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Cyclic Nucleotides, Thioldisulfide Status of Proteins, and Cellular Control Processes

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It is shown that cyclic nucleotides can have a variety of effects on cell division, cell shape, cell adhesion, and cell movement, depending on the cells selected and the conditions under which they are used. For example, while CHO cells elongate under the influence of exogenous dibutyryl CAMP, Y-1 adrenal tumor cells round up and polyoma-transformed 3T3 cells show no change in shape. The totality of experience with cyclic nucleotides suggests that where they have been used by cells as control elements involving the four processes listed above, they are superimposed on basic cellular processes that progress in their absence – that is, they must be acting indirectly. In attempting to understand the inhibitory action of methyl xanthines on egg development, we were forced to abandon the idea that they acted through cyclic nucleotides. We found that methyl xanthines inhibited the activation of glutathione reductase and that glutathione oxidizing agents act as mitotic inhibitors. Further, we found that tubulin polymerizability, NAD-kinase activity, and a mitotic apparatus associated Ca^{+2}-ATP-ase were all inhibited by oxidation of some of their sulfhydryls and were activated by reduction of the resulting disulfides. These results are discussed in terms of reported cycles and activations of glutathione reductase (GR) in cells and reports that mixed disulfides of glutathione and proteins can act as substrates for GR. Using the fact that a CAMP-dependent protein kinase has been reported to be activated by glutathione, we have suggested potential sites where sulfhydryl control processes and cyclic nucleotide control processes may interact in certain restricted cases.

Key words: **cyclic nucleotides, protein sulfhydryls, tubulin, glutathione**

INTRODUCTION

The regulation of mitosis is clearly a problem of profound importance with ramifications in cell, developmental, and molecular biology as well as in the understanding of pathological problems such as cancer. Such are the subtleties of language, however, that the preceding statement, which would hardly be contested by most people, contains a hidden attitude, namely that regulation is regulation and probably is to be universally

understood from knowledge of a small number of examples. If we could find a universal, naturally occurring stimulator or inhibitor of mitosis that could be applied to all cells, we would have a grand generalization and all further work would merely be commentary. Cyclic adenosine monophosphate (CAMP) has been proposed as a candidate for such a negative regulator, and cyclic guanosine monophosphate (CGMP) as a universal stimulator (1–4). A more sophisticated version of the idea is that the ratio of CAMP and CGMP controls cell division rather than the absolute quantities of these compounds (2) – the yin-yang hypothesis, to apply this eastern concept somewhat loosely (10). We shall show in the first part of this paper that such generalizations are not likely to be correct where cyclic nucleotides are concerned.

Our own disenchantment with this idea came after several years of attempting to show that CAMP was, in fact, the negative regulator of cell division in sea urchin eggs. This doubt arose from two sources. We had become suspicious of the idea that puromycin inhibits cleavage in marine eggs through its inhibitory effect on protein synthesis (5). In searching for an alternate mode of action for puromycin, we explored a report that showed that puromycin was an inhibitor of the phosphodiesterase for CAMP and, moreover, that the purine part of puromycin, dimethylaminopurine (DMAP), had a similar effect (6). Since DMAP also proved to be an inhibitor of mitosis in sea urchin and clam eggs without inhibiting protein synthesis, this idea looked attractive (5). Further, we were working on the mechanism of mitotic inhibition in sea urchin and clam eggs by methyl xanthines (MX) and since these were well-known inhibitors of the phosphodiesterase (PD) for CAMP in eggs (7, 8) as well as in other cells, the hypothesis was further supported. But it was false. When tested, puromycin and DMAP had no effect on the PD of the sea urchin eggs (7) and dibutyryl CAMP (DBCAMP), which was taken up by the egg in the form of N^6 monobutyryl CAMP, could attain levels of up to a thousand times that of endogenous CAMP with no inhibitory effects on cleavage or development through late gastrula stages (9, 10). DBCGMP similarly had no effect on cleavage, and in fact no detectible CGMP was found in the eggs [as contrasted with the sperm, where it is present in superabundant amounts (11)].

We were entangled in similar difficulties in attempts to apply some uniform schema to cells in culture, as will be seen below, and ultimately we were forced to withdraw from this hypothesis and with sober reflection (and mounting dread of the enormous task) reviewed the literature on cyclic nucleotides and cells, selecting the modalities of cell division, cell shape, cell adhesion, and cell motility as properties for examination (10). We concluded that, for each modality in which CAMP was concerned, anything was possible: a positive, a negative, or no effect, given the universe of cell types available. This immediately told us that the effects of cyclic nucleotides on these properties of cells must be indirect and could only be superimposed on whatever fundamental mechanisms exist in cells for control of the four selected processes insofar as cyclic nucleotides were concerned at all. That cyclic AMP was not in any way necessary for cellular life was indicated by the CAMP independent mutants of the S49 lymphoma isolated in Gordon Tomkins's laboratory (12): These divided with cell cycle parameters similar to those of the parental line (13) without the benefit of CAMP or of receptors therefor (14).

Our difficulties with the methyl xanthines in eggs were resolved, in part, by a different route: We found that their effects involved glutathione and NADP. This led to the hypothesis that a central process in the regulation of the polymerizability of tubulin in vivo is regulation of its sulfhydryl-disulfide status (15–18). This hypothesis will form the

second part of this report, where we will also attempt to identify the general areas that cyclic nucleotides may control in those cases in which microtubule assembly is part of the biological process that they may be directing – that is, to ascertain at what level they may be involved in regulation of microtubule-associated sulfhydryl processes

CYCLIC NUCLEOTIDES AND CELLS IN CULTURE

We used three cell lines in these studies: CHO cells, Py 3T3 cells, and Y-1 adrenal tumor cells. CHO cells were cultured by techniques devised by Puck (19, 20), Py 3T3 cells as described by Sheppard (21), and Y-1 adrenal tumor cells by protocols given by Donta et al. (22, 23).

Morphological Effects

Application of DBCAMP, cholera toxin, or the cytokinins (24) to CHO cells had morphological effects opposite to those obtained by application of the same reagents to Y-1 adrenal tumor cells. CHO cells showed the behavior well-described by Hsie and Puck (19) and Porter et al. (25), that is, they stretched out to considerable lengths. DBCAMP causes an increase in intracellular N^6 MBCAMP in CHO cells (26), which stimulates protein kinases (27) and because it inhibits the PD for CAMP, causes an increase in intracellular levels of CAMP. Cholera toxin leads to an increase in CAMP levels in CHO cells (28) and so the idea that CAMP leads to CHO cell elongation and microtubule assembly (29) is well supported. In the case of cytokinins, we do not have data for CHO or Y-1 cells, but it has been shown that there is no effect on CAMP levels in other cells (24). Y-1 adrenal cells also show effects that have been described in the literature: They round up (Fig. 1) on treatment with the same compounds that cause CHO cells to stretch out, namely DBCAMP (30), cholera toxin (23), and cytokinins (our observations).

These compounds, having showed opposite morphological effects on different cells with the same stimuli, we examined the third cell line available in the laboratory and found yet another effect on morphology due to DBCAMP and cholera toxin, that is, no effect at the light microscope level (Rebhun and Schnaitman, in preparation), a result that, although it was obtained on cells derived from the same source, contradicted that reported by Sheppard (21). The lack of effect on cell shape and colony morphology occurred on microbiological plates, tissue culture plates, and the latter coated with collagen, and thus appeared to be independent of substrate (plating efficiency increased in the order given). Growth rate and plateau level were, however, strongly affected by DBCAMP as reported by Sheppard (21).

Effects on Growth

Inhibitory effects of increase in intracellular CAMP levels on growth have been well documented for a number of cells (1, 3, 4, 5, 31, 32). Our own work, however, told us that this did not occur in sea urchin or clam eggs (8, 9) and thus could not be a general phenomenon. In the cells we were growing in the laboratory we found similar diversity. Hsie and Puck (19, 20) had reported that CHO cells treated with DBCAMP so as to cause great elongation did not show a decrease in growth rate, an observation that we were able to repeat. Further, CHO cells, which partially elongate when grown in 1% serum, grow in this medium as readily as in 10% serum, which also stresses the independence of growth rate and intracellular CAMP levels in these cells. CHO cells, therefore, allow a dissection

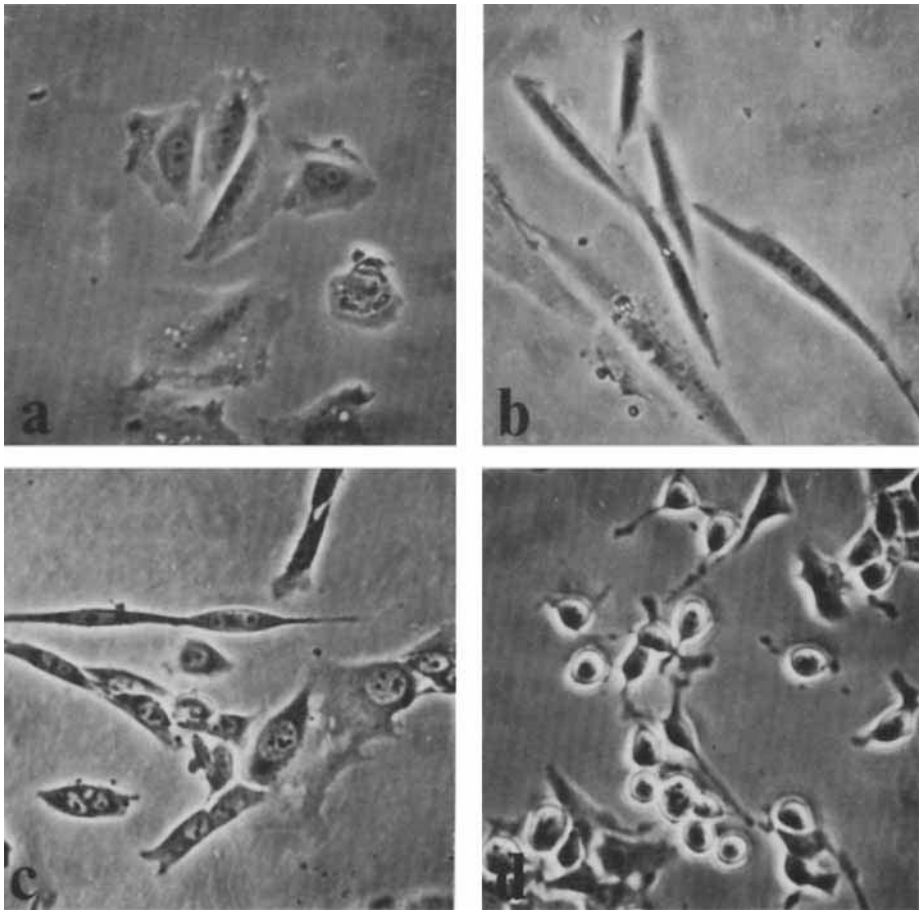

Fig. 1a. Normal CHO cells grown in Ham's F-12 medium plus 10% fetal calf serum. ×440.

Fig. 1b. CHO cells 24 hr after medium is made 1 mM in DBCAMP and 15 μM in testosterone. × 440.

Fig. 1c. Normal Y-1 adrenal tumor cells grown in Ham's F-10 medium plus 15% horse serum. ×440.

Fig. 1d. Y-1 adrenal tumor cells 24 hr after medium is made 0.5 mM in DBCAMP. × 440.

of effects of CAMP on morphology from those on growth. Py 3T3 cells, however, show the opposite effect; they show no morphological effects on the light microscope level due to application of DBCAMP at concentrations that decrease growth rate and cause the cells to saturate at densities (average density per plate) one-fourth that of control cultures. These cells, therefore, allow a study of decrease in growth potential with CAMP that is independent of effects on gross morphology of the cells. The decrease in growth rate and plateau density in no way can be considered to arise from a contact inhibition mechanism since cells in DBCAMP pile up in colonies indistinguishable from those in control cultures except, perhaps, for number and size.

Y-1 adrenal tumor cells show both morphological effects and a decrease in growth rate with DBCAMP (30) or cholera toxin (22) and thus demonstrate behavior different from either CHO or Py 3T3 cells.

TABLE I. Percent of Cells Detached After Trypsin Treatment

Y-1 adrenal tumor cells	Time after treatment (min)					
	2	10	15	20	25	30
+0.5 mM DBCAMP	48	49	75	92	93	91
Control (no DBCAMP)	18	23	53	65	81	85
CHO cells	2	4	6	8	10	12
+ 1 mM DBCAMP + 15 μM testosterone	21	41	56	75	82	85
Control (no DBCAMP or testosterone)	42	60	78	82	91	94

Cells were allowed to grow in complete medium for 24 hr, after which half the dishes were incubated in medium containing the appropriate stimulus for another 24 hr. They were then briefly rinsed in magnesium-calcium-free phosphate-buffered saline (PBS) containing the same stimulus that had been applied earlier (none in the case of controls). PBS was then replaced with 0.05% trypsin containing the stimulus and the cells were incubated at 37°C. Dishes were removed at the appropriate time and rolled gently four times. A 0.5 ml aliquot was removed and counted in a Coulter counter. More trypsin was added to the remaining cells which were removed with a rubber policeman and counted. Data is given as percent of cells dislodged to total cells in the dish. Table I–IV reprinted from Ref. 16, with permission.

Cellular Adhesion

Since Y-1 cells and CHO cells showed such different behavior in the presence of three stimuli (DBCAMP, cholera toxin, and cytokinins), we decided to examine one other parameter that has been discussed in connection with CAMP, namely, cellular adhesion to the substrate as measured by the rate of detachment of cells after treatment with trypsin or EDTA (33). The technique utilized is essentially that described by Johnson and Pastan (34). Trypsin was added to cells that were then gently rolled four times, and the supernatant was removed and counted for released cells. The remaining cells were then detached after treatment with higher trypsin concentrations and the use of a rubber policeman, and they were again counted. Data in Table I are given as a percent of control cells released as a function of time for Y-1 cells and for CHO cells. It can be seen that in the presence of DBCAMP, Y-1 cells are detached more easily than control cells, while the opposite occurs for CHO cells. While the trypsin or EDTA detachment procedure can be criticized as a true measure of adhesion, it still includes adhesion to the substrate among the phenomena it measures. It is clear, therefore, that the rounding up of Y-1 adrenal tumor cells is accompanied by a greater ease of detachment from the substrate; and thus, in this property, these cells again show response to DBCAMP opposite to that of CHO cells.

DISCUSSION

We have outlined some of the considerable variability that we have seen in cells when they are compared with respect to the effects of CAMP on several modalities. We wish to emphasize that the cells that we have used were not selected for this variability but were being studied for other purposes in the laboratory. This is simply to stress the possibility that such variability is rather widespread. We wish to add several more examples in order to fill out the inventory and to discuss another modality, cell movement, which has also been studied with respect to effects of CAMP.

While we have given examples in which CAMP decreases growth rate and in which no effect is seen on growth rate, a stimulation of cell division of CAMP has been reported in several cases. In explanted thymic lymphoblasts, a number of stimuli that cause increase in intracellular CAMP result in increased DNA synthesis and mitosis, although the results are quite complex with respect to dose rates and concentrations of extracellular calcium (35, 36). Some very interesting work showed that cell division could be stimulated in intact liver by a solution of triiodothyronine, amino acids, glucagon, and heparin and that glucagon could be replaced by DBCAMP and theophylline (37). In this system, neither CGMP nor any of its derivatives had any effect, stimulatory or inhibitory. Finally, Wood and Braun have identified materials in plants that they have called cytokinesins, which are supposedly the true plant mitotic hormones induced to form by cytokinins and which apparently act through CAMP. They appear to be inhibitors of the PD for CAMP (38) and the effects of cytokinins are mimicked by 8-Bromo-CAMP (39).

With respect to movement, Johnson et al. (40) reported that L929 cells are inhibited from moving by DBCAMP. On the other hand, Goldman et al. (41) have studied cells infected with human adenovirus 5, which do not move on the substrate. When DBCAMP is added the cells spread out on the substrate, form ruffles, and move. In our own movies of Py 3T3 cells (which, in fact, do not move very much in any case) no effect was seen on any movement that was present just as no effect was seen on morphology. Puck et al. (20) also report that DBCAMP does not affect movement in CHO cells. Thus, with respect to the four modalities which we have chosen to examine – cell division, cell movement, cell adhesion, and cell shape – we can find a cell that demonstrates either no effect, a stimulation, or an inhibition (the exception is cell adhesion, although we suspect that the Py 3T3 cells would fill this slot – i.e., show no effect).

It is quite clear from this brief survey (see Refs. 10 and 32 for more extensive discussion) that CAMP cannot be fundamentally involved in any of the above processes as a general condition for cell life. This is emphasized by the work from Gordon Tomkins's laboratory on variants of S49 lymphoma cells that were selected to be resistant to the killing effects of DBCAMP (12). These do not possess CAMP-binding proteins, so receptors for the nucleotide are not present (14). They nevertheless cycle with normal parental cell cycle parameters (13). Again, neither RLC normal liver cells nor HTC hepatoma cells show effects on growth with DBCAMP, while the hepatoma line H35 and MH_1C_1 are inhibited from growing (42). It is instructive that the basis for the latter effect appears to be a direct effect of CAMP on the enzymes of the deoxyribopyrimidine pathways: Thus, these cells will be halted in a different part of the cell cycle than would, for example, 3T3 cells with DBCAMP; again this emphasizes the fact that if cells require control by CAMP, the mode by which such control is exerted can vary with the phenotype of the cell.

An excellent illustration of this exists in the NRK line, which shows different effects on production of CAMP with infection by different viruses. When infected with a temperature-sensitive Kirsten virus, CAMP levels fall at the permissive temperature, illustrating the generalization that viral transformation is accompanied by a decrease in intracellular CAMP levels (43). However, when cells of the same line are infected with other viruses, the situation can be opposite. Thus Somers et al. (44) showed that on infection with a temperature-sensitive variant of mouse sarcoma virus (MSV line 1b) the cells increased their intracellular CAMP levels when the transformed phenotype was expressed at the permissive temperature. Further, this transformed phenotype was one in which the cells rounded up from a flattened state. Another similarity to the response of Y-1 cells: these cells will also

round up when treated with DBCAMP at the nonpermissive temperature. Finally, the authors state that neither the original NRK cells nor the cells transformed by the original (non-temperature-sensitive) MSV virus respond with morphological changes to DBCAMP at 36°C or 39°C, the temperatures used with the TS variant (line 1b). These cells, therefore, resemble the Py 3T3 cells in their lack of morphological response to CAMP.

The case for cyclic GMP as a stimulator of cell division has been made by a number of authors (45–51). The compound appears to increase after stimulation of cells into division from various states. For example, it has been shown that CGMP increases in amount when 3T3 cells are stimulated into division by serum or by purified pituitary growth factor (51; in the latter case, there were no changes in CAMP level). In a similar study of 3T3 cells and several serum and density revertants of SV3T3 cells (52, 53) it was shown that there was a reciprocal change in CAMP and CGMP levels which supported the idea that increases of CGMP were correlated with growth and decreases with cessation of growth. Moreover, 3T3 cells can be stimulated to DNA synthesis with 8-Br-CGMP (49). Stimulation of lymphocytes with plant lectins also resulted in great increases in concentrations of CGMP, and in the case of a purified PHA preparation, no change in CAMP level occurred (45).

These are impressive observations that suggest a strong relationship of CGMP and induction of cell division, since they have been obtained with fibroblasts and lymphocytes. However, we have found no detectable levels of CGMP in sea urchin eggs. Hovi et al. (54) found no changes of CGMP with stimulation of chick fibroblasts from the contact-inhibited state (but did find that CAMP could stimulate such cells to divide), and Short et al. (37) found no effect of CGMP on liver mitosis, whereas CAMP stimulated such cells (when used in the proper combination in a perfusing solution). Recently, Miller et al. (55) have presented evidence that CGMP falls on stimulation of 3T3 cells from G_0 and rises at contact inhibition, contradicting other workers (45–51). Thus, while the case for CGMP as a positive signal for induction of cell division has some evidence in its favor, other observations are beginning to accumulate that indicate the necessity for caution and suggest that the uses cells make of CGMP for cell division will vary with the phenotype in a manner similar to that seen for CAMP.

As a final example, which will lead us into our next topic, we examine the opposite effects of CAMP on microtubules in two types of cells. In CHO cells, the evidence is clear that CAMP increase in the cells leads to assembly of microtubules (25, 29) without necessity for protein synthesis or RNA synthesis (56). In polymorphonuclear leukocytes, the opposite is the case and CAMP appears to have a colchicine-like effect (57). Furthermore, CGMP or any phagocytic stimulus that generates it in the cytoplasm causes a rapid, almost explosive assembly of microtubules, in fact, within the first 15–30 sec of application of the signal (58). It is clear, therefore, that microtubule assembly or disassembly may be part of a biological process influenced by cyclic nucleotides, but which process and which nucleotide are matters determined by the phenotype of the cell. Cyclic nucleotides would appear to have no direct influence on the assembly or disassembly of microtubules themselves; this thesis can be verified by attempts to show the contrary with tubulin in vitro (unpublished observations of Borisy and Weisenberg; personal communication). This leads us, however, to a consideration of what processes may be involved in regulation of microtubules in vitro, and specifically leads us back to our work with inhibition of cleavage in marine eggs by methyl xanthines.

Methyl Xanthines and Cell Division in Marine Eggs

Inhibition of division in marine eggs by caffeine was first reported by Cheney (59, 60) and was of interest to us for several reasons despite the fact that high concentrations of the drug (5–10 mM) were necessary for its action. First, it caused a reversible decrease in the size of the in vivo mitotic apparatus, resembling in this respect the actions of colchicine. Second, the recovery was rapid (a matter of minutes after removal of the drug). Finally, caffeine is known to affect two systems, both of which might be important in regulation of the mitotic apparatus: CAMP and calcium (see Discussion, Ref. 61). Our results with methyl xanthines showed that they indeed increased the intracellular levels of CAMP in eggs (8), presumably through action as an inhibitor of egg PD (7, 8, 62). However, all of our other evidence suggested that this was not the mode of action of caffeine (see above and 8, 9, 16, 61) and that we would have to look elsewhere.

Influence of Caffeine on Oxidative Metabolism of Sea Urchin Eggs

Since Cheney (59, 60) had shown that caffeine inhibited cleavage in proportion to the inhibition of oxygen uptake in sea urchin eggs, we turned to these observations and were easily able to verify the results in eggs of the sea urchin Strongylocentrotus purpuratus, Table II (15). Complete inhibition of cleavage resulted when inhibition of oxygen uptake was about 50% of normal. Since this result is essentially the same as that obtained by Epel (63) for carbon monoxide poisoning of egg cleavage, we felt that the site of action of caffeine must be on some mitochondrial process, so we measured ATP and ADP levels in eggs after treatment with methyl xanthines from 5 min after fertilization through cleavage. After a dozen experiments we became convinced that our results are correct, namely that there is no change in the levels of these nucleotides in the presence of caffeine. This is quite contrary to results obtained in the same experiments with the uncoupling agent dinitrophenol (15, 61), which also is an antimitotic agent with effects similar to those of caffeine. This led us to seek other processes that might involve oxidation, and we were led to consider the possibility that caffeine could affect the pentose phosphate shunt pathway (PPSP) since about 50% of the oxidation of sea urchin eggs is associated with this pathway (64). Since the major product of this pathway is NADPH, we decided to measure both the tri- and dipyridine nucleotides in oxidized and reduced form in the presence and absence of methyl isobutyl xanthine (MIX), which is more potent than other methyl xanthines in our system, causing inhibition of division at 1 mM. As can be seen from Table III, we found no change in the levels of NAD and NADH but that NADP levels were depressed to 20% of normal and, suprisingly, NADPH levels were 50% of normal. If egg systems are similar

TABLE II. Effect of Methylxanthines on the Oxygen Uptake of Fertilized Sea Urchin Eggs

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

Fertilized sea urchin eggs were suspended in millipore-filtered sea water containing 0.1% penicillin and streptomycin. Oxygen uptake was measured manometrically in a Warburg apparatus, using conventional methods. Temperature was maintained at 20°C. Respiration was studied until first cleavage was reached in controls. The results are means ± S.E.M. of six experiments.

to those in other cells in that control of the PPSP is effected by the NADPH/NADP ratio (65) with inhibition occurring as the ratio increases (and not very sensitive to absolute values; Ref 66), this would be enough to understand the decrease in this portion of the oxidative processes of the cell. Since it was possible, however, that control came from within the PPSP, we asked whether MX could be acting on enzymes in the pathway and we examined levels of five key enzymes in control and MX-treated eggs (15). No effects were seen on activity of these enzymes in control versus treated eggs. This strongly suggested that control of pathway activity was by means of the NADPH/NADP levels and since these levels are regulated in many cells by the activity of glutathione reductase (GR) (67), we examined that enzyme with some care. The reactions catalyzed by GR and glutathione peroxidase (GP) are given in Fig. 2.

Glutathione, Glutathione Reductase, and Mitosis

Our first experiments showed us that the level of activity of GR in caffeine-treated versus control eggs was much depressed when the enzyme was assayed at the time when control eggs cleaved (Table IV). The inhibition was as much as 80%. However, when caffeine was added to 100,000 xg supernates (GR is more than 95% localized in the cytoplasm, there is a small amount of mitochondrial GR in eggs), no inhibition of the enzyme was obtained. Thus, the enzyme itself was not inhibited by MX, but its activity in whole eggs when treated with the drug was much reduced. That glutathione indeed appeared to be involved was shown by the ability of high concentration (10 mM) of exogenous glutathione (but not cysteine) to reverse the caffeine effect and allow two further cleavages (this limited reversal is consistent; see Rebhun et al. (16) for a discussion).

TABLE III. Effect of Methylxanthines on Intracellular Pyridine Nucleotide Levels in Fertilized Sea Urchin Eggs

Additions	% Relative concentration			
	NAD	NADH	NADP	NADPH
None	100	100	100	100
MX-treated*	95(±4.8)	94(±5.2)	22(±4.5)	51(±5.1)

*Methylxanthines were used at concentrations that inhibit cleavage in these eggs, i.e, 5 mM for caffeine, theophylline, and aminophylline and 1 mM for methyl isobutylxanthine. All assays were done enzymatically and were repeated at least three times.

TABLE IV. Glutathione Reductase Activity in Normal and Caffeine-Treated Sea Urchin Eggs

Experiment	GR activity
	(μ mole/mg protein/min)
Control (−EDTA)	0.40 – 0.50
Control (+EDTA)	0.50 – 0.65
Caffeine-treated (−EDTA)	0.10 – 0.15
Caffeine-treated (+EDTA)	0.10 – 0.15

GR activity was measured in the microsomal supernatant of fertilized eggs. Samples were collected when the eggs had just started to cleave (90–100 min). In experiments with caffeine, the eggs were allowed to develop in the presence of 10 mM caffeine for the same period of time.

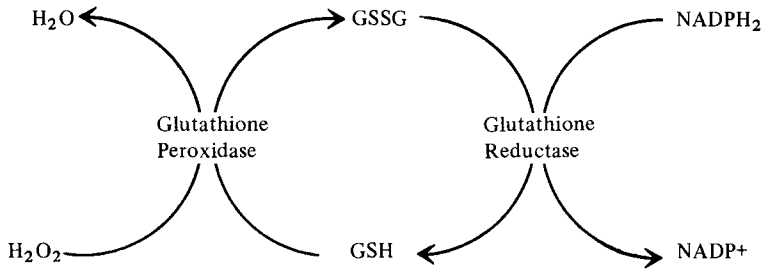

Fig. 2. Reactions catalyzed by GR and glutathione peroxidase.

At about the time we obtained these results two papers were published by Ii and Sakai (68, 69) that showed a cycle of glutathione reductase activity as a function of the cell cycle in sea urchin eggs. The enzyme activity was shown to reach a peak at a metaphase and to decrease to a minimum between first and second cleavage only to increase again to attain a maximum at the next metaphase. Furthermore, the activity of the enzyme at any point in the cycle could be increased by incubation with EDTA or EGTA (for several hours). After activation, the enzyme activity was approximately constant over the whole cell cycle, suggesting the existence of a cyclic process regulating the activity of the enzyme. A high-molecular-weight inhibitor of GR was found. In preliminary work with the GR enzyme of S. purpuratus, we have found a similar activation of the enzyme with EDTA in untreated eggs. However, in supernates of caffeine-treated eggs we have been unable to increase the activity of the enzyme with chelating agents. We suggest that the inhibitory effect of caffeine in vivo is to prevent dissociation of the inhibitor from the enzyme.

Given the apparent involvement of glutathione in maintenance of the MA, we turned to use of a compound that Kosower (70, 71) had introduced as a specific oxidant for glutathione in cells, namely diamide. The action of diamide as an oxidant for low-molecular-weight thiols is depicted in Fig. 3. While diamide reacts with other low-molecular-weight thiols and reducing agents such as NADH or NADPH, it reacts over 80 times faster with glutathione than with the pyridine nucleotides and ten times faster than with lipoic acid or cysteine (72). Further, diamide is reduced in the process of oxidizing substrates and is not reactive with other cell constituents in reduced form. It can be used, therefore, to remove glutathione stoichiometrically, a temporary condition from which many cells recover at rates depending upon the activity of their glutathione-generating systems (73). Application of 0.5–2 mM diamide to eggs containing a spindle caused disappearance of that structure at a rate proportional to the concentration of agent used and varying with the state of application – cells in anaphase are not as easily affected. Measurements of glutathione by Fahey's methods (74) verified that reduced glutathione disappeared and oxidized glutathione increased at these levels of diamide. In following up observations made by Okazaki et al. (75), we found that N-ethyl maleimide (NEM) caused a disappearance of the MA in 1 min at about 0.3 mM, and as discussed in Rebhun et al. (16), the MA can be made to disappear at concentrations of NEM that leave between 15–40% of the glutathione still present in eggs. Thus, a decrease in glutathione levels in eggs is correlated with the disappearance of MA. It came as a complete surprise, therefore, to find that levels of oxidized and reduced glutathione, which do not change as a function of cell cycle in either marine eggs (76) or cells in culture (77, 78), showed no change in caffeine-treated eggs (Table V). This unexpected result, which we verified in four separate

TABLE V. GSH and GSSG Levels in Sea Urchin Eggs After Treatment With Diamide or Caffeine

Treatment*	GSH concentration+	GSSG concentration+
Control	6 – 10 mM	2.5 – 8 μM
10 mM caffeine	5 – 10 mM	3–10 μM
1 mM diamide	5 – 10 μM	5–10 mM

*Eggs of the sea urchin Stronglyocentrotus purpuratus were fertilized and immersed in the desired solution 5 min later. Eggs were harvested at about the time of metaphase of first cleavage (in controls). GSH and GSSG were estimated by the methods of Fahey et al. (74).

+Concentrations are given in molarity. This is calculated based on the known volume of the sample, the glutathione levels per sample, and the known volume of the egg. The numbers give the range in four experiments. For a given batch of eggs, the levels in control and caffeine-treated eggs are essentially identical.

experiments (in which diamide-treated eggs showed marked decreases in glutathione), was especially difficult to accept since exogenous glutathione partially overcomes the caffeine inhibition; however, we are stuck with our data. In retrospect, however, if caffeine is not inhibiting GR activity but simply inhibiting its activation, we should not be suprised that glutathione levels do not change with caffeine since they do not change in normal eggs during periods in which GR activity shows cyclic variation (76). To summarize, we can eliminate the MA, or prevent it from forming, either by depletion of 60–85% of the cellular glutathione or by inhibition of the activation of the enzyme glutathione reductase.

Sulfhydryls and Tubulin

If diamide affects cells solely through the reduction of glutathione levels, it should not react with cellular proteins, and in particular it should not affect the polymerizability of tubulin. This was easily tested and found to be untrue: Diamide inhibited the polymerizability of tubulin (17, 18), an inhibition easily reversed by all reducing agents tried – mercaptoethanol, dithiotheitol, and reduced glutathione, although the latter had to be used at about 10 mM. Treatment of tubulin with diamide resulted in loss of the 20S and 30S fast-sedimenting peaks, as seen in the optical ultracentrifuge, and corresponding rings, as seen with the electron microscope (17, 18). Both ultracentrifuge pattern and EM pattern returned on reduction of the tubulin and return of the polymerizability. Measurement of the free sulfhydryls in brain tubulin isolated by Shelanski's glycerol method (79) showed the presence of 7.1–7.3 free sulfhydryls per 55,000 dalton monomer (not correcting for the presence of high-molecular-weight protein), which agrees with measurements made by Kuriyama and Sakai (80) (Table VI). In recent experiments 2x-cycled tubulin isolated with glycerol was treated with radioactive NEM, run on SDS gels, sliced, and counted. At levels of C^{14} NEM that gave $(4–5) \times 10^3$ CPM at the tubulin bands, no counts above background were found between tubulin and the top of the gel, that is, neither tau-factor nor high-molecular-weight proteins (81) possess –SH groups that react with NEM. If we use this result to correct the estimates of free –SH on tubulin for the amount of HMW protein, we come very close to eight free sulfhydryls per monomer of tubulin (in tubulin prepared with glycerol). In tubulin prepared without glycerol the amounts of free sulfhydryls are lower (17, 18).

Of considerable importance is the fact that treatment with diamide lowers the number of free sulfhydryls to about four if corrections are made for HMW proteins, indicating

TABLE VI. Sulfhydryl Titers of Polymerizable and Diamide-Treated Tubulin

Tubulin	No. of sulfhydryls/55,000 MW
Prepared with Glycerol:	
DTNB	7.3 ± 0.3 (6)*
4-TP	7.2 ± 0.2 (4)
DTNB + 6 M GuHCl	7.0 ± 0.9 (2)

Preincubations		Sulfhydryl assay (sulfhydryls/55,000 MW)	
Additions	Conditions	DTNB	4-TP
Prepared with glycerol:			
None	15–30′; 25°C	7.4 ± 0.5 (3)*	7.1 ± 0.1 (3)
Diamide 1 mM	15–30′; 25°C	3.8 ± 0.7 (3)	
Diamide 1 mM + 6 M GuHCl	15–30′; 25°C	3.6 (1)	
Diamide 1 mM	1 hr; 37°C	2.0 (1)	
Diamide 1 mM	15′; 25°C dialyzed 2 hr‡		3.5 (1)
Diamide 1 mM	15′; 25°C dialyzed 20 hr‡	4.0 (1)	
Prepared without glycerol:			
None	15–30′; 25°C	3.4 ± 0.3 (2)	
Diamide 1 mM	15–30′; 25°C	2.0 ± 0.3 (2)	

Tubulin was prepared through three complete cycles of polymerization and analyzed for sulfhydryls with 4-TP or DTNB. DTNB reaction mixtures contained 2–5 μM tubulin, 0.1 mM DTNB, and 0.1 M Tris-HCl, pH 7.5, in a final volume of 1 mM. 4-TP reaction mixtures contained 2–5 μM tubulin, 1 mM 4-TP, and 0.1 M phosphate buffer, pH 6.5, in a final volume of 1 ml. Where present, 8 M urea or 6 M GuHCl were included in the reaction mixtures.

*Number of different preparations on which determinations were made.
‡After diamide addition, tubulin solutions were dialyzed against PEM buffer at 0°C. Conditions for DTNB and 4-TP assays are as described in Fig. 1. Diamide was added to 0.1–0.2 mM solutions of tubulin. Aliquots of each sample were taken to give 2–5 μM protein in the final assay mixture. All tubulin was prepared through three cycles of polymerization either with or without glycerol.

that four sulfhydryls have disappeared (17, 18) (Table V). Given the fact that the mechanism of action of diamide on low-molecular-weight thiols is a two-step process in which the intermediate compound (see Fig. 3 and Ref. 71) is unstable, transferring its sulfhydryl adduct to another free thiol compound and forming a disulfide, diamide will probably not bind to protein sulfhydryls but most likely will cause oxidation of adjacent free sulfhydryls, becoming reduced in the process (17, 18) expecially in the absence of low-molecular-weight thiols in the medium. Thus, formation of two disulfides, which can be reversed with reducing agents, will inhibit interaction of the tubulin dimer with itself (but not break this dimer apart) and prevent formation of microtubules or rings. Whether formation of two disulfides is necessary to prevent polymerization or whether one will do is not known. However, in the work of Kuriyama and Sakai (80) blockage of two sulfhydryls with NEM, PCMB, or DTNB prevented polymerization, suggesting that formation of one disulfide (presumably a specific one) will block polymerization.

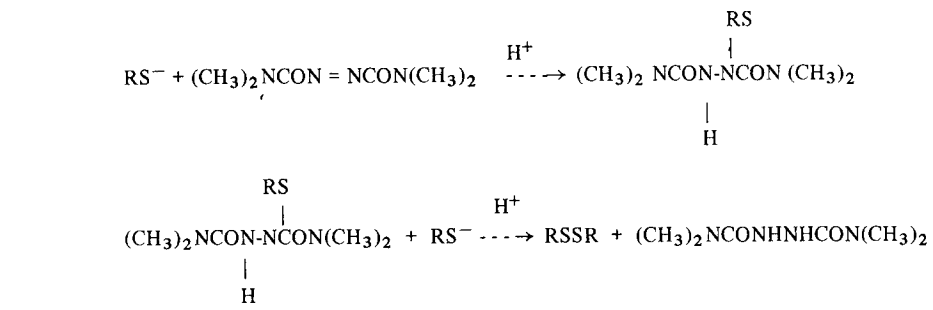

Fig. 3. Mechanism of action of diamide with low-molecular-weight thiols (71).

Diamide and a Ca^{+2}-ATP-ase

Part of our initial exploration of possible processes by which caffeine could effect the MA was through the release of calcium from intracellular stores (61), since MX have this effect on muscle sarcoplasmic reticulum (82). Mazia et al. (83) and Petzelt (84, 85) had described a calcium-stimulated ATP-ase that appeared to be more concentrated in the spindle than in the cytoplasm when the spindle formed and that showed an increase at the beginning of prophase, reached a peak at metaphase, and declined through anaphase. We investigated the levels of this ATP-ase in caffeine-treated eggs (16) and found that it was considerably decreased (Table VII). Further, it was returned to control levels by addition of the reducing agent dithiothreitol (DTT). When diamide-treated eggs were examined for the ATP-ase, no activity could be obtained, but again activity was restored to control levels by addition of DTT. Finally, the in vitro Ca-ATP-ase could be totally inhibited by diamide and restored to full activity by DTT, suggesting to us that the enzyme could be regulated by reversible oxidation of adjacent sulfhydryls as was found in the case of tubulin. The ATP-ase, however, is quite impure and is only identified by its ability to be stimulated by calcium. Nevertheless, it suggested that a dual mode of inhibition of cell division occurred with both diamide and caffeine, namely an inhibition of the polymerizability of tubulin by oxidation of its sulfhydryls and by an increase in the levels of calcium above those at which tubulin will polymerize.

TABLE VII. Ca^{2+}-activated ATP-ase Activity in Fertilized Sea Urchin Eggs

Sample	Ca^{2+}-ATP-ase activity
	N mole/mg/protein/min
Control	900 (± 60)
Control – Ca^{2+} (in vitro)	45 (± 10)
Control + DTE (10^{-3} M)*	1600 (± 80)
Control + Diamide (10^{-3} M)*	50 (± 20)
Control + Caffeine (10^{-2} M)*	872 (± 60)
Caffeine treated (10^{-2} M)$^{+}$	225 (± 30)
Diamide treated (10^{-3} M)$^{+}$	No detectable activity
Diamide treated + DTE (10^{-3} M)	920 (± 70)

Ca^{2+}-ATP-ase was assayed in water-homogenates of fertilized sea urchin eggs after cold-ethanol extraction of the eggs as described by Petzelt.

*DTE, diamide, or caffeine were added in vitro in the assay tube.

$^{+}$In the case of caffeine-treated and diamide-treated eggs, the eggs were incubated for 60–90 min with the compounds before they were collected for the enzyme assay.

Does Diamide Act on Proteins in Vivo?

The problem raised by these observations is whether the effects of diamide in vivo can be considered to arise from lowering of glutathione levels or can result from direct interaction of diamide with tubulin or with the Ca^{+2}-ATP-ase. Two arguments can be made for the suggestion that the effects are likely to be due to a lowering of glutathione levels. First, from the preceding discussion of effects of NEM on mitosis, it can be seen that spindles will disappear at glutathione concentrations 15–40% of normal; that is, not all of the glutathione is depleted when the MA disappears. Second, in studies on isolated tubulin and the Ca^{+2}-ATP-ase, addition of glutathione prior to addition of diamide protected the tubulin from oxidation. These arguments suggest – although they clearly do not prove – that disappearance of the MA is a consequence of reduction of glutathione levels rather than direct effect on the proteins themselves. The same argument was used by Oliver et al. (86) in studies of the induction of capping in lymphocytes by diamide and will be discussed below.

These results suggest, however, that a number of proteins may be capable of regulation by oxidation and reduction of adjacent sulfhydryls. In addition to tubulin and the Ca^{+2}-ATP-ase, it has been shown that a CAMP-dependent protein kinase from kidney is inhibited by diamide in a manner reversed by reducing agents (87). Further, in a study of the NAD-kinase of sea urchin eggs (88), we have found a complete inhibition by diamide and reversal by DTT (89). In a study of an ATP-ase in the cortex of sea urchin eggs, Mabuchi (90) has found that it is activated in the reduced state and inactivated on oxidation. That not all sulfhydryl-containing enzymes are affected by diamide has been shown by Srivastava et al. (91). Similarly, glutathione reductase, which has oxidation-reduction adjacent sulfhydryls in its active site, is not affected by diamide presumably because they are protected by interaction with a flavin ring (92). These results suggest that only certain proteins containing sulfhydryls will be affected by diamide, and we submit that these are proteins that contain adjacent sulfhydryl groups that are not in the active site of the enzyme. It should also be noted that the five examples we possess are all intracellular proteins active in the reduced state and inactive in the oxidized state. This is precisely the opposite of enzymes such as RNA-ase (93), DNA-ase (94), and lysozyme (95), which are extracellular proteins inactive in the reduced state and active in the oxidized state.

It is possibly a coincidence that the intracellular proteins are all active in the reduced state and possess easily accessible sites that can activate or inactivate the protein by change in the oxidation-reduction state of its sulfhydryls. However, it is tempting to suggest that this is not accident and that this possibility, open to manipulation by the investigator, has not been neglected by evolution in designing the cell. It should also be noted that the proteins we are dealing with are those that are not necessarily active at all times in cellular life. They are proteins that can be rapidly turned on or off as needed to meet the necessities of fertilization, mitosis, hormone action, etc. We will now consider possible modes by which these proteins may be controlled in the intracellular environment.

Possible Mechanisms for Control of the Sulfhydryl-disulfide Status of Tubulin, NAD-kinase, CA^{+2}-ATP-ase, Protein Kinase, and Cortical ATP-ase

So far we have been skirting the edges of hypothesis and staying relatively close to a (possibly disparate) set of facts. We now take the plunge wholeheartedly and speculate on what might be the regulatory mechanisms that alter the state of oxidation and reduction of intracellular proteins that require control. We will present two possible models. The

first assumes that the oxidized state of the protein is one in which intramolecular disulfides are formed. In the second the oxidized state is one in which a mixed disulfide of protein and glutathione (or cysteine) is the oxidized form. In both, the strategy of the cell is to keep the inactive (oxidized) form present until there is a stimulus to activation and then to reduce the protein to active form. Since a number of disulfides in the cell are reduced by obtaining reducing equivalents via NADPH (more rarely NADH) and a reductase, we will assume that this is also true for the proteins we are discussing (for more complete discussion, see Rebhun et al., Ref. 16). For activation of the proteins we therefore assume the existence of an NADPH- or NADH-dependent reductase that can be activated on demand and that converts the disulfide group to sulfhydryls. It would be tempting to suggest that glutathione reductase is such a tubulin, NAD-kinase, reductase etc. In initial experiments with tubulin oxidized by diamide, we did not find oxidation of NADPH in the presence of yeast or sea urchin glutathione reductase (when all traces of diamide, which can oxidize NADPH, are removed). However, Srivastava and Beutler (96, 97) have found that the mixed disulfides of glutathione and lens crystalline proteins, hemoglobin, and albumin can serve as substrates for yeast GR that cleaves the glutathione by using NADPH (or NADH) as source of reducing power. Since yeast GR can hardly have developed the ability to reduce the mixed disulfide of hemoglobin or lens crystalline protein by specific selection pressures, these results indicate that the enzyme may function to uncover needed sulfhydryls on enzymes that are normally protected from oxidative stress by combination with glutathione (97).

We thus have some precedent for the energy-dependent part of the protein-activating process under consideration and evidence that GR could act as the needed reductase. The question is What returns them to the oxidized state? and more specifically What keeps them in this state? The problem is somewhat acute since the intracellular environment is largely a reducing one because of the greater concentration (20–50 times) of reduced over oxidized glutathione, at total glutathione levels of 1–11 mM (73, 74, 76). One would expect that under these conditions disulfides would be reduced, as occurs, for example, in the pancreatic trypsin inhibitor which depends on three disulfides for its activity (98). However, precedent exists for suggesting that this excess of reducing power in the form of reduced over oxidized glutathione drives some protein sulfhydryls into disulfide form. Detailed analyses have been given for the renaturation of the reduced (inactive) forms of the extracellular proteins lysozome (95) and serum albumin (99, 100). In both cases, the goal was to find conditions that would favor the most rapid rates of return of native configuration, which to a large extent meant formation of the proper disulfide bonds. In both cases, maximum rates of renaturation occurred when the buffers contained low-molecular-weight thiols in which the reduced form was in 10-fold to 20-fold excess over the oxidized form and the absolute concentration of the thiol was about 1 mM. However, the results were more sensitive to the ratio of reduced to oxidized form than to the absolute quantities of agent. Glutathione was the agent of choice, although cysteine-cystine would also work. The mechanisms involved in renaturation involved sulfhydryl-disulfide interchanges, which allow any incorrect disulfides to be reduced so that "hunting" for the proper disulfide can continue. The stability of native disulfides appears to be partly conferred on the bond by the native configuration of the protein (95), which is then "fixed" by the disulfide bond. There is considerable question, of course, as to whether this is a legitimate model for application to intracellular proteins, since in the latter case we are presumably not talking about a denatured, random coil configuration in the oxidized state, but are talking about a two-state system – both states, and the transition between them, pre-

sumably being well defined. Nevertheless, the work offers a precedent for the possibility that a reducing atmosphere can drive a protein disulfide into stable form in competition with a low-molecular-weight disulfide.

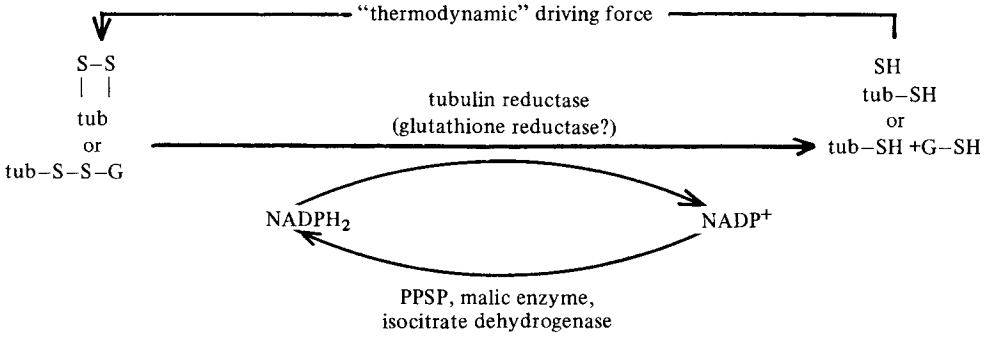

Fig. 4. Hypothetical schema for regulation of protein sulfhydryl-disulfide status using tubulin as illustration.

Of considerable importance are the observations concerning the second model we have proposed, in which the oxidized state is a mixed disulfide with a low-molecular-weight thiol, of which glutathione is a prime candidate. In the work of Srivastava and Beutler (96, 97), it was shown that the mixed disulfide of protein and glutathione was not reduced by glutathione in the case of lens protein and only reduced at very high glutathione concentration in the case of hemoglobin-S-S-G. Again, this indicates that the disulfide bond in the protein is highly stable in the presence of low-molecular-weight thiols and that the intervention of an enzymatic system for cleaving the glutathione from the protein is necessary.

A possible model for the case where the target protein is in the disulfide form has been proposed (15, 16). The case where the oxidized form is a glutathione-protein mixed disulfide is summarized in Fig. 4. In both of these models an NADPH-dependent protein reductase, probably glutathione reductase, is the central control element. The cyclic variation of GR in sea urchin eggs (68, 69) or its activation at the inception of phagocytosis (101) makes it an important enzyme to study for its control properties. However, there are other potential control sites involving the regulation of small molecules: NADPH (or, possibly, NADH) is an interesting candidate, and we remind the reader that either oxidizing NADPH or utilizing the NADP analogue amino pyridine adenine dinucleotide (15) results in disappearance of the MA. Thus, our central model requires a reductase for the disulfide or mixed disulfide form of the protein, a method to control this reductase, and a source of power, usually NADPH. The latter can be the pentose phosphate shunt pathway (via glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, both of which produce NADPH from NADP); or it can be the malic enzyme (102) or cytoplasmic isocitrate dehydrogenase (103), both of which also produce NADPH from NADP. These appear to be the only sources of NADPH that have been investigated and may be important in systems in which the PPSP is not present (for example, Rosenstraus and Chasin (104) have reported on a mutant of CHO cells that does not possess glucose-6-phosphate dehydrogenase but nevertheless lives and divides). The system is complex but does not present an inordinately large number of control sites (Fig. 4). It will be most interesting to see whether the postulated processes occur in cells and

what surprises are in store as we attempt to investigate this. That we are close to an important cell regulatory system is clear from the wealth of classical data suggesting that the approach of mitosis is heralded by an increase in the state of reduction of cellular proteins (105, 106).

Glutathione and Microtubules in Other Systems

The question arises whether the relation of glutathione, NADPH, and GR, that we have been developing in marine eggs can be applied to any other system. This appears to be so in two other processes, both of which are associated with polymorphonuclear leukocytes (PMNs) and involve phagocytosis on the one hand and the induction of capping on the other. It is possible to dissociate two aspects of phagocytosis by the use of the sulfhydryl alkylating agent NEM. In its presence PMNs are able to phagocytose particles but are unable to digest them (107). In this study it was also shown that NEM at the effective concentrations reduced glutathione levels to 20–30% of normal and inhibited the activity of the PPSP. No EM morphological studies were reported with this system, but from other work it is clear that a microfilament-associated phagosome formation process can be dissociated from a lysomal secretion process which appears to require the assembly of microtubules for its activity. The work of Reed (107) suggests, therefore, that NEM affects the assembly of microtubules possibly through the reduction of glutathione levels, as we have suggested above for the effects of NEM on spindles. A further phenomenon that suggests the relation of the glutathione system to microtubule assembly is that reported by Strauss et al (101) in which glutathione reductase was shown to double its activity within the first 15 sec of a phagocytic stimulus. Strauss et al. (101) attempt to relate this activation to the needs of the cell for NADP, the substrate for the first two enzymes of the PPSP and used for production of NADPH needed for NADPH oxidase and H_2O_2 production. However, the oxygen increase associated with activation of that pathway occurs about 1.5 min after a phagocytic stimulus (at least 1 min later than GR activation) and must make use of NADPH already in the cell; that is, the NADP utilized by the PPSP can at most replace the NADPH converted by GR to NADP and cannot involve any net increase in the reduced nucleotide. However, the assembly of microtubules takes place within 15–30 sec after stimulation (58) and thus correlates well with the period in which glutathione reductase activation occurs: We suggest that this is the significant correlation, rather than that with activation of the PPSP; this calls to mind again the activation of GR in sea urchin eggs and its correlation with spindle formation.

A second process involving microtubules in PMNs and lymphocytes also appears correlated with sulfhydryl processes, as shown in the work of Oliver et al. (86). It was shown by this group that diamide induced capping in these cells in conditions in which it was inhibited by Con A. Further, the capping occurred at intracellular levels of glutathione that were 30–70% of normal and occurred in more cells and in shorter time than was seen with colchicine. This group also worried about the possibility that diamide could induce formation of protein disulfides in the living cell, or more to the point, tubulin-glutathione complexes as shown in erythrocytes for hemoglobin-glutathione by Srivastava and Beutler (108), therefore they used the hydroperoxides introduced by Srivastava et al. (97) to avoid this problem. These compounds bleed off glutathione by using its reducing equivalents to reduce the peroxides enzymatically through glutathione peroxidase, and they do not affect tubulin polymerization directly (86). With these peroxides, capping is again induced in Con A-blocked PMNs and lymphocytes. In electron micrographs it was shown that

the induction of microtubule assembly that normally accompanies treatment of these cells with Con A did not occur when the cells were treated with diamide or hydroperoxides, thus completing the link between glutathione and microtubule assembly.

SUMMARY

We now have evidence in three processes that microtubule assembly can be affected by the glutathione system in two ways: 1) by reduction of glutathione levels to 15–70% of normal (depending on the system) and 2) by variation in the level of glutathione reductase either normally – as shown in the work of Ii and Sakai (68, 69) in sea urchin eggs and by Strauss et al. (101) in PMNs – or by experimental inhibition in caffeine-treated eggs (15, 16, and this paper). It is of considerable importance to recognize that in sea urchin eggs, normal fluctuation of GR is not accompanied by variation in reduced or oxidized glutathione levels, and in caffeine-treated eggs glutathione levels are also equal to control. These results strongly suggest that it is the enzymatic uses to which glutathione is put that are of importance as the control system in normal cells, and that control under physiological conditions (as opposed to those imposed by exogenous inhibitors) is through glutathione reductase (or an NADPH-dependent protein reductase with tubulin, NAD-kinase, etc. as substrate) rather than through regulation of GR levels. It is these considerations that lead us to postulate the schemes in Fig. 4.

Cyclic Nucleotides and Sulfhydryl Processes: Where May the Relation Occur?

Our introductory remarks suggested that there could be a relation between the effects of cyclic nucleotides and microtubule assembly-disassembly processes. It is also clear from that discussion that cyclic nucleotides can only affect such processes in a most indirect fashion and only in some cells. We emphasize this again by pointing out that some cells do not require a prior period of translation and transcription for effects of cyclic nucleotides to occur (CHO cell; Patterson and Waldren, Ref. 56), whereas others do (109). Also, in some cells a CAMP increase signals assembly of microtubules (CHO cells), while in others such an increase is associated with disassembly (but CGMP is a positive signal) (PMNs; Refs. 57, 58). While the potential relations of cyclic nucleotides, sulfhydryls, and microtubules can be myriad, there are some simple observations that are indicated. The great rapidity of CGMP production in PMNs as a result of a positive stimulus and the rapid activation of glutathione reductase and assembly of microtubules (all within 15 sec) suggest that not too many steps are involved. Our model would dispose one to look for modulation of GR activity either directly by cyclic nucleotides (which would clearly violate the thesis that CN act through protein kinases) or more likely by phosphorylation of GR. Since CAMP protein kinases may be partially regulated by reduction of disulfides (88) (either intramolecular or mixed disulfides with glutathione) and GR may be involved as discussed above, one can envision a further activation mechanism whereby PK is activated by GR, which further activates PK. Such a mechanism would ensure a rapid and complete activation of both enzymes. These ideas are readily tested.

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