Accumulation of Adrenocorticotropin Secretory Granules in the Midbody of Telophase AtT20 Cells: Evidence That Secretory Granules Move Anterogradely Along Microtubules

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Abstract. During the cell cycle the distribution of the ACTH-containing secretory granules in AtT20 cells, as revealed by immunofluorescence labeling and electron microscopy of thin sections, undergoes a cycle of changes. In interphase cells the granules are concentrated in the Golgi region, where they form, and also at the tips of projections from the cells, where they accumulate. These projections contain many microtubules extending to their tips. During metaphase and anaphase the granules are randomly distributed in the cytoplasm of the rounded-up mitotic cells. On entry into telophase there is a rapid and striking redistribution of the granules, which accumulate in large numbers in the midbody as it develops during cytokinesis. This accumulation of secretory granules in the midbody is dependent upon the presence of microtubules. The changing pattern of distribution of the secretory granules during the cell cycle fulfills the predictions of a model envisaging first that secretory granules associate with and move along interphase microtubules in a net anterograde direction away from the centrioles, and secondly that they do not associate with microtubules of the mitotic spindle during metaphase and anaphase.

In nerve cells during the anterograde transport of synaptic vesicles along the axon from the cell body to the synapse, microtubules act as rails (1, 25). Although most of the key experiments to date have been done using the squid giant axon, there is evidence that microtubules provide rails for the movement of organelles in other cell types (14, 18, 19).

We have been studying the formation of the ACTH-containing secretory granules in AtT20 cells (27), a line of pituitary tumor cells that secrete ACTH (10, 12, 21, 24). In these cells the secretory granules, which enter a regulated exocytic pathway (13), are formed at the trans side of the Golgi apparatus (27). However, the granules characteristically accumulate at the tips of projections from the cell (16). Under the electron microscope, Kelly et al. (16) observed microtubules in thin sections of projections from AtT20 cells. Moreover, Buckley and Kelly (5) have identified a transmembrane protein common to secretory vesicles of both neurons and endocrine cells, including the ACTH-containing granules of AtT20 cells. There is reason, therefore, to think that the migration of secretory granules in AtT20 cells might, like the movement of synaptic vesicles down axons, be both dependent on microtubules and anterograde.

If this were the case one would expect microtubules to extend down the projections of interphase AtT20 cells to their tips where the secretory granules accumulate. But what would be the distribution of secretory granules in the cytoplasm of mitotic AtT20 cells? During metaphase and anaphase, after the depolymerization of the interphase microtubules, one would predict that the granules would become randomly distributed in the peripheral cytoplasm, outside the mitotic spindle of the rounded-up cell, as are other organelles in mitotic cells, for example, vesicles derived from the Golgi apparatus, elements of the endoplasmic reticulum, and mitochondria (23, 32). One might also venture to predict that during telophase, as soon as the interphase state is restored, secretory granules might accumulate in the midbody region. The reason for such a prediction is that, in the region of the midbody, microtubules emanating from the daughter cells overlap and interdigitate. If at telophase the secretory granules were to associate with these microtubules and begin a net anterograde migration, they would reach the ends of the microtubules at the midbody. Secretory granules reaching the midbody from each daughter cell should not then be able to migrate further, for example, into the opposite daughter cell, if the overall movement along the microtubules is vectorial. Here we present evidence that at telophase secretory granules do indeed accumulate in the midbody of AtT20 cells as predicted.

Materials and Methods

Cell Culture

The AtT20 cells (clone D16V) were cultured as described previously (27) on Falcon plastic culture vessels (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) or on glass coverslips. In our hands the cell doubling time is ~40 h (26).
Figure 1. AtT20 cells immunolabeled for ACTH. (A) AtT20 cell with a long process labeled with anti-ACTH antiserum and a rhodamine-conjugated second antibody. Note secretory granules along the length of the process and accumulated at its tip and also in the Golgi region. There are very few granules in other parts of the cytoplasm of this particular cell. (Inset) Another example of a row of granules migrating to the tip of a process. (B) The same fields viewed under Nomarski optics. Note that the Golgi region is clearly seen. (C) Electron micrograph of the tip of an AtT20 cell in a cryo-thin section after labeling with anti-ACTH antiserum and protein A conjugated to gold. Note that the labeling is restricted to the cores of the secretory granules and dissolving granules that have been released into the medium (arrow). Bars in A and B, 10 μm; in C, 0.1 μm.
To increase the population of mitotic cells, cultures were exposed to a thymidine block followed by a nocodazole block. Briefly, cultures were incubated in culture medium containing 2 mM thymidine for 20-24 h. They were then washed twice with medium and incubated for 4-5 h in normal medium. At this point nocodazole was added to the medium to a final concentration of 40 ng/ml and the cultures were incubated for 2 h to accumulate metaphase cells. The nocodazole medium was then removed and the cells washed and given normal medium. Samples of cells were then fixed immediately and at 15-min intervals thereafter over a period of 1½ h for either immunofluorescence microscopy or electron microscopy. In some experiments cultures at 35, 40, and 45 min after the release of the mitotic block were exposed to 4 μg/ml of nocodazole in culture medium. This concentration of nocodazole rapidly depolymerizes the microtubules. At 50 min after the mitotic block these cultures were fixed and processed for immunocytochemistry using anti-ACTH antiserum.

**Antisera**

We raised in rabbits an antiserum against swine ACTH (Serva Ltd., Heidelberg) by immunizing the animals with the swine ACTH cross-linked to keyhole limpet hemocyanin (Sigma Chemical Co., Munich) following a published procedure. The specificity of the antiserum for ACTH was tested by (a) immunoblotting of both swine ACTH and extracts of AtT20 cells, (b) immunoprecipitation of AtT20 cell extracts, and (c) immunofluorescence labeling of AtT20 cells. The antiserum proved to be specific for ACTH and its precursor pro-opiomelanocortin.

**Immunofluorescence Microscopy**

Cells growing on glass cover slips were fixed in 3% paraformaldehyde in phosphate-buffered saline and labeled with either the antiserum raised against ACTH (see above) or with a rabbit antitubulin antibody (generously provided by Dr. E. Karsenti, European Molecular Biology Laboratory, Heidelberg) using the procedure described by Ash et al. The second antibody was a sheep anti-rabbit antibody conjugated to rhodamine. Cells were observed using a Zeiss photomicroscope III with a planapo ×63 oil immersion objective and appropriate filters. The cells were also stained with Hoechst dye 33258 to reveal the nuclei or chromosomes.

**Electron Microscopy**

Cells growing on petri dishes were fixed for 1 h in a 1% solution of glutaraldehyde in 100 mM Pipes buffer (pH 7.0), then washed in buffer and postfixed for 1 h in 2% OsO4 in 0.1 M cacodylate buffer (pH 7.3). The cells were then dehydrated in ethanol and embedded in Epon 812. Thin sections were contrasted with uranyl acetate and lead citrate and viewed in Philips electron microscopes.

Some cultures were fixed and prepared for cryosectioning as described previously. Frozen sections were then labeled with the anti-ACTH antiserum followed by protein A conjugated to 8-10 nm gold particles.

**Results**

**Accumulation of ACTH Granules in Interphase Cell Processes**

On tissue culture plastic surfaces AtT20 cells grow in large clumps many cells deep rather than as a monolayer of well-spread cells. However, at low cell densities individual cells can be observed. Characteristically, cells in direct contact with the plastic substratum have one or more projections that vary in geometry from short fingerlike protrusions to very thin processes >100 μm long ending in a spread growing tip. The latter are very reminiscent of the growth cones of cultured nerve cells (e.g., see Figs. 1 and 2). When labeled for immunofluorescence microscopy with antisera raised against ACTH (see Materials and Methods), the tips of these processes are seen to contain large accumulations of ACTH-positive granules (Fig. 1 and reference 16). Granules can also be seen along the length of the fine processes extending from the cell body (Fig. 1). Within the cell body the Golgi region is heavily labeled for ACTH but elsewhere in the cytoplasm granules occur at a much lower concentration (Fig. 1). Electron microscopic immunogold labeling of the granules at the tips of projections confirms that ACTH is restricted to the cores of the granules (Fig. 1 C); in fact, the only ACTH-positive structures detectable by immunogold labeling in AtT20 cells are the secretory granules and condensing secretary protein in the trans-Golgi network (27). When cells are labeled for immunofluorescence microscopy using an antitubulin antibody, microtubules can be seen extending into the spread tips of cell processes where they fan out to form a dense network (Fig. 2).

**Distribution of ACTH Granules during Mitosis**

To increase the population of cells in mitosis cultures were subjected to a thymidine block followed by a nocodazole block (22, 33). This resulted in ~5-10% of the cells going semisynchronously through mitosis while remaining attached to other cells in the culture or to the substratum. We did not recover and examine any mitotic cells which detached and floated in the medium.

In early prophase cells, identified by the condensation of
Figure 3. Mitotic AtT20 cells immunolabeled for ACTH. (A–C) Cell in early prophase. The condensing chromosomes are revealed by staining with Hoechst dye (A). The corresponding Nomarski image is shown in B whereas the distribution of secretory granules revealed by immunofluorescence labeling with anti-ACTH antiserum is shown in C. Note that secretory granules are concentrated in the perinuclear Golgi region and at the tip of a short cell process, the characteristic interphase distribution. (D and E) Two cells at metaphase. The Nomarski image (D) shows that the cells have completely rounded up and sit on top of interphase cells. The labeling with anti-ACTH antiserum (E) reveals the random distribution of the secretory granules throughout the cytoplasm. (Inset to D) The metaphase plate of the cell immedi-
chromosomes revealed by staining with Hoechst dye, the distribution of ACTH granules resembles that in interphase cells (Fig. 3, A–C). As the cells round up and enter metaphase, however, the granules become randomly distributed in the cytoplasm, which is intensely fluorescent after labeling for ACTH (Fig. 3, D and E). During anaphase, while the chromosomes move apart, the cytoplasm remains uniformly intensely fluorescent as in metaphase (Fig. 3, F–H). However, as the cells enter telophase and cytokinesis begins, there is a striking redistribution of the fluorescently labeled granules. Early in telophase the granules accumulate in the cytoplasm of the two daughter cells adjacent to the developing cleavage furrow. In favorable micrographs the granules appear to be in rows radiating from the midbody (Fig. 4, A and B) or in a rod-shaped cluster spanning the midbody (Fig. 4, C–E). By late telophase many secretory granules have accumulated precisely in the midbody between the two daughter cells (Fig. 5), which becomes much more intensely fluorescent than the rest of the cytoplasm. As can be seen from the immunofluorescence labeling, the extent of this accumulation of ACTH granules in the midbody is similar to that at the tips of processes extending from interphase cells (Fig. 5 B).

Under the conditions we use, a significant number of mitotic cells have reached late telophase 45 min after the release of the metaphase block. To test whether or not microtubules are essential for the accumulation of secretory granules in the midbody, we exposed cultures at 35, 40, and 45 min after the release of the mitotic block to 4 μg/ml of nocodazole. This high concentration of the drug results in the rapid depolymerization of most of the microtubules. At 50 min after the mitotic block the cells were fixed and labeled for ACTH. As Fig. 6 shows, when most of the microtubules were depolymerized in this way, the secretory granules were no longer concentrated in the midbody region of late telophase cells. In contrast, the labeling of the cell cytoplasm had increased.

Figure 4. AtT20 cells at early telophase. (A and B) An early telophase cell stained with Hoechst dye and labeled with anti–ACTH antiserum, respectively. In B the secretory granules labeled for ACTH are seen aggregating in the cytoplasm of the daughter cells in the region of the cleavage furrow. The developing midbody region is too intensely fluorescent to allow resolution of individual granules. However, in the region indicated with an arrow, the granules appear to be aligned in rows focussing into the midbody. (C–E) Another early telophase viewed under Nomarski optics, after staining with Hoechst dye, and after labeling for ACTH, respectively. The ACTH labeling is concentrated in a rod extending across the cleavage furrow and parallel to the long axis of the midbody (arrow in E). The developing midbody is visible in the Nomarski image (arrow in C). All micrographs at the same magnification. Bar, 10 μm.
We conclude that intact microtubules are required for the accumulation of secretory granules in the midbody.

The rabbit anti-ACTH antiserum that we have used immunoprecipitates only ACTH and its precursor polypeptide pro-opiomelanocortin from extracts of AtT20 cells (Tooze et al., manuscript submitted for publication). Furthermore, this antiserum does not label the cytoplasm of Chinese hamster ovary (CHO) cells, which we used as a control, at any
stage of the cell cycle including telophase (data not shown). There is, therefore, no reason to doubt that the antiserum is completely specific for ACTH, and that the labeling of the midbody of telophase AtT20 cells reflects the accumulation of secretory granules there.

Electron Microscopy of Secretory Granules in Mitotic AtT20 Cells

In thin sections of metaphase and anaphase AtT20 cells under the electron microscope, secretory granules can be seen, together with mitochondria and membrane-bound vesicles and sheets of rough endoplasmic reticulum, in the cytoplasm between the chromosomes and the plasma membrane (not shown). At these stages of mitosis there is no obvious preferential distribution of the granules within the cytoplasm outside the mitotic spindle. By contrast in the dumbbell-shaped pairs of late telophase daughter cells, the secretory granules are concentrated in the midbody (Fig. 7), lying in rows between the microtubules. In sections longitudinal to the midbody the secretory granules can be seen up to where the microtubules interdigitate. Sometimes they are absent from the region of overlap itself (not shown) but in other cells they appear to penetrate it (Fig. 7 C). The electron microscopic images are exactly as expected from the pattern of immunofluorescence staining. They show that the secretory granules are indeed accumulated only within the midbody itself. In the cytoplasm immediately adjacent to the midbody, there is no accumulation of granules (Fig. 7 A). In other words, there is no gradual gradient of granules leading into the midbody but rather a high concentration of granules in the midbody and then abruptly a change to the average cytoplasmic concentration of granules. We have never seen, in sections of late telophase cells with a fully developed midbody, large numbers of granules associated with those parts of the midbody microtubules that radiate into the daughter cell cytoplasm.

As Fig. 7, A and C show, the number of secretory granules seen in individual thin sections of midbodies varies considerably. There are two explanations for this variation. First, single thin sections, ~50 nm thick, sample only a small fraction of the total volume of a midbody which is a cylinder ~1.7–2 μm in diameter and 3–4 μm long. Secondly, as judged from the intensity of immunofluorescence labeling with anti-ACTH antiserum, the number of secretory granules can vary significantly from cell to cell.

Counts of the secretory granules in the midbody and in the cytoplasm of the paired daughter cells, as revealed in electron micrographs, indicate that the concentration of the granules per unit volume of thin section is some 10–15-fold higher in the midbody than in the rest of the telophase cytoplasm. This order of magnitude difference in the concentration of the granules is consistent with the indirect immunofluorescence labeling (Fig. 5). However, counts of granules in thin sections of the midbody of late telophase cells and in the tips of cytoplasmic projections of interphase cells indicate comparable accumulations at these two locations, as is apparent from a comparison of Fig. 7 and Fig. 1 C.

Apart from secretory granules the only other structures repeatedly seen within the midbodies are flattened, usually smooth-membraned vesicles (Fig. 7 C), whose origin remains to be determined, although some must be derived from the rough endoplasmic reticulum (see Fig. 7 B). Unfortunately, none of the antibodies against integral proteins of the Golgi apparatus that we have available (6) specifically label the Golgi apparatus of AtT20 cells. We have been unable, therefore, to investigate whether any of the smooth vesicles or secretory granules in the midbody have Golgi markers in their membranes. Mitochondria do not enter the midbody whereas they are present at the tips of projections from interphase cells, as first noted by Kelly et al. (16).

Discussion

In AtT20 cells secretory granules form in the trans-Golgi network (27), but they accumulate at the tip of processes extending up to >100 μm from the interphase cell bodies. This distribution of the secretory granules means that there must be a net anterograde migration of the granules in interphase cells. In the absence of dynamic studies, we cannot say whether the movement of the granules is exclusively anterograde, or whether individual granules can move in both anterograde and retrograde directions. The net migration, however, must be anterograde. The presence of microtubules along the fine processes, and forming a dense net at their flattened tips, is consistent with the notion that in AtT20 cells secretory granules move along microtubules in a manner...
analogous to the movement of synaptic vesicles in axons (1, 25, 29).

Although this distribution of microtubules and secretory granules in interphase AtT20 cells is compatible with vectorial, net anterograde transport of the granules, it is the rapid redistribution of granules during telophase that provides the really compelling, albeit still circumstantial evidence for this idea. During mitosis the Golgi apparatus vesiculates (30–32), transport of proteins from the rough endoplasmic reticulum to the Golgi region is inhibited (11, 30) and endocytosis (3, 4, 9) and exocytosis (15) cease. The secretory granules in the metaphase mitotic AtT20 cells, which were accumulated during a 2-h exposure of the cultures to nocodazole must, therefore, have been formed before mitosis began. Moreover, in the absence of exocytosis, none should be secreted before the restoration of the interphase state in the daughter cells. It follows that the movement of the secretory granules during the successive stages of mitosis in AtT20 cells represents the successive redistribution of granules from their previous interphase locations.

The difference in the distribution of the secretory granules in interphase and metaphase cells (compare Fig. 1 with Fig. 3, D and E) is striking. It establishes that both the long cytoplasmic projections and the clusters of granules at their tips are lost when interphase microtubules become reorganized into the mitotic spindle and the cell completely rounds up. The fact that the clusters of granule at the tips of interphase projections do not remain at metaphase as clusters, but instead disperse to become randomly distributed in the cytoplasm, is particularly significant. It means that there is no special "sticky" matrix at the tips of the interphase projections which holds the granules in a cluster, or, if such a putative matrix exists, it does not survive the reorganization of the cytoskeleton at mitosis. When interphase cells are exposed to 4 μg/ml of nocodazole for 60 min, which depolymerizes essentially all of their microtubules, the secretory granules become progressively more uniformly distributed throughout the cytoplasm, mimicking the metaphase situation. At the same time many of the cell projections become much shorter and fatter (data not shown). We suggest that a net anterograde migration along the microtubules is both the necessary and sufficient event to cause the granules to accumulate at the tips of interphase projections.

The scattered distribution of secretory granules throughout the peripheral metaphase and anaphase cytoplasm indicates that they do not associate with microtubules of the mitotic spindle at these stages of mitosis. The rapid relocation of the secretory granules into the midbody in the few minutes required for the cleavage furrow to develop is both striking and unusual; we know of no other example of cytoplasmic organelles specifically relocating to the midbody of animal cells. Moreover, our colleague Clive Walter, who has recently examined under the electron microscope thin sections of several hundred mitotic baby hamster kidney (BHK) and CHO cells, found no evidence of identifiable organelles within any of the many midbodies he examined (personal communication). This relocalization of secretory granules in AtT20 cells is, however, precisely what would be expected if the granules suddenly became able to telophase once again to associate with microtubules and migrate along them in a direction away from the centrioles. At cytokinesis the granules would automatically accumulate in the midbody where the microtubules, emanating from the two daughter cells, interdigitate. Because the microtubules of the two daughter cells have opposite polarities migration beyond the midbody would be precluded if the net migration was anterograde.

Although the microtubules of the mitotic spindle are characterized by their dynamic instability, (see review in reference 17), two subsets are apparently stabilized. They are the microtubules captured by kinetochores of the chromosomes and the microtubules that overlap at the equator of the spindle. Kirschner and Mitchison (17) propose that lateral cross-linking interactions between the overlapping and antiparallel microtubules might serve to cap them. Whether or not the midbody is formed from these preexisting microtubules of the mitotic apparatus or from newly polymerized microtubules at telophase, or a mixture of both is still debated (8). At the very least, however, preexisting microtubules appear to be necessary for the accumulation of microtubules in the midbody (8). Irrespective of their origin, it is the midbody microtubules which, we propose, act as the rails for the migration of secretory granules into the midbody.

Without specific markers for the secretory granules which could be introduced into living cells, dynamic studies of granule movement are precluded. Even if such markers were available, dynamic studies would be restricted to the projections of interphase cells. In the fully rounded-up metaphase cells, it would not be feasible to follow individual granules. We cannot, therefore, directly prove our interpretation of the changing distribution of secretory granules as cells pass from interphase through mitosis and to telophase. However, the model that we propose accounts for the pattern of distribution of the granules at all stages of the cell cycle and alternative simple hypotheses are not compatible with our observations. For example, the fact that accumulation of granules in the midbody is dependent on microtubules rules out the suggestion that some putative "sticky protein," unrelated to microtubules, accumulates in the midbody and adventitiously captures secretory granules. The fact that mitochondria are excluded from the midbody, while secretory granules and only a few smooth-membraned vesicles are concentrated there, makes highly unlikely any suggestion that, as microtubules are constricted into the midbody, they

Figure 2. Electron micrographs of midbodies between telophase daughter AtT20 cells. (A) Large numbers of secretory granules are seen accumulated in rows between the microtubules but mitochondria are excluded. Note the newly formed nucleus (N). For purposes of orientation, the arrowheads mark the secretory granules that are shown at higher magnification in B. (B) At higher magnification it is clear that apart from the secretory granules the only other structures that have migrated into the midbody are membrane-bound vesicles. Some of these must be derived from the endoplasmic reticulum because of the presence of budding intracisternal A-type particles (long arrow), which are morphologic markers of the rough endoplasmic reticulum in these cells (28). (C) Another midbody. The smooth-membraned vesicles that apparently migrate into the midbody with the secretory granules are indicated by arrows. This micrograph also shows secretory granules within the region of overlapping microtubules (arrowhead). Bars in A, 1 μm; in B and C, 0.1 μm.
simply sweep the secretory granules out of the cytoplasm. If such sweeping occurred one would expect to find mitochondria in the midbodies and also secretory granules along the lengths of the microtubules extending from the midbody into the daughter cell cytoplasm, which is not the case at late telophase. To achieve a 10–15-fold concentration of secretory granules within the midbody itself, with no obvious accumulation of granules along the microtubules fanning out from it, must, in our view, mean migration of the granules into the midbody along the microtubules.

In short, our observations fulfill the predictions of a model that envisions the following: (a) secretory granules associate with and migrate along interphase microtubules in a net anterograde direction and as a result accumulate in the tips of cell projections; (b) this association is inhibited when the interphase microtubules reassemble into the mitotic spindle and the clusters of granules, previously at the tips of the projections, are free to disperse in the metaphase cytoplasm; (c) immediately upon restoration of the interphase state at telophase, the association and net vectorial movement along microtubules are again possible for the secretory granules. As a result within the few minutes needed for the formation of the cleavage furrow and midbody, there is a 10–15-fold concentration of granules within the latter.

The exclusion of mitochondria from the midbody merits comment because mitochondria are abundant amongst the secretory granules at the tips of interphase AtT20 cells (16 and our unpublished results). We have no direct evidence bearing on this question but it should be borne in mind that the migration of secretory granules into the midbody occurs rapidly; formation of the cleavage furrow and midbody takes <10 min in mammalian cells (8). The exclusion of mitochondria from the midbodies containing secretory granules could be taken as evidence that secretory granules move vectorially much more rapidly than the larger mitochondria. Because AtT20 cells divide only every 40 h (26), any difference in the rate of migration would be of much less significance during interphase.

Burke et al. (6) reported that, in telophase NRK cells, Golgi membranes accumulate in a region that corresponds to the cytoplasmic ends of the midbody microtubules in the daughter cells. Unfortunately, the antibodies used by Burke et al. proved not to label specifically the Golgi apparatus of AtT20 cells. This precluded a double-labeling experiment with AtT20 cells and anti–ACTH and anti–Golgi antibodies. However, in the light of the findings reported here, we suggest that the accumulation of Golgi membranes at the region where midbody microtubules end in the cytoplasm may well result from the migration of Golgi vesicles in the mitotic cytoplasm along the midbody microtubules, but in a direction opposite to that of the secretory granules.

The biochemical basis of the inhibition of the association of secretory granules with spindle microtubules during metaphase and anaphase remains to be elucidated; it could, for example, result from the presence (or absence) of a particular microtubule associated protein during mitosis, or to a transient change in the level of some secondary modification to a microtubular protein during mitosis. Alternatively, the surface of the secretory granules may be transiently modified possibly by phosphorylation. Whatever its cause, the inhibition of association of cytoplasmic organelles with mitotic microtubules presumably facilitates the efficient and accurate segregation of the chromosomes during anaphase. Once that crucial event is achieved the microtubules can then assume their interphase state and function. Like other events restoring interphase, for example, reconstitution of the nuclear envelope (23) and the Golgi apparatus (32), the accumulation of secretory granules in the midbody occurs in a few minutes. These diverse processes may well be under the same general control mechanisms, which for the reassembly of the nuclear envelope have been shown to involve dephosphorylation reactions (7).

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