrated that a single unidentified factor was responsible for the growth effects it gave. They also found that the LBF factor existed in several chromatographically distinct forms, one of which could be in either a thiol or a corresponding disulfide form.

Dewey (1941 and 1944), while studying the nutrition of Tetrahymena geleii also noted that at least two unknown factors were required for optimal growth of the cultures, which he called Factor I and Factor II. Factor II was further subdivided into Factor IIA and Factor IIB. Factor IIA consisted of two forms of the same unknown substance, and was called Protogen in 1949. Factor IIB was composed of Factor IIB' which could be, for a large part, replaced by increased pyridoxine, and Factor IIB'' which could be replaced by

Cu^{++} and Fe^{+++} ions in relatively high concentrations.

O'Kane and Gunsalus (1947 and 1948) studied the pyruvate oxidation by Streptococcus faecalis and reported that an accessory factor was required by the cells for this process. This factor which they called the Pyruvate Oxidation Factor (POF), was found in yeast extract.

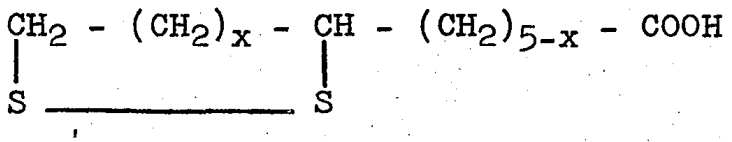
Kline and Barker (1948) reported that a new factor was required for the growth of Butyribacterium rettgeri. The active substance which they called the B.R.Factor seemed to be a weak acid.

It is Snell and Broquist (1949) who presented experimental evidence indicating that the acetate-replacing or LBF factor, Protogen, the Pyruvate Oxidation Factor and the B.R.Factor were identical. This encouraged research toward the isolation and identification of the unknown factor.

Reed et al (1951) were able to crystallize the active substance, calling it α-lipoic acid because of its high solubility in lipid solvents, its acidic nature and its involvement in the formation of acetate through the oxidative decarboxylation of pyruvate which is an α-keto acid. They extracted α-lipoic acid from diverse biological sources such as acid-hydrolized liver residue and yeast extract. Due to its presence

in a wide variety of biological sources and the fact that it has an extremely high biological activity and possesses a catalytic role in the oxidative decarboxylation of α-keto acids, they considered α-lipoic acid as a member of the B-vitamins. Their analysis showed the presence of a disulfide linkage, of easily reducible groups which they did not relate to the disulfide linkage, and of an acidic group. Moreover, in addition to lipoic acid, they found several distinct compounds possessing acetate-replacing activity in biological preparations. These compounds were mixed disulfides formed from reduced lipoic acid and naturally-occurring thiols such as glutathione. Acid hydrolysates also contained a substance chemically related to lipoic acid, but more polar. Lipoic acid was always partially converted to this more polar substance which led Reed et al to suggest that it could be an oxidation product of lipoic acid.

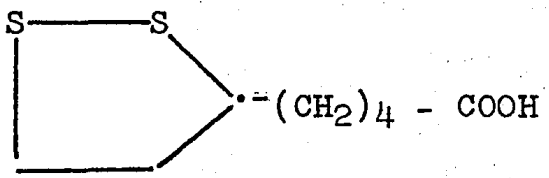
After desulfurization of lipoic acid, Reed et al (1952) established that its carbon skeleton is a straight eight-carbon chain and that the lipoic acid molecule is the intramolecular disulfide of a mercaptooctanoic acid unsubstituted in the α and β position. In the same year, Brockman et al suggested the following structure:



They proposed the name thioctic acid for x = 2, as this value gave the more stable ring in constructed models. Bullock et al (1952) supported the hypothesis that the value of 2 for x might be the true value by determining the relative pyruvate oxidation activity of the three possible isomers:

Isomer	Relative POF activity
DL-6 Thioctic acid	100.0
DL-5 Thioctic acid	0.3
DL-4 Thioctic acid	0.1

These workers also synthesized lipoic acid as a yellow oil, but still could not state the position of the secondary sulfur unequivocally. It was Mislow and Meluch (1956) who established the absolute configuration of (+)-α-lipoic acid as following:



The optical activity of lipoic acid was described in detail by Walton et al (1955). They synthesized

(+), (-), and DL-α-lipoic acid and showed that in the pyruvate oxidase assays, the activity of the (+)- form was double that of DL-α-lipoic acid, while the activity of the (-)-α-lipoic acid was essentially zero. Their conclusion was that (+)-α-lipoic acid is identical with the natural compound.

As mentioned earlier, lipoic acid received several names. Brockman et al (1954) have suggested that to clarify the nomenclature, the name Thioctic acid should be used for the synthetic material, and the names Protogen and Lipoic acid reserved for naturally-occurring materials possessing the appropriate biological activity.

Biological effects of lipoic acid.

As noted previously, microorganisms such as Tetrahymena geleii, Butyribacterium rettgeri, and many lactic acid bacteria including Lactobacillus bulgaricus exhibit an absolute requirement for lipoic acid. Others such as Streptococcus faecalis and Lactobacillus delbruckii, require lipoic acid as a growth stimulant.

According to DeBusk and Williams (1955), lipoic acid stimulates growth and food utilization in chicks and rats. This was negated by Jowsey (1959).

In contrast with the fact that lipoic acid is necessary for the growth of several microorganisms and might influence positively the growth of other organisms as rats and chicks, it also has several specific inhibitory effects on growth and morphogenesis.

A marked antimitotic effect on the cells of the onion Allium cepa is obtained using lipoic acid (DiCarlo, 1957). This effect consists of a prophasic block accompanied by a pre-prophasic block. Growth of the filamentous fungus Allomyces nacrogymus is also inhibited by remarkably low concentrations of lipoic acid (Machlis, 1957).

Morphogenesis of amphibian embryos is affected by lipoic acid which strongly inhibits gastrulation and the closure of the neural tube (Brachet, 1962 and 1963). Its morphostatic effect is 100 times stronger than that of mercaptoethanol. Tail elongation is also inhibited. This effect which is specific to lipoic acid is also observed in the frog as well as in the Xenopus embryo.

The gastrulation and neurulation of chick embryos also is inhibited by lipoic acid (Pohl and Brachet, 1962). The embryos become fragile, suggesting a possible modification of the cellular membranes.

In sea urchin eggs, an animalizing effect is induced by oxidized lipoic acid and to a lesser extent by reduced lipoic acid (Runnstrom, 1956). During gastrulation, the morphogenetic movements of the echinoderm embryos are abnormal if earlier stages had been treated with lipoic acid (Wolfson and Fry, 1965).

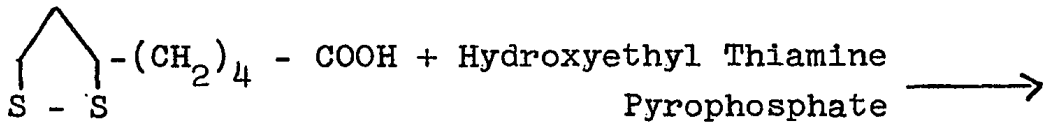
Lipoic acid acts as an inhibitor of the regenerative capacity of Hydra and Planaria. In Hydra, the blockage of regeneration induced by lipoic acid may be reversed by compounds which cause an increase in the number of tentacles (Spangenberg and Eakin, 1961). In Planaria, regeneration of the head is permanently blocked by lipoic acid, which does not affect the regeneration of the tail (Eakin and Henderson, 1959).

Aside from its effects on growth, morphogenesis and regeneration, lipoic acid also has a role in therapeutics as it protects against ionizing radiations (Genazzani et al, 1958 and 1959). Furthermore, lipoic acid has a protective action against potassium cyanide poisoning in mice (Cutolo and Reduzzi, 1956), muscular and liver damage by CCl_4 in mice and guinea pigs (Hashimoto, 1961), and heavy-metal intoxication in mice and dogs (Grumert, 1960).

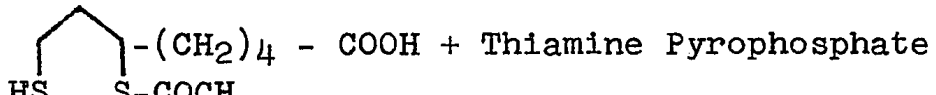
Role of lipoic acid in the oxidative decarboxylation of α-keto acids.

In the course of metabolism, α-keto acids are decarboxylated. In the case of pyruvic acid, the decarboxylation yields acetyl CoA which is an essential component of the Citric Acid Cycle and is also used in the synthesis of fatty acids and cholesterol and for acetylation reactions. The decarboxylation of each molecule of pyruvic acid yields four molecules of ATP.

The decarboxylation of pyruvic acid takes place in a multienzyme complex which contains lipoic acid in a protein-bound form. Due to its ability to interchange between the oxidized and the reduced form, lipoic acid acts as a coenzyme for the acetylation reaction:



(Oxidized lipoic acid)



(Acetyl lipoic acid)

The acetyl group is then transferred to CoA to give Acetyl CoA, and the reduced form of lipoic acid is reoxidized by FAD (Schmidt and Freiburg, 1965).

Materials and Methods

Cultures.

The complementary mating types A and B of *Paramecium multimicronucleatum* used in these investigations were obtained from the Carolina Biological Supply Company.

The paramecia were grown in hay infusion made as follows: 5 grams of dry timothy hay were boiled for 10 minutes in a pyrex beaker containing 1,000 cc of glass-distilled water. This infusion was then left uncovered for three days to allow bacterial growth. Enough glass-distilled water was then added to compensate for evaporation. The pH was subsequently adjusted to 7.0 with 1.0 and 0.1 N NaOH when necessary, and an inoculum of approximately 1,000 paramecia was introduced into the medium. The cultures were usually flourishing after 8-10 days and remained so for approximately 10 days before declining.

Light Cycle.

In order to get a strong mating reaction around noon, we maintained the paramecia cultures 14 inches from 100 watts of fluorescent light on a rhythm of 14 hours of light and 10 hours of darkness at 23° C. Under these conditions, mating competence appeared around the

middle of the light period, which we adjusted to occur at noon. In order to be sexually active the paramecia must also be starved for at least three days. In our cultures, mating competence appeared around the twelfth day after inoculation and remained for about five days.

Procedure.

Fission number.

Plastic boxes measuring 11.5 x 10.5 cm were used as moisture chambers in all the experiments. These boxes contained three pyrex glass depression slides, each with three 1 ml depressions.

Prior to an experiment, a stock solution of the reagent to be tested was made and the desired concentrations prepared from it. In all cases, three day old hay infusion was used as the solvent as it presumably contained enough bacteria to sustain good growth. Paramecia from a flourishing culture were then washed three times in one of the concentrations of reagent (by successive transfers of single paramecia with a micropipet in three depressions containing 0.8 ml of washing fluid). After washing, nine paramecia were isolated, each in a depression containing 0.8 ml of the same concentration of reagent as the washings. With this procedure, the initial concentration of reagent

was maintained in the depression even after the introduction of the cell.

The paramecia in each spot were counted 24 and 48 hours after isolation and the fission number was recorded, using the formula $n = 2^x$ where x is the fission number and n is the number of paramecia counted per depression. All the experiments were performed twice (therefore involving 18 cells), except the lipoic acid experiments which were performed four times (36 cells).

To test the reversibility of the effects induced by the different concentrations of a reagent, nine cells (usually one from each depression) were removed after 24 and 48 hour treatments of the reagent concentration to be tested, washed by successive transfer in three depressions containing 0.8 ml of normal medium and isolated, each in 0.8 ml of normal medium. The fission number was recorded after 24 and 48 hours in each case. The effects of a concentration of a reagent were considered reversible when the fission number of the cells was statistically comparable to that of the control. The control consisted of nine non-treated cells which were washed and isolated in the same normal medium as that used for the treated cells.

Statistical methods.

The mean fission number of the paramecia subjected to a certain concentration of a reagent was calculated by the following formula:

$$\overline{X} = \frac{1}{n} \sum f_i x_i$$

where $\overline{X}$ is the mean fission number, n is the total fission numbers recorded (usually 18), $\sum$ is the sum of f_i, the frequency of each fission number, times its value, x_i.

The standard deviation (S.D.) of the mean was then calculated by the following formula:

$$S.D. = \sqrt{\frac{1}{n-1} \sum (x_i - \overline{X})^2 f_i}$$

The mean fission number of the treated cells was then compared to that of the corresponding control to see if the difference was random or due to the reagent. The results were considered significantly different at the 5% level ($P \leq 0.05$).

Conjugation.

Before an experiment, the population density of the cultures of mating types A and B to be used was determined by the following method: several 0.1 samples of the well-mixed cultures were fixed with one

drop of 2% formaldehyde, and the number of paramecia counted. The population density per ml was calculated from the mean number of paramecia present in the samples. Then, the population density of the cultures A and B were made approximately equal by concentration of the less dense culture through centrifugation of this culture and removal of the necessary amount of fluid from the supernatant, followed by the resuspension of the cells in the new volume of fluid.

Our aim was to find the effects of different concentrations of each reagent on the mating reaction in the following conditions:

1. Reagent added to the paramecia two hours before conjugation.

2. Reagent added to the paramecia just before conjugation.

We placed a desired number of 4.5 ml aliquots of each culture A and B in 15 ml centrifuge tubes. One tube of each culture (A_1 and B_1) was kept without reagent, being the control. To a second tube of each culture (A_2 and B_2) we added 0.5 ml of the reagent to be tested, in a concentration ten times the desired one. The tubes were immediately mixed and the 1:10 dilution brought the concentration to the desired one.

This was done at 10:00 a.m., and the tubes were left until noon. A third tube of each culture (A_3 and B_3) was kept without reagent until noon, and the reagent was added as above at noon.

It is to be noted that all these procedures were carried under the same light which was used to keep the light cycle going.

At noon, the tubes were centrifuged at 1,000 rpm (160 g) for 1.5 minutes, and the cells collected from the bottom of the centrifuge tube in 0.5 ml of fluid. The cells were placed in depressions determined in advance.

Then, a number of drops known to contain approximately 500 paramecia was taken from cultures A and B having received the same treatment (A_1 and B_1, A_2 and B_2, A_3 and B_3), and these animals were mixed in a new depression.

The paramecia were examined every 15 minutes. The observations were recorded concerning the percentage of conjugation in each depression. This percentage was recorded using the following quantitative system:

Grade of the reaction	Percent of conjugation
I	0 - 25
II	25 - 50
III	50 - 75
IV	75 - 100

Reagents.

The following sulfhydryl reagents were used in the present investigations:

Lipoic acid in the oxidized form (Mann Research Laboratory).

Reducing reagents:

Cysteine (Mann Research Laboratory), reduced glutathione (General Biochemicals Inc.), mercaptoethanol (Calbiochem) and mercaptoethylgluconamide (Cyclo Chemical Corp.).

Oxidizing reagents:

Cystine (General Biochemicals Inc.), oxidized glutathione (General Biochemicals Inc.), dithiodiglycol (Calbiochem) and iodobenzoic acid (Columbia Organic Chemicals Co.).

Alkylating reagent:

N-ethylmaleimide (Columbia Organic Chemicals Co.).

Among the above substances, cysteine, reduced glutathione, mercaptoethanol and mercaptoethylgluconamide are freely soluble in distilled water and hay infusion. The other substances had to be dissolved in 0.2 ml of 1.0 N NaOH. This stock solution was then neutralized with HCl and diluted with hay infusion to

the desired concentration. The pH was adjusted to 7.0 $\pm$ 0.2 with a Metrohm pH meter. All solutions were prepared immediately prior to use.

Results

The effects of sulfhydryl reducing reagents on the fission number.

As shown in Tables I and II, cysteine and reduced glutathione affect the fission number at similar concentrations. At 10^{-2} M which is the highest non-lethal concentration for both, fission is inhibited. No significant inhibition of fission is caused by lower concentrations. The inhibition caused by the two reagents at 10^{-2} M is completely reversible after 24 and 48 hour treatments.

Mercaptoethanol also inhibits fission at 10^{-2} M. But the inhibition is total, and the fission number remains lower than in the control after removal of the cells from the reagent (Table III). At 10^{-3} M, fission is also inhibited but in a completely reversible manner.

Mercaptoethylgluconamide is a substance which presumably does not penetrate the cells because of the gluconamide group. Table IV shows that this substance inhibits fission at 10^{-1} M and 5×10^{-2} M. The fission number of the cells subjected to 10^{-1} M remains significantly lower than that of the control after removal from the reagent while the cells subjected to 5×10^{-2} M recover completely when placed in normal medium. No

visible effects are noted when the cells are subjected to a concentration of 10^{-2} M, contrary to results for the above reducing reagents.

The effects of sulfhydryl oxidizing reagents on the fission number.

Among the four oxidizing reagents used, cystine, oxidized glutathione and dithiodiglycol did not affect the fission number at concentrations below and including 10^{-4} M (Tables V, VI, and VII). Higher concentrations could not be used because of the difficulty of dissolving these substances.

Iodobenzoic acid inhibits fission at 10^{-2} M (Table VIII). After 24 hour treatments, the cells placed in normal medium recover completely while the cells treated for 48 hours remain significantly inhibited after removal from the reagent.

The effects of an alkylating reagent on the fission number.

N-ethylmaleimide inhibits fission at 5×10^{-3} M (Table IX). This inhibition is reversible after 24 hour treatments while the fission number of cells treated for 48 hours remains significantly lower than that of the control after removal from the reagent.

The effects of lipoic acid on the fission number.

A low concentration of lipoic acid (2×10^{-5} M or 4 µg/ml) increases the fission number of the cells while higher concentrations (5×10^{-4} M or 100 µg/ml and more) have the opposite effect (Fig. 1). The inhibiting action of lipoic acid increases with increasing concentrations from 5×10^{-4} M to 1.75×10^{-3} M (350 µg/ml). The concentrations of 1.75×10^{-3} M and 3×10^{-3} M (500 µg/ml) block fission completely.

After 24 hour treatments, the effects are reversible for all the concentrations, but the cells which had been subjected to 1.75×10^{-3} M and 3×10^{-3} M recover completely only after the first 24 hours (Fig. 2).

If the cells are left in lipoic acid for 48 hours, it is again those which were subjected to 1.75×10^{-3} M and 3×10^{-3} M which show a long-lasting effect. About 19% and 50% of the cells from 1.75×10^{-3} M and 3×10^{-3} M respectively die during the 24 first hours. The others remain inhibited during the same 24 hours. Subsequently, they divide at different rates. Approximately one third of the paramecia of each group remains inhibited. Another third of the cells of each group recovers completely and has a fission number of the

same order as the control, while the last third of the cells divides at a higher fission number than the control. Because of this difference of response of the cells, the mean fission number of the live cells in the two groups is comparable to that of the control but with a larger standard deviation (Fig. 3).

Figure 5 summarizes the results of this section and shows the reagents which significantly affect fission in order of decreasing effectiveness. Only the effective concentrations of each reagent are plotted with their corresponding control.

Effects of the sulfhydryl reagents on conjugation.

Mercaptoethanol at 10^{-2} M and mercaptoethylgluconamide at 10^{-1} M inhibit conjugation. In the two cases, inhibition of conjugation is total during 24 hours. Lower concentrations of the two reagents do not affect conjugation (Table X).

Lipoic acid has no effect on conjugation at concentrations below and including 5×10^{-4} M. Above this concentration, inhibition of conjugation increases with increasing concentrations of lipoic acid (Fig. 4).

Paramecia subjected to the above reagents for two hours before conjugation and paramecia to which the reagents were added just before conjugation showed no

difference in the effects observed.

None of the other sulfhydryl reagents used had any effect on conjugation.

In all the experiments, the control had a mating reaction of Grade IV (75 - 100% conjugation). Each concentration of each reagent was tested at least twice.

Table I. Animals were isolated in depressions containing different concentrations of cysteine. The total number of individuals were counted in each depression after 24 and 48 hours. The mean fission number and its standard deviation are given for each concentration and period of exposure. The asterisk indicates a significant difference from the control at the 5% level.

The recovery after the treatment was followed by transferring single cells to new medium after 24 and 48 hours exposure to the reagent, and observing the fission as above.

Table I.

The effect of several concentrations of cysteine on fission

Time of count	Fission number			
	Control	Concentration of cysteine		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	1.19 ± 0.14	1.20 ± 0.20	1.15 ± 0.12	*0.72 ± 0.19
48 h	2.71 ± 0.18	2.75 ± 0.24	2.57 ± 0.17	*1.78 ± 0.24

Recovery after 24 hour treatments.

Time of count	Fission number			
	Control	Concentration of cysteine		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	1.35 ± 0.19	1.37 ± 0.22	1.40 ± 0.18	1.35 ± 0.21
48 h	2.32 ± 0.24	2.29 ± 0.26	2.45 ± 0.21	2.32 ± 0.25

Recovery after 48 hour treatments.

Time of count	Fission number			
	Control	Concentration of cysteine		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	1.29 ± 0.17	1.31 ± 0.18	1.22 ± 0.20	1.35 ± 0.16
48 h	2.81 ± 0.23	2.70 ± 0.25	2.78 ± 0.22	2.69 ± 0.18

Table II. Animals were isolated in depressions containing different concentrations of reduced glutathione. The total number of individuals were counted in each depression after 24 and 48 hours. The mean fission number and the standard deviation are given for each concentration and period of exposure. The asterisk indicates a significant difference from the control at the 5% level.

The recovery after the treatment was followed by transferring the organisms to new medium after 24 and 48 hours exposure to the reagent, and observing the fission as above.

Table II.

The effect of several concentrations of reduced glutathione on fission

Time of count	Fission number			
	Control	Concentration of reduced glutathione		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	1.29 ± 0.15	1.31 ± 0.20	1.22 ± 0.19	*0.85 ± 0.12
48 h	3.81 ± 0.18	3.70 ± 0.24	3.78 ± 0.22	*2.59 ± 0.18

Recovery after 24 hour treatments.

Time of count	Fission number			
	Control	Concentration of reduced glutathione		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	1.92 ± 0.20	1.78 ± 0.20	1.89 ± 0.22	1.83 ± 0.19
48 h	2.97 ± 0.25	2.87 ± 0.23	2.92 ± 0.25	3.04 ± 0.22

Recovery after 48 hour treatments.

Time of count	Fission number			
	Control	Concentration of reduced glutathione		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	2.04 ± 0.17	2.10 ± 0.18	2.15 ± 0.21	2.12 ± 0.19
48 h	3.66 ± 0.21	3.59 ± 0.20	3.70 ± 0.23	3.54 ± 0.24

Table III. Animals were isolated in depressions containing different concentrations of mercaptoethanol. The total number of individuals were counted in each depression after 24 and 48 hours. The mean fission number and the standard deviation are given for each concentration and period of exposure. The asterisk indicates a significant difference from the control at the 5% level.

The recovery after the treatment was followed by transferring the cells to new medium after 24 and 48 hour exposure to the reagent, and observing the fission as above.

Table III.

The effect of several concentrations of mercaptoethanol on fission

Time of count	Fission number			
	Control	Concentration of mercaptoethanol		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	2.04 ± 0.19	1.92 ± 0.17	*1.16 ± 0.11	*0.00 ± 0.00
48 h	3.19 ± 0.22	3.32 ± 0.21	*2.35 ± 0.18	*0.00 ± 0.00

Recovery after 24 hour treatments.

Time of count	Fission number			
	Control	Concentration of mercaptoethanol		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	2.04 ± 0.14	2.00 ± 0.10	1.95 ± 0.18	*1.32 ± 0.09
48 h	3.00 ± 0.17	3.07 ± 0.20	3.15 ± 0.22	*1.62 ± 0.15

Recovery after 48 hour treatments.

Time of count	Fission number			
	Control	Concentration of mercaptoethanol		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	1.92 ± 0.19	2.00 ± 0.00	1.81 ± 0.15	*0.00 ± 0.00
48 h	2.87 ± 0.21	2.81 ± 0.11	2.57 ± 0.23	*0.26 ± C.09

Table IV. Animals were isolated in depressions containing different concentrations of mercaptoethylgluconamide. The total number of individuals were counted in each depression after 24 and 48 hours. The mean fission number and its standard deviation are given for each concentration and period of exposure. The asterisk indicates a significant difference from the control at the 5% level.

The recovery after the treatment was followed by transferring the cells to new medium after 24 and 48 hours exposure to the reagent, and observing the fission as above.

Table IV.

The effect of several concentrations of mercaptoethylgluconamide on fission

Time of count	Fission number				
	Control	Concentration of mercaptoethylgluconamide			
		10^{-3} M	10^{-2} M	5×10^{-2} M	10^{-1} M
24 h	2.00 ± 0.00	2.00 ± 0.00	1.95 ± 0.16	*1.35 ± 0.20	*0.53 ± 0.24
48 h	3.97 ± 0.06	3.96 ± 0.15	3.86 ± 0.21	*1.96 ± 0.18	*0.82 ± 0.19

Recovery after 24 hour treatments.

Time of count	Fission number				
	Control	Concentration of mercaptoethylgluconamide			
		10^{-3} M	10^{-2} M	5×10^{-2} M	10^{-1} M
24 h	2.59 ± 0.18	2.64 ± 0.17	2.50 ± 0.17	2.58 ± 0.22	*1.00 ± 0.00
48 h	4.05 ± 0.09	4.00 ± 0.00	3.95 ± 0.10	3.95 ± 0.20	*2.09 ± 0.04

Recovery after 48 hour treatments.

Time of count	Fission number				
	Control	Concentration of mercaptoethylgluconamide			
		10^{-3} M	10^{-2} M	5×10^{-2} M	10^{-1} M
24 h	1.48 ± 0.18	1.59 ± 0.23	1.32 ± 0.14	1.45 ± 0.21	*0.00 ± 0.00
48 h	2.81 ± 0.14	2.92 ± 0.09	2.73 ± 0.15	2.92 ± 0.09	*0.00 ± 0.00

Table V. Animals were isolated in depressions containing different concentrations of cystine. The total number of individuals were counted in each depression after 24 and 48 hours. The mean fission number and its standard deviation are given for each concentration and period of exposure.

Table V.

The effect of several concentrations of cystine on fission

Time of count	Fission number		
	Control	Concentration of cystine	
		10^{-5} M	10^{-4} M
24 h	1.64 ± 0.21	1.74 ± 0.20	1.67 ± 0.19
48 h	2.69 ± 0.25	2.59 ± 0.29	2.56 ± 0.26

Table VI. Animals were isolated in depressions containing different concentrations of oxidized glutathione. The total number of individuals were counted in each depression after 24 and 48 hours. The mean fission number and its standard deviation are given for each concentration and period of exposure.

Table VI.

The effect of several concentrations of oxidized glutathione on fission

Time of count	Fission number		
	Control	Concentration of oxidized glutathione	
		10^{-5} M	10^{-4} M
24 h	1.83 ± 0.24	1.86 ± 0.16	1.74 ± 0.21
48 h	3.32 ± 0.26	3.20 ± 0.25	3.39 ± 0.29

Table VII. Animals were isolated in depressions containing different concentrations of dithiodiglycol. The total number of individuals were counted in each depression after 24 and 48 hours. The mean fission number and its standard deviation are given for each concentration and period of exposure.

Table VII.

The effect of several concentrations of dithiodiglycol on fission

Time of count	Fission number		
	Control	Concentration of dithiodiglycol	
		10^{-5} M	10^{-4} M
24 h	1.52 ± 0.16	1.58 ± 0.18	1.41 ± 0.16
48 h	3.40 ± 0.23	3.58 ± 0.27	3.26 ± 0.24

Table VIII. Animals were isolated in depressions containing different concentrations of iodobenzoic acid. The total number of individuals were counted in each depression after 24 and 48 hours. The mean fission number and its standard deviation are given for each concentration and period of exposure. The asterisk indicates a significant difference from the control at the 5% level.

The recovery after the treatment was followed by transferring the cells to new medium after 24 and 48 hour exposure to the reagent, and observing the fission as above.

Table VIII.

The effect of several concentrations of iodobenzoic acid on fission

Time of count	Fission number			
	Control	Concentration of iodobenzoic acid		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	1.64 ± 0.24	1.64 ± 0.24	1.64 ± 0.24	*1.00 ± 0.00
48 h	3.15 ± 0.17	3.18 ± 0.17	3.42 ± 0.23	*2.64 ± 0.13

Recovery after 24 hour treatments.

Time of count	Fission number			
	Control	Concentration of iodobenzoic acid		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	1.32 ± 0.12	1.32 ± 0.11	1.43 ± 0.12	1.22 ± 0.16
48 h	2.81 ± 0.20	2.83 ± 0.15	2.98 ± 0.09	2.60 ± 0.20

Recovery after 48 hour treatments.

Time of count	Fission number			
	Control	Concentration of iodobenzoic acid		
		10^{-4} M	10^{-3} M	10^{-2} M
24 h	1.32 ± 0.11	1.22 ± 0.14	1.36 ± 0.13	*0.81 ± 0.08
	2.76 ± 0.16	2.83 ± 0.13	2.59 ± 0.21	*2.09 ± 0.17

Table IX. Animals were isolated in depressions containing different concentrations of N-ethylmaleimide. The total number of individuals were counted in each depression after 24 and 48 hours. The mean fission number and its standard deviation are given for each concentration and period of exposure. The asterisk indicates a significant difference from the control at the 5% level.

The recovery after the treatment was followed by transferring the cells to new medium after 24 and 48 hours exposure to the reagent, and observing the fission as above.

Table IX.

The effect of several concentrations of N-ethylmaleimide on fission

Time of count	Fission number			
	Control	Concentration of N-ethylmaleimide		
		10^{-4} M	10^{-3} M	5×10^{-3} M
24 h	1.92 ± 0.18	2.15 ± 0.17	2.00 ± 0.00	*1.22 ± 0.16
48 h	3.51 ± 0.28	3.59 ± 0.25	3.66 ± 0.22	*2.00 ± 0.00

Recovery after 24 hour treatments.

Time of count	Fission number			
	Control	Concentration of N-ethylmaleimide		
		10^{-4} M	10^{-3} M	5×10^{-3} M
24 h	2.22 ± 0.20	2.16 ± 0.19	2.32 ± 0.20	2.15 ± 0.19
48 h	3.42 ± 0.23	3.40 ± 0.24	3.52 ± 0.26	3.42 ± 0.17

Recovery after 48 hour treatments.

Time of count	Fission number			
	Control	Concentration of N-ethylmaleimide		
		10^{-4} M	10^{-3} M	5×10^{-3} M
24 h	2.20 ± 0.21	2.32 ± 0.20	2.15 ± 0.18	*1.59 ± 0.21
48 h	3.92 ± 0.11	4.00 ± 0.05	3.81 ± 0.23	*2.15 ± 0.18

Table X. Cells of mating type A and B were put in the desired concentration of the reagent to be tested before mixing. The sign "-" indicates an absence of effect of the corresponding concentration of the reagent, while the sign "+" indicates an inhibition of conjugation by the reagent.

Table X.

The relation between the effects on fission and conjugation of six sulfhydryl reagents

Reagent	Concentration allowing total recovery after 24 hour treatments	Effect on conjugation	Concentration having a long-lasting effect on fission after 24 hour treatments	Effect on conjugation
Cysteine	10^{-2} M	-		
Reduced glutathione	10^{-2} M	-		
Mercapto-ethanol	10^{-3} M	-	10^{-2} M	+
Mercaptoethyl-gluconamide	5×10^{-2} M	-	10^{-1} M	+
Iodobenzoic acid	10^{-2} M	-		
N-ethyl-maleimide	5×10^{-3} M	-		

Figure 1. Animals were isolated in depressions containing different concentrations of lipoic acid. The total number of individuals were counted in each depressions after 24 and 48 hours.

The vertical bars represent the standard deviation.

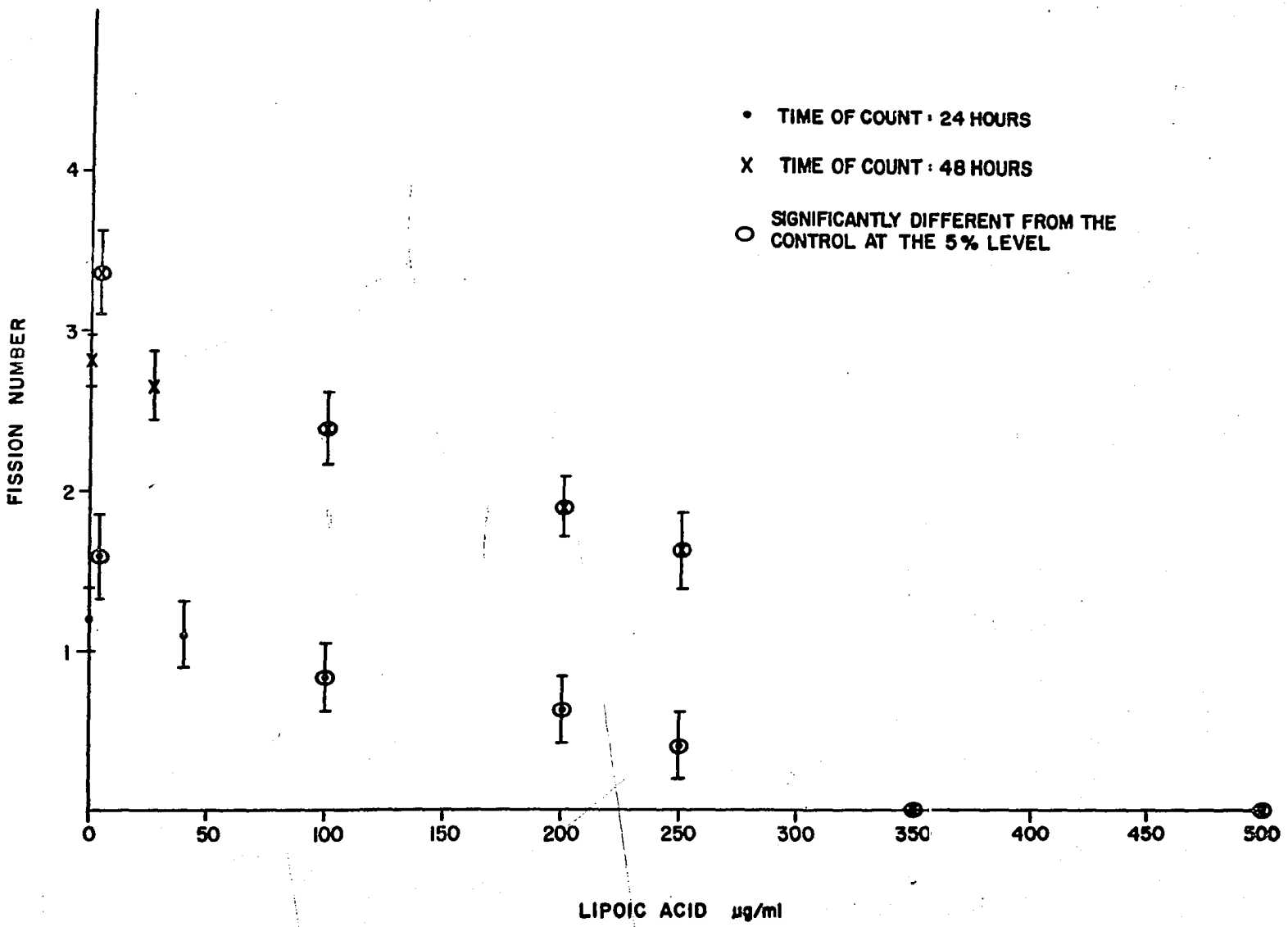

• TIME OF COUNT : 24 HOURS
X TIME OF COUNT : 48 HOURS
O SIGNIFICANTLY DIFFERENT FROM THE CONTROL AT THE 5% LEVEL
FISSION NUMBER
1
2
3
4
0
50
100
150
200
250
300
350
400
450
500
LIPOIC ACID µg/ml

Figure 2. Animals subjected to several concentrations of lipoic acid for 24 hours were transferred to new medium. The total number of individuals were counted in each depression after 24 and 48 hours.

The vertical bars represent the standard deviation.

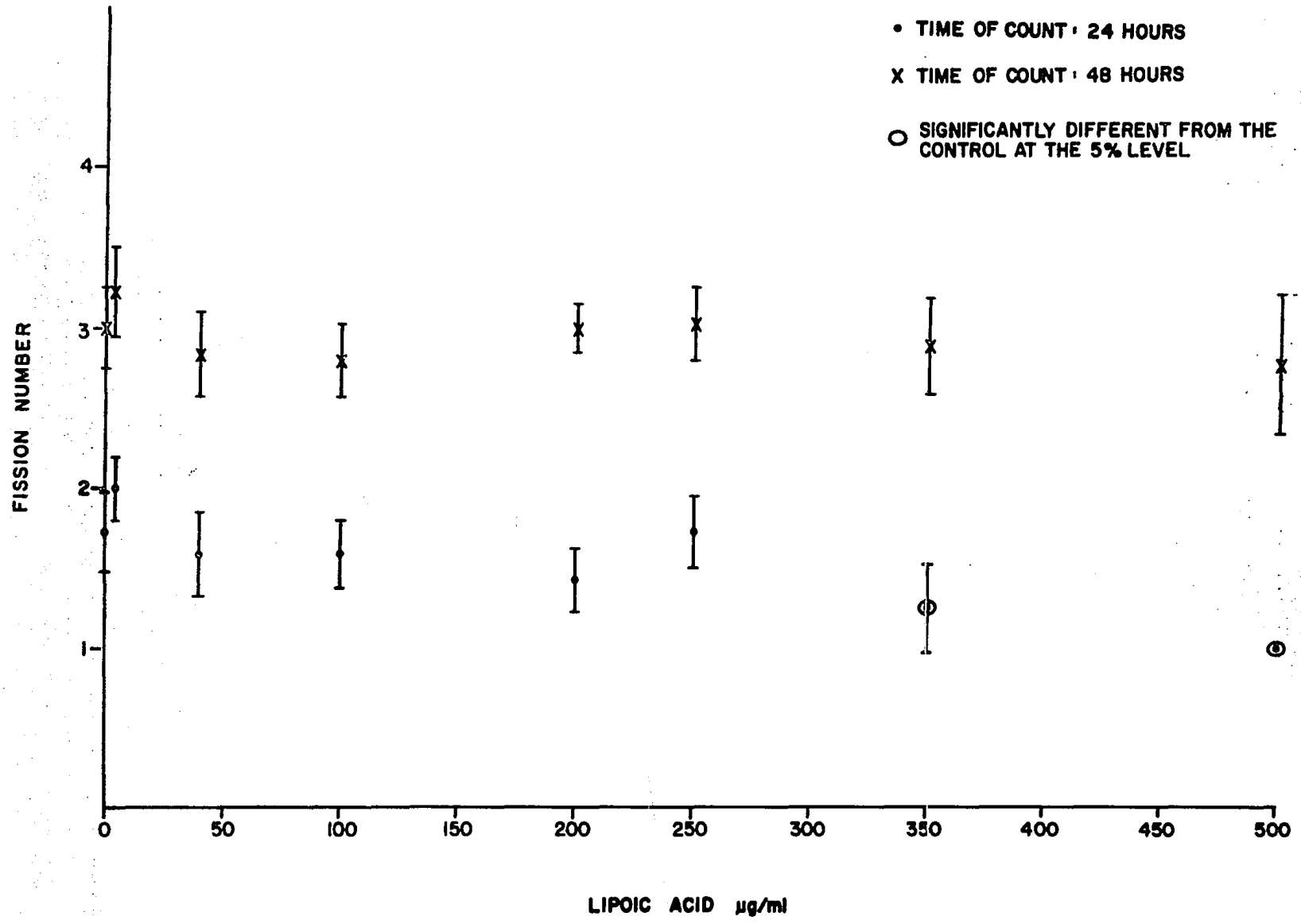

• TIME OF COUNT : 24 HOURS
X TIME OF COUNT : 48 HOURS
O SIGNIFICANTLY DIFFERENT FROM THE CONTROL AT THE 5% LEVEL
FISSION NUMBER
1
2
3
4
0
50
100
150
200
250
300
350
400
450
500
LIPOIC ACID µg/ml

Figure 3. Animals subjected to several concentrations of lipoic acid during 48 hours were transferred to new medium. The total number of individuals were counted in each depression after 24 and 48 hours. The percentage of dead cells found after 24 hours was also counted.

The vertical bars represent the standard deviation.

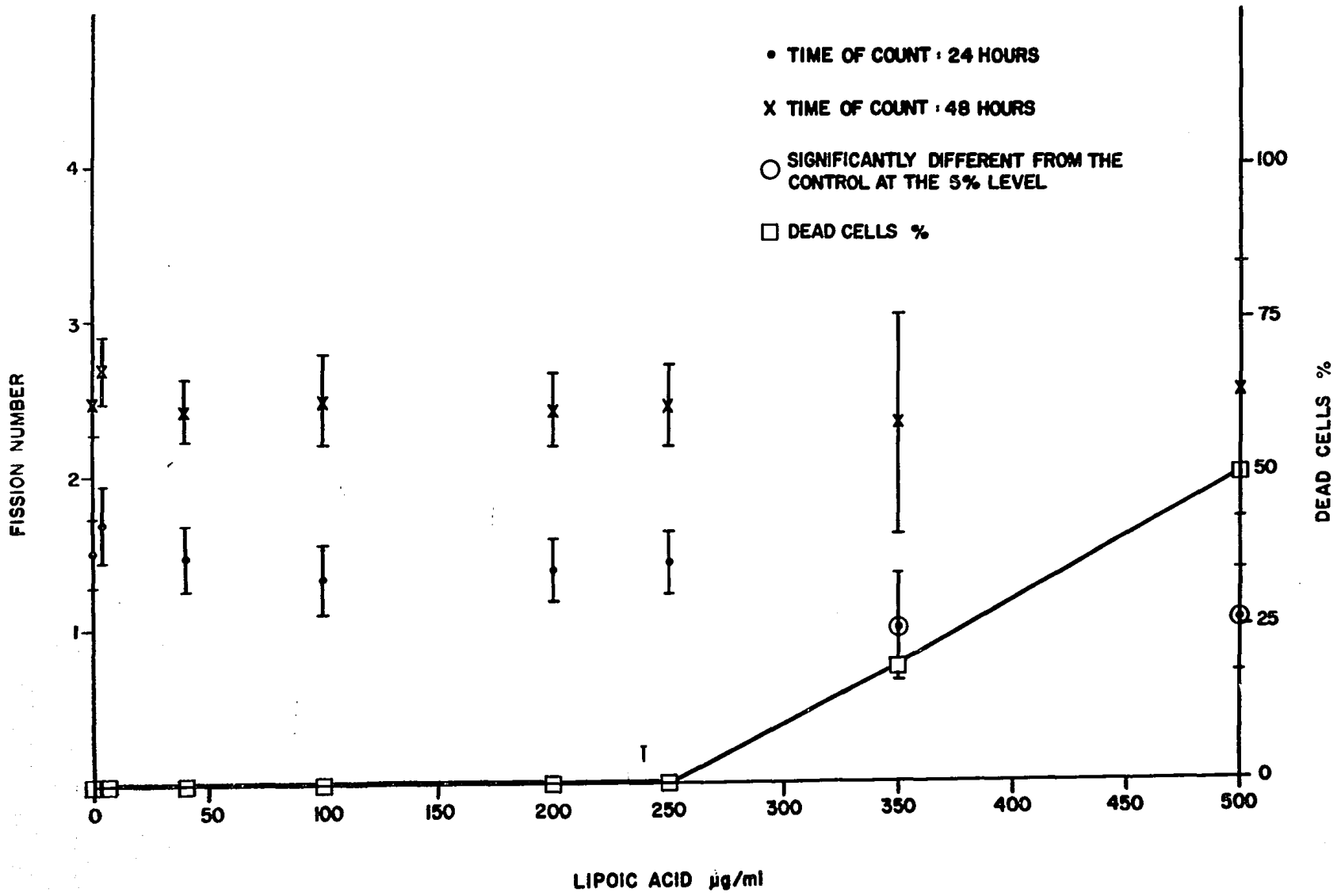

• TIME OF COUNT : 24 HOURS
X TIME OF COUNT : 48 HOURS
○ SIGNIFICANTLY DIFFERENT FROM THE CONTROL AT THE 5% LEVEL
□ DEAD CELLS %
FISSION NUMBER
DEAD CELLS %
LIPOIC ACID µg/ml
0
1
2
3
4
0
25
50
75
100
0
50
100
150
200
250
300
350
400
450
500

Figure 4. Cells of mating type A and B were subjected to several concentrations of lipoic acid and mixed. The percentage of conjugation was recorded. A reaction of Grade I represents a percentage of conjugation between 0 and 25. Similarly, Grades II, III and IV represent respectively a percentage of conjugation of 25 to 50, 50 to 75 and 75 to 100.

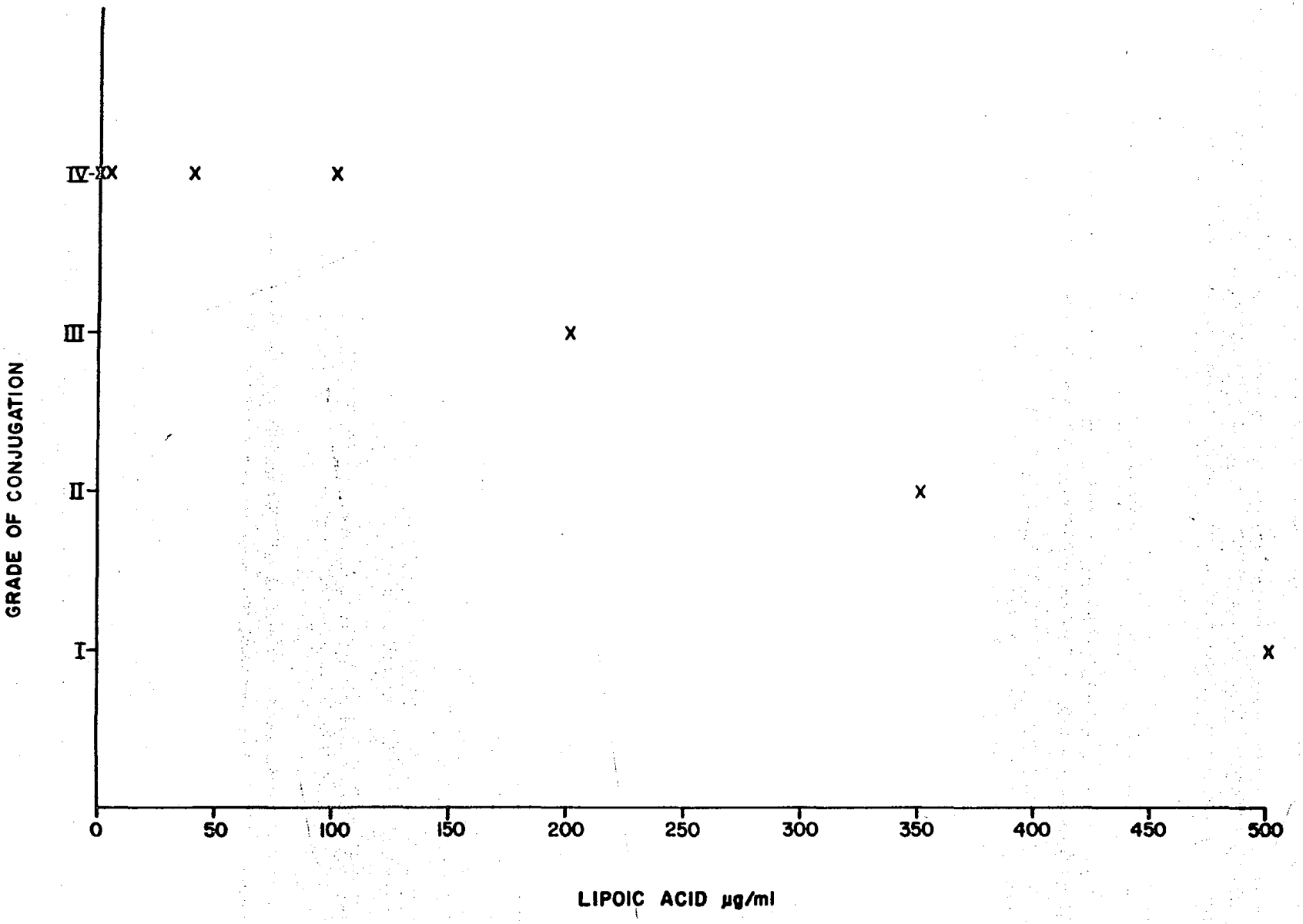
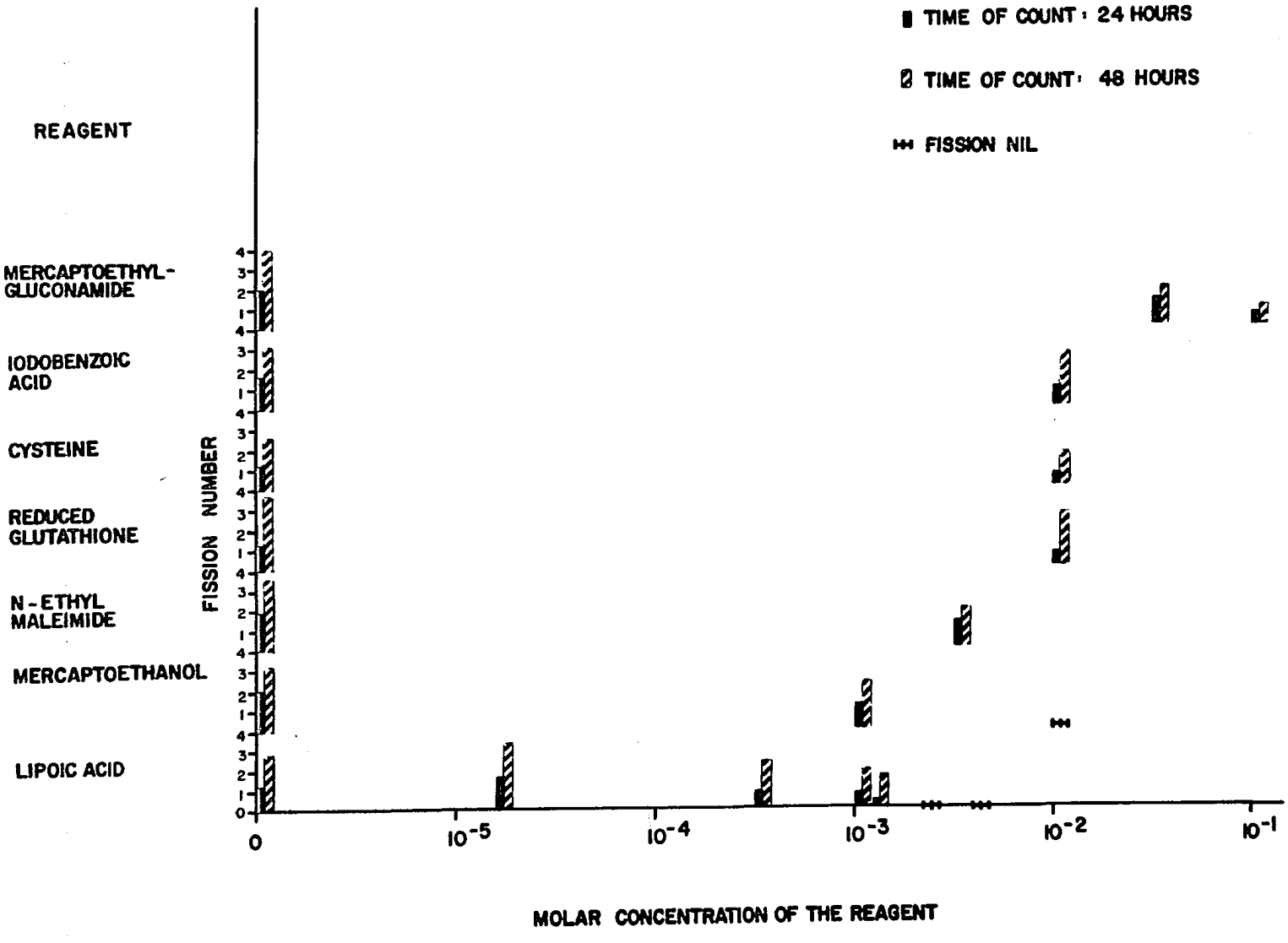

GRADE OF CONJUGATION
IV
III
II
I
0
50
100
150
200
250
300
350
400
450
500
LIPOIC ACID µg/ml

Figure 5. This figure is an attempt to summarize the effects of the sulfhydryl reagents tested on fission. Only the effective concentrations of the reagents are plotted, together with their corresponding control.


TIME OF COUNT : 24 HOURS
TIME OF COUNT : 48 HOURS
FISSION NIL
REAGENT
MERCAPTOETHYL-GLUCONAMIDE
IODOBENZOIC ACID
CYSTEINE
REDUCED GLUTATHIONE
N-ETHYL MALEIMIDE
MERCAPTOETHANOL
LIPOIC ACID
FISSION NUMBER
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
MOLAR CONCENTRATION OF THE REAGENT

Discussion

Fission.

In normal conditions of temperature and pH, the fission number of Paramecium multimicronucleatum varies between one and two per 24 hours, depending on the amount of food available. The following sulfhydryl reagents affect the fission number of the cells, and are listed in order of decreasing effectiveness: lipoic acid, mercaptoethanol, N-ethylmaleimide, reduced glutathione, cysteine, iodobenzoic acid and mercaptoethylgluconamide. The decreasing effectiveness of these reagents may be partly due to the degree of permeability of the cell membrane to each one of them. In fact, the less effective reagent, mercaptoethylgluconamide, presumably cannot enter the cell because of the gluconamide group of its molecule, while the more effective reagent, lipoic acid, being a liposoluble substance, is likely to penetrate easily into the cell.

The first site which comes in contact with the reagents is the cell membrane which covers the body of the paramecium. Like other membranes, the cell membrane is a complex of lipids and proteins (Sjostrand, 1967), the latter containing sulfhydryl groups (Rothstein and Weed, 1963). Sulfhydryl reagents are

known to disturb cell membrane functions. For example, mercury and chlormerodrin cause an inhibition of sugar transport, a specific increase in K^+ permeability with no change in permeability to Na^+, a general increase in cation permeability and a non-specific increase in permeability (Rothstein and Weed, 1963). Although no two reagents necessarily attack the same sulfhydryl groups because of the large difference of reactivity of these groups, it is possible that each of the sulfhydryl reagents we used in the present work affect the paramecium membrane by altering its permeability to one or more species of ions or molecules.

If these alterations occur in paramecium, they seem to be immediately reversible in all cases except with 10^{-1} M mercaptoethylgluconamide and 10^{-2} M mercaptoethanol where the effects of the reagents last during at least 48 hours following the treatment. In the case of mercaptoethylgluconamide where the effects of the reagent are primarily if not entirely on the cell membrane, the long-lasting effects may be due to the high concentration of the molecules which could react with sluggish sulfhydryl groups after saturation of the reactive sites. Disturbance of the sluggish groups would be more difficult to reverse. Mercaptoethanol at 10^{-2} M might also attack masked membrane

sulfhydryl groups, but the long-lasting effects of this reagent may be due to a similar intracellular action as well. Lipoic acid, aside from its action as a sulfhydryl reagent, could affect the membrane by dissolving in its lipid phase.

Alteration of membrane permeability would influence the cell metabolism indirectly by allowing free penetration in the cell of some ions or molecules which are usually kept outside selectively. By the same mechanism, ions or molecules which might be necessary for the cell would be able to cross out the membrane or might be prevented from getting into the cell. A change in cell membrane permeability of amphibian blastulae and gastrulae is induced by mercaptoethanol which causes a collapse of the blastocoele (Brachet and Delange-Cornil, 1959). In paramecium, a change in membrane permeability, by influencing indirectly the cell metabolism, would disturb life processes in general, and therefore also fission.

When they have crossed the cell membrane, the reagents may attack numerous sites inside the cell. The role of disulfide bonds in the stability of the mitotic apparatus was demonstrated by Mazia and Dan (1952). Introduction of reducing substances such as

cysteine, glutathione or mercaptoethanol in the cytoplasm might inhibit the spindle formation by preventing the formation of the spindle disulfide linkages or breaking these linkages if they are formed, thereby causing a disorganization of the spindle. In Paramecium multimicronucleatum, four spindles form during prophase. Although the spindle size is small, it is possible that blockage of the four spindles would influence fission. Studies at the microscopic level of treated paramecia might tell us if spindle disorganization occurs in the cells under the influence of the reducing reagents used. In sea urchin eggs, where the spindle size is very large as compared to the micronuclear spindles of paramecium, mercaptoethanol inhibits mitosis. Its effect seems to involve a loosening in the spindle structure which appears highly disorganized at the microscopic level (Mazia and Zimmerman, 1958). This disorganization is completely reversible for the sea urchin eggs and might be so in the case of paramecium, where the effects of glutathione and cysteine at 10^{-2} M and mercaptoethanol at 10^{-3} M are reversible.

Other possible mechanisms by which the sulfhydryl reagents might influence cell division are: reduction or oxidation of otherwise oxidized or reduced

sulfhydryl groups on enzymes having a catalytic function in providing the energy necessary for the mitotic process; inhibition of enzymes requiring a redox potential within fixed limits for normal activity. Inhibition of these enzymes might induce a disturbance in the general metabolism and influence mitosis indirectly. Interference with the balance between the oxidized and the reduced forms of coenzymes such as DPN, TPN and lipoic acid might also influence mitosis indirectly.

These effects are likely to be reversible by the reestablishment of the normal redox potential, and may partly account for the reversible inhibition of fission induced by glutathione and cysteine at 10^{-2} M, mercaptoethanol at 10^{-3} M and lipoic acid above 10^{-4} M. Iodobenzoic acid and N-ethylmaleimide might also affect the cells by the same mechanisms. The difference in the reactivity of the cellular sulfhydryl groups may account for the slow reversibility of the effects of 48 hour treatments of the two reagents: the molecules of iodobenzoic acid and N-ethylmaleimide might attach to hindered sulfhydryl groups which would be indispensable to the cell on a long range basis. These groups might be unattainable by the other sulfhydryl reagents cited above.

N-ethylmaleimide which is an alkylating agent and presumably remains attached to the blocked sites was expected to have stronger effects than iodobenzoic acid which may detach from the binding sites. The lack of difference may be due to the slowness of the alkylation reaction or to the dissociation of the N-ethylmaleimide complex in vivo.

Oxidized lipoic acid, in addition to the mechanisms discussed above, is likely to affect the fission indirectly by causing a disequilibrium between the free and bound form as well as between the oxidized and reduced form of the substance within the oxidative decarboxylation complex.

Lipoic acid might also influence nucleic acid incorporation. Heilporn-Pohl and Quertier (1964) studied the metabolism of nucleic acids under the influence of lipoic acid, using labelled precursors, in amphibian and chick embryos. They reported that lipoic acid inhibits 20 to 50% of uridine and cytidine incorporation but does not influence the incorporation of desoxyuridine in the embryos. According to the authors, lipoic acid might inhibit pentose reduction. The thymidine incorporation appears to be complementary to that of uridine and cytidine. A profound disturbance

of DNA metabolism seems to occur. Autoradiographic studies using the same labelled precursors as those of the above work would tell us whether lipoic acid influences nucleic acid incorporation in Paramecium multimicronucleatum.

Lipoic acid has been reported as a growth factor for many microorganisms. In fact, very low concentrations of the substance (4 µg/ml) enhance growth of our cells. Lipoic acid might therefore be a growth factor for paramecium as well. This effect of lipoic acid may be related to its role as a cofactor in the oxidative decarboxylation complex: enhancement of the functioning of this complex would result in an increase in the rate of general metabolism, and therefore in the fission rate. The enhancing effect of lipoic acid on growth does not last after removal of the cells from the medium containing 4 µg/ml of lipoic acid. This indicates that a continuous supply of the factor must take place in order to allow increased growth.

In considering the results given by the different reagents discussed on the fission number of paramecia, we must not forget that the cells we are dealing with are not synchronized, as no successful methods for synchronizing paramecia have yet been found (Whitson,

1964). At any given moment, each cell was at its own mitotic phase. Although we tried to deal with cells of approximately the same size during the initial single isolations, we may have isolated in adjacent spots, a cell which was in prophase and one which was in interphase. It is evident that after 24 hours, the first cell would probably have a higher fission number than the second cell. This would have some influence on the standard deviations of fission numbers.

Because of the non-synchrony of the cells, we were not able to determine the sensitive period of the cell cycle at which the effects of sulfhydryl reagents were strongest. In synchronized cultures of Tetrahymena pyriformis, the effects of mercaptoethanol on cell division are strongest during a sensitive peak which occurs aroung 40 minutes after the last heat shock (Mazia and Zeuthen, 1966).

Other sources of variation include the bacterial flora of the cultures which may have influenced the sulfhydryl-disulfide balance of the experimental milieu. Also, the amount of bacteria varying from an experiment to another, the fission number may have been influenced by the amount of bacteria which served as food. It is also probable that the reducing reagents were increasingly oxidized by air during the experiments, although the

redox potential was not measured.

Conjugation.

At concentrations which affect fission in an immediately reversible manner, none of the sulfhydryl reagents except lipoic acid has an influence on conjugation. This is supported by the conclusions of Metz (1954) who reported that oxidizing and reducing substances do not affect mating reactivity in Paramecium calkinsi. He concluded that mating type substances probably contained proteins but that sulfhydryl and disulfide groups were not essential to their activity. The effects produced on conjugation by mercaptoethylgluconamide and mercaptoethanol at concentrations having a long-lasting effect on fission, and by lipoic acid above 5×10^{-4} M, must therefore be due to action on another site than the mating substances.

As discussed in the section on fission, sulfhydryl reagents may alter the permeability of the cell membrane. We hypothesized that mercaptoethylgluconamide and mercaptoethanol at high concentrations would react with sluggish sulfhydryl groups. It is possible that reaction with sluggish sulfhydryl groups would cause a larger disturbance of membrane functions and a more profound alteration of membrane permeability than reaction with reactive sulfhydryl groups. As a

mating inhibitor substance is known to be present in the paramecia (Metz and Butterfield, 1950), alteration of the membrane permeability by mercaptoethylgluconamide and mercaptoethanol might allow the inhibitor molecules to move to the outside of the cell membrane where they would combine with the molecules of the mating substance and prevent mating. Conversely, the molecules of the mating substance, instead of staying on the cilia of the cells, might migrate inside, thereby inhibiting mating. The mating substances contain proteins and are therefore complex molecules of relatively large size. The inhibitor molecules may also be proteins, although their chemical properties have not yet been described. Here appears the necessity of a profound alteration of the membrane permeability which would enable these molecules to cross the membrane. The reason why the other reagents used, as well as lower concentrations of mercaptoethylgluconamide and mercaptoethanol, do not affect conjugation would therefore be an insufficient increase in membrane permeability.

The increasing inhibition of conjugation by increasing concentrations of lipoic acid may also be explained in regard of an alteration of membrane permeability.

If lipoic acid affects the membrane structure by

dissolving in its lipid phase, the structural change would be proportional to the amount of lipoic acid dissolved in the lipid phase of the membrane. If the increase in permeability of the membrane is proportional to the structural change induced by lipoic acid, the number of molecules of the mating substance able to cross in or the number of inhibitor molecules able to move out of the membrane would be proportional to the permeability, and therefore to the lipoic acid concentration.

Alteration of membrane permeability would then appear to be a relatively fast process, as no difference is seen between the percentage of conjugation of the paramecia treated for two hours before conjugation and the paramecia placed in the reagent just before conjugation.

To test our interpretation of mating inhibition, it would be necessary to have a method which would enable us to locate the mating type and the inhibitor molecules in the cell. But this direct approach is still impossible as no structural or specific histochemical methods are yet known for the mating type and inhibitor molecules. To overcome this difficulty, one could induce the formation of labelled antibodies specific for the mating-type and inhibitor molecules, and

subject the paramecia to each of these labelled antibodies before and after the treatment with the reagents which inhibit conjugation. The location of these antibodies would tell us if the mating type or the inhibitor molecules have migrated through the membrane under the influence of the reagents tested.

If our interpretation is negated by the above experiments, one could attempt to relate the effects of the inhibiting reagents with a disturbance of the nucleic acid metabolism, such as has been reported in the case of lipoic acid (Heilporn-Pohl and Quertier, 1964) and of mercaptoethanol (Quertier, 1962). The effects of mercaptoethylgluconamide which presumably cannot penetrate into the cell are unlikely to be due to an intracellular mechanism, although they may have an indirect influence on metabolism.

Summary

Lipoic acid and a range of sulfhydryl reagents were tested for their effects on the fission number and conjugating ability of Paramecium multimicronucleatum. Lipoic acid was given special attention because of its specific biological effects on many organisms.

It was found that the reagents affect fission in the following order of decreasing effectiveness: lipoic acid, mercaptoethanol, N-ethylmaleimide, reduced glutathione, cysteine, iodobenzoic acid and mercaptoethylgluconamide. The effects of these reagents on fission are immediately reversible after 24 hour treatments with each concentration used, except for 10^{-1} M mercaptoethylgluconamide and 10^{-2} M mercaptoethanol. The significance of these results is discussed in relation to the possible sites of cellular action of these reagents.

Conjugation is inhibited by the same concentration of mercaptoethylgluconamide and mercaptoethanol which has a long-lasting effect on fission. The inhibition of conjugation by lipoic acid increases with increasing concentrations of the substance, starting at 5×10^{-4} M. The other sulfhydryl reagents have no

effect on conjugation. It is hypothesized that the common site of action of the three reagents which inhibit conjugation is the structure of the cell membrane. The implications of this hypothesis are discussed.

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