EFFECTS OF CAFFEINE AND OTHER METHYLXANTHINES ON THE DEVELOPMENT AND METABOLISM OF SEA URCHIN EGGS

Involvement of $NADP^+$ and Glutathione

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ABSTRACT

Methylxanthines (MX) inhibit cell division in sea urchin and clam eggs. This inhibitory effect is not mediated via cAMP. MX also inhibit respiration in marine eggs, at concentrations which inhibit cleavage. Studies showed that no changes occurred in ATP and ADP levels in the presence of inhibitory concentrations of MX, indicating an extra-mitochondrial site of action for the drug. Subsequent studies revealed decreased levels of $NADP^+$ and NADPH, when eggs were incubated with inhibitory concentrations of MX, but no change in levels of NAD^+ and NADH. MX did not affect the pentose phosphate shunt pathway and did not have any effect on the enzyme NAD^+-kinase. Further studies showed a marked inhibitory effect on the glutathione reductase activity of MX-treated eggs. Reduced glutathione (GSH) could reverse the cleavage inhibitory effect of MX. Moreover, diamide, a thiol-oxidizing agent specific for GSH in living cells, caused inhibition of cell division in sea urchin eggs. Diamide added to eggs containing mitotic apparatus (MA) could prevent cleavage by causing a dissolution of the formed MA. Both MX and diamide inhibit a Ca^{2+}-activated ATPase in whole eggs. The enzyme can be reactivated by sulfhydryl reducing agents added in the assay mixture. In addition, diamide causes an inhibition of microtubule polymerization, reversible with dithioerythritol. All experimental evidence so far suggests that inhibition of mitosis in sea urchin eggs by MX is mediated by perturbations of the in vivo thiol-disulfide status of target systems, with a primary effect on glutathione levels.

Our earlier studies have shown that neither cAMP nor its butyrated derivatives, at concentrations up to 2×10^{-2} M, affect early cleavage of sea urchin or clam eggs, at least through the late gastrulation and feeding stages (47). Incorporation experiments with both radioactive cAMP and dibutyryl cAMP demonstrated uptake of exogenously added nucleotides in sea urchin eggs. Identification and quantitation of the intracellular compounds showed that intracellular levels of cAMP or N^6-monobutyryl cAMP (the converted intracellular product of dibutyryl cAMP) reached at least 10^{-5} M by first cleavage (at an external concentration of 10^{-3} M), two orders of magnitude higher

than the cAMP levels normally found in sea urchin eggs (38, 39). These observations indicated that, unlike the results in some higher organisms, cAMP does not participate in regulation of mitosis in early cleavage stages of sea urchin or clam eggs.

However, caffeine, aminophylline and methyl isobutylxanthine, all reversibly inhibit mitosis in sea urchin and clam eggs by causing a shrinkage of the mitotic apparatus and, at high enough concentrations, cause its disappearance. They were also shown to be inhibitors of phosphodiesterases in sea urchin eggs and to cause an increase in intracellular cAMP (38–40). Since our incorporation experiments made it unlikely that methylxanthines (MX) inhibited mitosis by acting through cAMP, we began a more detailed study of other possible effects which might lead to the understanding of their antimitotic effects.

As early as 1945, Cheney reported an inhibition of respiration in sea urchin eggs in the presence of caffeine, with mitotic inhibition being proportional to the deficit in O_2 uptake. Complete inhibition of cleavage occurred when egg respiration was decreased to 50% of normal (6). The present work is an extension of Cheney's original observations, which we readily confirmed. Further biochemical studies were undertaken to explore the relation of inhibition of oxygen uptake to inhibition of cell division by MX and the results are presented in this paper.

MATERIALS AND METHODS

Eggs of *Strongylocentrotus purpuratus* were used in the present study. The sea urchins were purchased from Pacific Bio-Marine Supply Co., Venice, Calif., and maintained at 10–12°C in an aquarium. The various nucleotides, enzymes, and biochemicals used in this study were obtained from either Sigma Chemical Co., St. Louis, Mo., or from Boeringer Mannheim Corp., New York. All other chemicals were of reagent grade.

Eggs of *S. purpuratus* were collected and washed as described previously (38). The eggs were fertilized with an appropriately diluted suspension of fresh sperm and only those batches of eggs which showed 95–100% fertilization were used for experimental purposes.

O_2-Uptake Measurements

The eggs were fertilized in Millipore-filtered sea water containing 0.01% penicillin and streptomycin to avoid contamination by microorganisms. O_2 uptake was measured manometrically in a Warburg apparatus, using conventional procedures. Temperature was maintained at 20°C. The various MX were added to different samples of fertilized eggs, at concentrations at which they inhibited cleavage, and egg respiration was measured in experimentals and controls. Respiration was generally studied until first cleavage in controls (100–110 min after fertilization), after which each sample of eggs was examined under the microscope to ensure inhibition of cleavage.

Measurement of ATP

Intracellular concentration of ATP was measured enzymatically, according to the method described by Adam (1). The nucleotide was extracted with 0.5 N cold perchloric acid immediately before the assay. The assay involves phosphoglycerate kinase (PGK) in the following reaction:

$$\text{3-phosphoglycerate} + \text{ATP} \underset{}{\overset{\text{PGK, } Mg^{++}}{\rightleftharpoons}} \text{1,3,-diphosphoglycerate} + \text{ADP}.$$

The 1,3-diphosphoglycerate thus formed was then reduced by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and NADH, the indicator reaction being:

$$\text{1,3-diphosphoglycerate} + \text{NADH} + \text{H}^+ \underset{}{\overset{\text{GAPDH}}{\rightleftharpoons}} \text{glyceraldehyde-3-phosphate} + \text{NAD}^+ + \text{phosphate}.$$

Thus, the reaction can be followed by measuring the decrease in optical density at 340 nm, which stoichiometrically measures the concentration of ATP present in the added acid extract.

Measurement of ADP

ADP was also measured enzymatically by a method described by Adam (2). ADP present in the perchloric acid extract was stoichiometrically phosphorylated with phosphoenolpyruvate and pyruvate kinase. Pyruvate formed was then reduced with NADH and lactic dehydrogenase. Again, the reaction was followed by oxidation of NADH at 340 nm.

Measurement of NAD^+, NADH, $NADP^+$, NADPH

The pyridine nucleotides were measured enzymatically, using principles and methods as described by Klingenberg (26). NAD^+ and $NADP^+$ were extracted with 0.5 M perchloric acid. NADH and NADPH need very careful extraction with alcoholic KOH, since the reduced nucleotides are destroyed by acid conditions. For further details see Klingenberg (26).

NAD^+ was measured by following the reaction:

$$NAD^+ + \text{Ethanol} \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} \text{acetaldehyde} + NADH + H^+.$$

NADH was assayed by using the following enzymatic

reaction:

$$NADH^+ + H^+ + \text{pyruvate} \xleftrightarrow[\text{dehydrogenase}]{\text{lactic}} NAD^+ + \text{lactate}.$$

NADP was assayed by reducing it by glucose-6-phosphate and glucose-6-phosphate dehydrogenase (G6PDH), i.e. by the following reaction:

$$G6P + NADP^+ \xleftrightarrow{G6PDH} 6\text{-phosphogluconate} + NADPH + H^+.$$

This can be quantitatively measured by following the increase in optical density at 340 nm.

NADPH was assayed by the enzymatic reduction of α-oxoglutarate by glutamic dehydrogenase (GIDH) as represented in the following reaction:

$$\alpha\text{-oxoglutarate} + NH_4^+ + NADPH \xleftrightarrow{GIDH} \text{glutamate} + NADP^+$$

Thus, due to the characteristic extinction coefficients of reduced pyridine nucleotides at 340 nm, all the pyridine nucleotides could be conveniently assayed by following either decrease or increase in optical density at 340 nm.

The pentose phosphate shunt pathway (PPSP) enzymes were assayed spectrophotometrically by following standard enzymatic procedures as described in reference 26. A 100,000 *g* supernate of egg homogenate was used for the assay of these enzymes.

NAD^+ – Kinase Assay

Enzymatic activity was determined spectrophotometrically. NAD^+ – kinase catalyzes the following reaction:

$$NAD^+ + ATP \rightarrow NADP^+ + ADP.$$

Since divalent cations are necessary for enzymatic activity, the assay was performed in the presence of Mg^{++}. $NADP^+$ formed in the reaction was converted to NADPH by the addition of glucose-6-phosphate and glucose-6-phosphate dehydrogenase. Since the latter reaction was specific for $NADP^+$, there was a quantitative reduction of $NADP^+$ formed by NAD^+-kinase and, thus, it could be easily measured by following the increase in absorption at 340 nm. A 20,000 *g* supernate of egg homogenate was used as the source of the enzyme. Other details of the assay were as those described by Blomquist (4).

Glutathione Reductase Assay (GR)

The enzyme was assayed spectrophotometrically by following the rate of NADPH oxidation (i.e., a decrease in absorption at 340 μm) in the following reaction:

$$GSSG + NADPH \xleftrightarrow{(GR)} GSH + NAD^+.$$

The details of the assay are based on those described by Ii and Sakai (19, 20). The assay mixture contained EDTA (1 mM), GSSG (0.8 mM), NADPH (0.1 mM), and an appropriate amount of the enzyme in a final vol of 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.2.

Eggs were collected and fertilized as described above. In experiments with caffeine (and other methylxanthines) the eggs were allowed to develop in the presence of 10 mM caffeine for 90–100 min. Eggs were then collected by low-speed centrifugation and the packed eggs were homogenized in a suitable volume of ice-cold distilled water with an all glass Dounce homogenizer. The homogenate was checked under the microscope for complete egg breakage and then centrifuged at 100,000 *g* for 60 min. The postmicrosomal supernate obtained thereafter was used as the source of the enzyme. Activity was expressed as μmole of NADPH oxidized/mg protein/minute.

Glutathione Peroxidase Assay

This enzyme was assayed by coupling it with GR. The GSSG formed by the peroxidase was reduced by GR and NADPH. Oxidation of NADPH was followed by a decrease in absorbance at 340 nm. Other details were the same as those described by Hosoda and Nakamura (18).

Proteins were determined by the microbiuret method of Itzhaki and Gill (22), using bovine serum albumin as the standard. All spectrophotometric assays were performed in a Beckman Du spectrophotometer (Beckman Instrument, Inc., Fullerton, Calif) with Gilford Electronics (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

RESULTS

One of the initial experiments done in the present investigation was to examine the effects of caffeine and other MX on the respiration of fertilized sea urchin eggs. As reported by Cheney (6), we found a 50–60% inhibition of O_2 uptake in fertilized eggs in the presence of concentrations of MX which inhibited cleavage in these eggs (Table I). At this point we hypothesized that inhibition of respiration was related to the inhibition of mitosis and, as with dinitrophenol (DNP), which is an uncoupler of oxidative phosphorylation in these eggs (7), the MX acted through a decrease in ATP levels (59). As a control for our methods, we used DNP and azide and found significantly lower levels of ATP, as would be expected. However, when ATP and ADP levels were measured in the presence of caffeine and other MX, no changes in levels of the adenine nucleotides could be detected (Fig. 1). Measurements were repeated in 12 separate experiments before we finally accepted the results. The normal level of ATP found in these eggs was around 2–3 mM, which was similar to that re-

ported by Epel and others (11). Thus, the MX, although they depress respiration, seemed to act through an extra-mitochondrial process.

Since sea urchin eggs have a prominent pentose phosphate shunt pathway, which is reportedly activated upon fertilization (21) and is responsible for at least 50% of the oxygen uptake of the eggs (14), we decided to see whether the levels of triphosphopyridine nucleotides $NADP^+$ and NADPH were affected by MX. To obtain a more complete picture, we also measured the NAD^+ and NADH levels, since inhibition of NAD^+-kinase, which produces large amounts, of $NADP^+$ from NAD^+ within seconds after fertilization (11), could account for inhibition of oxygen uptake by restricting $NADP^+$, the co-factor for the first enzyme in the PPSP.

TABLE I

Effect of Methylxanthines on the Respiration of Fertilized Sea Urchin Eggs

Additions	Inhibition of O_2-uptake
	%
Caffeine (5 mM)	50 ± 2.5
Aminophylline (5 mM)	53 ± 3.2
Theophylline (5 mM)	51 ± 4.5
Methyl iso-butylxanthine (1 mM)	58 ± 4.5

Eggs were fertilized in Millipore-filtered sea water, containing 0.01% penicillin and streptomycin. O_2-Uptake was measured at 20°C, in a Warburg apparatus. All the above methylxanthines effectively inhibited cleavage at the concentrations added. Normal respiration (i.e., no additions) was routinely measured. The results are means ± SEM of six experiments.

NAD^+ and NADH levels were not changed in the presence of MX, whereas a very marked decrease in levels of $NADP^+$ and NADPH were detected. The effect was more pronounced for $NADP^+$ (70–80% lower) than for NADPH (40–50%). The results of these experiments are summarized in Fig. 1. When NAD^+-kinase was assayed in the egg supernates, no difference in activity of the enzyme could be detected between untreated and caffeine-treated eggs. Thus, the decrease in $NADP^+$ levels was not brought about by a decrease in the activity of NAD^+-kinase. Since the NADPH level was also lower in caffeine-treated eggs, we examined the possibility that this resulted from an inhibition of the PPSP in these eggs.

The key enzyme in the PPSP, glucose-6-phosphate dehydrogenase (which drives the oxidation of glucose towards the PPSP and is highly specific for $NADP^+$) was measured in fertilized egg homogenate, after the eggs have been incubated in sea water with 10 mM caffeine. No change in the activity of the enzyme could be detected when compared with the activity of control egg homogenate. Subsequently, the following major PPSP enzymes were assayed in control and caffeine-treated egg homogenates: 6-phosphogluconate dehydrogenase, phosphopentose isomerase, transketolase, and transaldolase. To our disappointment, no differences in activities of any of the above

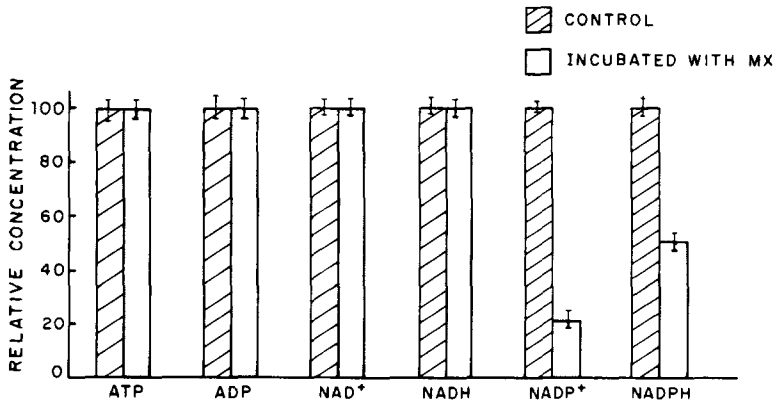

FIGURE 1 Effect of methylxanthines on various adenine and pyridine nucleotide levels in sea urchin eggs. Fertilized eggs at first cleavage were harvested and used for extraction and estimation of various nucleotide contents. Methylxanthines were used at concentrations which would inhibit cleavage in these eggs, i.e., 5 mM for caffeine, theophylline and aminophylline, and 1 mM for methylisobutylxanthine. All assays were done enzymatically and have been repeated at least three times.

enzymes could be detected in caffeine-treated eggs compared to those in normally fertilized eggs.

At this stage it was obvious that control of $NADP^+$ (and NADPH) levels by caffeine (and other MX) must come from outside the PPSP. A possible site was the glutathione system, since NADPH is involved in the production of reduced glutathione (GSH) by the enzyme glutathione reductase, and in some cells the activity of GR is enough to supply the $NADP^+$ required by G-6-P (18). The oxidation reduction cycle involving GSH and $NADP^+$ is as represented in Fig. 2. Thus, it was necessary to examine the activity of GR in these eggs in the presence and absence of caffeine. Moreover, Ii and Sakai (20) have recently reported GR from fertilized sea urchin eggs, the activity of which varies at different stages of the cell cycle. Indeed, when we measured GR activity in the microsomal supernate of homogenates of caffeine-treated eggs, a 70–75% inhibition was found after 90 min (approximate cleavage time) in the presence of 10 mM caffeine (Fig. 3). On the other hand, if 10 mM caffeine was added directly into the cuvette while assaying the enzyme in the supernates from control (untreated) eggs, no change in activity was found. Thus, while caffeine does not inhibit the activity of glutathione reductase when directly added in vitro, it does inhibit the appearance of GR in eggs incubated with caffeine (or other methylxanthines). The activity of GR at cleavage in control eggs was found to be around 0.4–0.5 μmol/mg protein/min.

Ii and Sakai also showed that the enzymatic activity of GR increases after fertilization and reaches a maximum around metaphase, after which it decreases again (19, 20). This cycle continues with each mitosis. Furthermore, they reported that if egg extracts (at any stage of mitotic cycle) are incubated for long periods (2–5 h) with EDTA or EGTA, there is an enhancement of the enzymatic activity the maximal activity of GR thus obtained was the same throughout the mitotic cycle. Thus, the total activity of glutathione reductase in these eggs was constant, when activated by EDTA or EGTA. Ii and Sakai have also demonstrated an "inhibitor" of glutathione reductase, which could be recombined with purified enzyme, thus inhibiting its in vitro activity. We were able to verify some of their observations. When the postmicrosomal supernate from control (90 min after fertilization) eggs was incubated with 5 mM EDTA for 2 h, a 20–25% increase in the activity of glutathione reductase was obtained. However, the activity of the enzyme in caffeine-inhibited eggs could not be further activated in vitro and always remained at the preincubated level (Fig. 3). These results suggest that caffeine prevents the dissociation of glutathione reductase from its inhibitor, thus resulting in a decreased amount of active enzyme. Glutathione peroxidase, the enzyme involved in oxidation of GSH in cells, was assayed in control (untreated) and caffeine-treated fertilized eggs and its activity was not affected by caffeine.

The above results strongly suggested the involvement of glutathione in cell division and some confirmatory experiments were carried out to test this possibility. When fertilized eggs were incu-

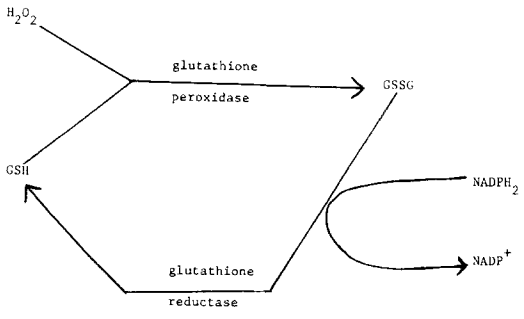

FIGURE 2 Schematic representation of the biological oxidation-reduction cycle involving GSH and $NADP^+$.

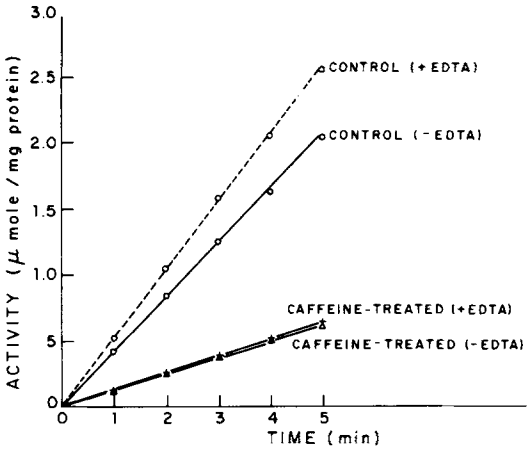

FIGURE 3 Glutathione reductase activity in control and caffeine-treated fertilized eggs. The rate of GR activity was measured in the microsomal supernate of the eggs. Where indicated, the microsomal supernate was incubated with 5 mM EDTA for 2 h before the enzyme assay. Other details of the assay are given in the text. O—O, control; O---O, control (+EDTA); Δ—Δ, caffeine-treated; +—+, caffeine-treated (+EDTA).

bated in sea water with 5 mM caffeine, cleavage inhibition could be overcome by simultaneous incubation with 10 mM reduced glutathione (GSH), but not with oxidized glutathione (GSSG). This would be expected if only the reducing enzyme (i.e. glutathione reductase) is inhibited by caffeine and not the oxidizing enzyme (i.e. glutathione peroxidase). Moreover, incubation with 10 mM GSSG inhibited cleavage of these eggs. Inhibition of cleavage by GSSG probably could be attributed to its inhibitory effect on protein synthesis (29, 58). The high concentration of GSH (i.e. 10 mM) necessary to overcome the caffeine inhibition is not surprising, since the in vivo levels of GSH have been shown to be about 6–11 mM in sea urchin eggs (8). The reversal of cleavage inhibition by caffeine in the presence of 10 mM GSH was only partial, i.e. the eggs did not develop beyond the four-cell stage. This was true even when 10 mM GSH additions were repeated three to four times at intervals of 20 min during the first 90–100 min of development, or, repeated after first and second egg cleavages. Thus, it seems that the damage caused by caffeine cannot be repaired beyond the second cleavage by additions of GSH (although removal of caffeine results in resumption of cleavage).

Further confirmatory experiments were performed using a Kosower reagent, diamide (27). This is a thiol-oxidizing agent, which reacts much more rapidly with GSH, when compared with other cellular constituents like NADH, CoASH, and lipoic acid (28). Kosower's group have further shown by a combination of rate data and concentration data that GSH is the major target for diamide within the cell and that conversion of GSH to GSSG is an important immediate consequence of diamide treatment. Diamide, at 10^{-3} M, was therefore added to fertilized sea urchin eggs after the mitotic apparatus (MA) had formed. Indeed, there was complete inhibition of division in the presence of 10^{-3} M diamide, accompanying disappearance of the MA. Complete inhibition of division was effective up to 5×10^{-4} M diamide. At 10^{-4} M a partial inhibition (about 50%) was noted and at 10^{-5} M the eggs cleaved normally. Thus, diamide added to eggs from fertilization to the time the MA formed prevented MA formation or caused a dissolution of the MA and prevented the eggs from cleaving, in a manner similar to that of caffeine (47, 49).

Further, if inhibition or formation of GSH is involved in caffeine effects, one should be able to obtain mitotic inhibition with oxidizers of NADPH, the co-factor for enzymatic formation of GSH. Phenazine methosulfate, an artificial acceptor of H^+ ions (from NADPH), was also found to be a mitotic inhibitor in sea urchin eggs, at a concentration of 10^{-4} M. Moreover, the NADP analog AADP (3-aminopyridine adenine dinucleotide phosphate), which was kindly provided by Dr. R. H. Garrett of this department, was also found to inhibit cleavage in these eggs when added 2–3 min after fertilization, whereas the corresponding NAD-analog AAD, (3-aminopyridine adenine dinucleotide) had no effect on cleavage. These results strongly and clearly suggest the involvement of GSH and $NADP^+$ (via the oxidation-reduction cycle as outlined above) in cell division. Thus, all experimental evidence so far suggest that inhibition of mitosis in sea urchin eggs by caffeine is mediated by perturbations of the intracellular thioldisulfide status.

DISCUSSION

It is likely from the results reported above that maintenance of GSSG and GSH levels are of crucial importance for continuation of cell division in marine eggs. If, however, GSG and GSSG are directly involved in mitotic events, they might be expected to show cyclic variation during the mitotic cycle. Despite Rapkine's (45) original report of such variation, all later workers have reported that GSG and GSSG levels are constant during mitosis in sea urchin eggs (51), HeLa cells (25), Ehrlich ascites cells (16), CHO cells (17), and rat parotid gland cells stimulated to divide by injection of isoproteranol (17). While variations in levels of GSH and GSSG have been found in lily by Nasitir and Stern (37), the patterns do not allow a simple interpretation or a direct relationship to cell division. An increase in GSSG and a decrease in GSH occur during sporulation in Neurospora (12), but these most likely relate to inhibition of protein synthesis (58), rather than directly to inhibition of cell division.

The fact that GSH and GSSG do not vary as a function of cell cycle could mean that they are unrelated to mitotic events, or, more likely, that they are maintained at given levels by other processes, whatever might be the varying demands on them during mitosis. One may recall that while ATP is the immediate source of energy for movement during muscle contraction, its levels do not change even after many contractile events (5), the ATP being efficiently resynthesized via creatine

kinase. Smilarly, ATP, ADP, and AMP levels do not change in early sea urchin embryogenesis (11), despite the fact that at least 40% of the egg ATP must be consumed in the first 10 min of development in the production of $NADP^+$ from NAD^+ by the enzyme NAD^+kinase (11). The results reported in this paper strongly suggest that maintenance of GSH (and/or GSSG) levels is necessary for the normal continuation of mitosis in sea urchin eggs; to what quantitative extent these compounds can be varied and still support normal cell division is under investigation in this laboratory. The work of Okazaki et al. (41) suggests that at levels between 15 and 40% of normal, cleavage will stop.

The mechanism responsible for maintenance of GSH level are unknown but two schemes suggest themselves. One involves the possibility of an interchange between free GSH and protein-bound GSH, P-S-S-G (36), and several glutathione transferases have been described (15). The second will be discussed below after we examine the glutathione reductase cycle.

We wish to examine now the question of whether GSH acts directly on the ultimate target systems involved in mitosis, and what, in fact, these systems are. Several possibilities exist for both questions. We will start with the second, namely, what are the target systems involved in glutathione effects on cell division.

There are at least two possible targets directly related to mitosis which can be regulated in vitro by oxido-reduction of their sulfhydryls and preliminary results from this laboratory suggest that both may be involved. One is the Ca^{2+}-activated ATPase, which Petzelt and his collaborators have shown to undergo cyclic variation correlated to mitotic events in sea urchin eggs (42, 43) and possibly in cells in culture (44). This ATPase is probably related to the Ca^{2+}-uptake vesicles described by Kinoshita and Yazaki (24). The case for the necessity of regulating calcium during cell division has been outlined except in detail and will not be repeated here except to point out that calcium can cause depolymerization of microtubules and disappearance of in vitro and in vivo MA (48). The idea that calcium uptake and release may be regulated by reduction and oxidation of sulfhydryls of an ion transport system has been examined in detail by Dikstein (9), who has shown that diamide causes contraction in protozoa, specifically in Vorticella (34). Since recent work by Amos et al. (3) strongly supports the idea that such contraction involves Ca^{2+} and a contractile protein, spasmin, the results with diamide are likely to be due to inhibition of intracellular Ca^{2+}uptake systems (24) in the protozoa, with concomitant release of Ca^{2+}. In the case of marine eggs, the ability of diamide to cause a disappearance of an already formed MA is dramatic (49) and therefore, by analogy to the protozoan case, could occur because of release of stored calcium (24). In support of this idea we have reported a marked inhibition of the Petzelt Ca^{2+}-ATPase in both caffeine and diamide-treated eggs, an inhibition which can be reversed in vitro by the addition of dithioerythritol (49). Moreover, the ATPase can be inhibited in vitro by diamide (but not by caffeine) and reversed with dithioerythritol (49).

Another potential target for reduction/oxidation processes is tubulin itself, which is known to have eight (31) or ten (10) free sulfhydryls. Indeed, we have shown that diamide will inhibit polymerization of tubulin in vitro and that ability to polymerize returns on treatment with dithioerythritol or mercaptoethanol (35). Similar inhibition of polymerization occurs with GSSG, although only at high concentrations. We have recently reported details of this work (35) and have discussed it in relation to the work of Kuriyama (30) which demonstrated inhibition of tubulin polymerization by NEM and PCMB.

The actual site at which diamide acts in the cell is thus not definitely clear, since inhibition of tubulin polymerization will obviously lead to antimitotic effects. However, the work of Kosower et al. (28) makes it clear that diamide reacts very rapidly with GSH compared to its rate of reaction with proteins; they, in fact, claim it unlikely that diamide will significantly interact with proteins in living cells. Whatever effects there are on tubulin directly will likely be secondary to those arising from depletion of the GSH pool—and the NADPH pool also; diamide also reacts with NADPH, although only at 1/80 the rate with which it reacts with GSH (28). Further, in the test tube, GSH protects tubulin from oxidation by diamide. (35). Thus, we suggest that diamide, applied to an egg when a MA is present, acts by reduction of the GSH pool. The effects of such reduction could be twofold: release of calcium by inhibition of Ca^{2+}-uptake systems and inhibition of tubulin polymerization by oxidation of tubulin. We will now discuss the enzymatic systems which may intervene between GSH and its targets.

In a number of cells (56), including sea urchin

eggs (20), enzymes known as transhydrogenases occur, which catalyze the exchange of hydrogens between sulfhydryls and disulfides. The general form of such reactions is as follows:

$$2GSH + P{-}S{-}S{-}P' \xleftarrow{\text{transhydrogenase}} G{-}S{-}S{-}G + P{-}SH + P'SH$$

where we have used glutathione as the H^+ donor, although it could have been cysteine or a variety of other sulfhydryl compounds. P and P′ may be parts of a given protein, the reaction then representing a reversible reduction of a protein disulfide. Sakai (52) has presented evidence that mitotic apparatus tubulin may act as a sulfhydryl donor through such a transhydrogenase and Kimura (23) has shown that sperm tail tubulin can donate hydrogen to a cortical protein through a transhydrogenase. While this work is not without fault, (54) it strongly suggests that tubulin can participate in hydrogen exchange reactions, which require a transhydrogenase in order to proceed. The extent to which such reactions proceed depends upon the equilibrium constant for the reaction and is thus relatively fixed. In order to be of interest in cell division, the reaction must be driven by some cell cycle-dependent energy source. It is of great interest, therefore, that reactions such as that outlined above can be coupled to reductases such as glutathione reductase (56) which can result in recycling of G—S—S—G to G—SH (Fig. 2), the energy deriving ultimately from NADPH. There could be two interesting results from such a coupled reaction. First, the reaction is pulled towards the right, i.e., towards the reduced state of the protein, the extent of reduction being determined in part by the rate of reduction of oxidized glutathione. Second, the levels of oxidized and reduced glutathione are not changed by the coupled reaction, although their turnover rates clearly vary. Thus, the result of a periodic variation in glutathione reductase, such as that reported by Ii and Sakai (19, 20) in sea urchin eggs, is to provide a periodic variation in the thiol-disulfide status of those systems which are substrates for transhydrogenases and which utilize glutathione (and ultimately, NADPH) as their source of reducing power. On the basis of results recently presented elsewhere (49, 35) we suggest that the Petzelt Ca^{+2}-ATPase and tubulin are likely to be substrates for such transhydrogenases. In addition to these, there appear to be two further possible candidates in eggs which are relevant to cell division.

In a series of papers summarized in a review (52) and in two more recent reports (32, 33), Sakai and Mabuchi have shown that a cell cycle-dependent variation in thiol-disulfide status occurs in targets in the cell cortex, one of which may be a dynein-like ATPase (33). It is thus possible that processes associated with cytokinesis (46) as well as those associated with karyokinesis may be regulated by periodic variation in thiol-disulfide status. Finally, we return to an interesting observation described in the section on results in which we reported the inability of eggs to progress for more than two cleavages when supplied by exogenous glutathione in the presence of caffeine (if both are washed out the eggs continue cleavage, so no essential damage has been done). Kosower has shown that oxidized glutathione inhibits protein synthesis both in vivo and in vitro even in the presence of reduced glutathione (58, 30). Since we have shown that eggs possess a glutathione peroxidase activity which is not inhibited by caffeine, it seems reasonable to suggest that exogenous glutathione is converted to G—S—S—G, which accumulates when GR is inhibited, and which shuts off protein synthesis thus inhibiting cell division (57). If this interpretation is correct, we have yet a fourth intracellular system which when oxidized through the glutathione system, results in an inhibition of cell division.

It is also likely that other key sulfhydryl systems related to cell division exist in eggs. This is indicated by the fact that cysteine cannot reverse caffeine inhibition whereas it can reverse the inhibition of mitosis by tosyl lysine chloroketone (TLCK), a proteolytic enzyme inhibitor (50); we have repeated these observations with the eggs used in this study. The TLCK effects are thought to be due to the generation of α-keto aldehydes in eggs (50); these compounds are known to be inhibitors of mitosis and probably act on sulfhydryl systems (55). A more thorough discussion of enzymes involved in sulfhydryl regulation in cells, details of mechanisms of nomenclature for transhydrogenases, and possible implications of variation of thiol-disulfide status of tubulin in relation to cell surface phenomena have been presented elsewhere (49).

It is not known whether caffeine (or other methylxanthines) will have similar effects on GR activity in other systems. The details may vary

significantly and indeed GR has been reported to decrease in activity during mitosis in Ehrlich ascites tumor cells (13), although the timing of mitotic events was much less accurate than in sea urchin eggs (19, 20). However, in view of the extensive literature relating sulfhydryl groups to cell division (41, 51–53), it is likely that some of the general ideas discussed here will pertain to other systems. Thus, it is probable that the regulation of calcium by a sulfhydryl-controlled Ca^{+2}-ATP-ase [reported to occur in mammalian cells as well as in marine eggs, (42–44)] will be widespread in mitotic systems and that regulation of tubulin polymerization by means of sulfhydryl-disulfide balance is also likely to be ubiquitous. Our results, therefore, pinpoint two defined target systems and two other potential systems (cortex and protein synthesis) with which to study the elusive nature of the involvement of sulfhydryls in cell division.

This work has been supported by grants from the National Science Foundation and the National Institutes of Health.

Received for publication 4 August 1975, and in revised form 17 November 1975.

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