ators of inflammation, such as migration inhibition factor (*8*) and eosinophil stimulation promoter (*9*), isolated schistosomal granulomas from mice can elaborate substances that influence fibroblast activation. We have provided evidence here for the presence of molecules within granulomas and their culture supernatants which can stimulate fibroblast proliferation in vitro. In related studies, we have found that these granuloma supernatants induce changes in the intracellular concentrations in fibroblasts of adenosine 3′,5′-monophosphate and prostaglandins, and alter the ability of fibroblasts to incorporate [^{3}H]proline into collagen. Although it seems likely that the source of the stimulating factor is the inflammatory cells, since mononuclear cells have been shown capable of producing related substances (*2–5*), we cannot at present exclude the possibility that it might derive from the schistosomal egg.

These data suggest a possible molecular link between granuloma formation and the subsequent development of hepatic fibrosis in *S. mansoni* infections. Since the egg granuloma in this disease appears to represent a chronic inflammatory response to egg antigen (*1*), the biological implications of these findings potentially extend to fibrosis resulting from chronic inflammation in a variety of other conditions as well.

DAVID J. WYLER
Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014
SHARON M. WAHL
LARRY M. WAHL
Laboratory of Microbiology and Immunology, National Institute of Dental Research

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Selective Inhibition of Tubulin Synthesis by Amiprophos Methyl During Flagellar Regeneration in *Chlamydomonas reinhardi*

Abstract. *Amiprophos methyl (APM) is a strong, readily reversible and highly selective inhibitor of tubulin synthesis in* Chlamydomonas reinhardi. *The extensive induction of tubulin synthesis that accompanies flagellar regeneration in this organism is prevented by 3 to 10 μM APM. When applied after induction has begun, APM causes a rapid cessation of tubulin synthesis. Translation studies in vitro indicate that the lack of tubulin production in APM-treated cells is not due to a direct inhibition of tubulin messenger RNA translation but rather to a selective depletion of tubulin messenger RNA.*

Microtubules play a central role in several cellular processes (*1*, *2*) including cell division, motility, cell surface organization, secretory functions, cellular differentiation and, perhaps, control of cell proliferation (*3*). A great deal has been learned regarding the molecular properties of microtubules and their assembly (*1*), yet relatively little is known concerning the mechanisms responsible for controlling the synthesis of the tubulin subunits from which microtubules are assembled. One process in which tubulin synthesis has been shown to be intimately involved is the regeneration of flagella in *Chlamydomonas reinhardi* (*4*, *5*). In this system the removal of flagella leads to a rapid and extensive induction of tubulin synthesis. An early observation relating to the control of tubulin synthesis in these cells was that treatment with colchicine or other compounds which prevented flagellar regeneration, and thus blocked utilization of cytoplasmic tubulin reserves (a potential feedback signal), did not interfere with the induction of tubulin synthesis (*4*, *5*). We have now tested for its effects on tubulin synthesis, another compound which has recently been shown to inhibit flagellar outgrowth (*6*). In contrast to the previous inhibitors, this compound, APM (amiprophos methyl) (Fig. 1) (*7*), had a marked inhibitory influence on tubulin production in deflagellated cells. Indeed, subsequent experiments have shown that APM is a rapid, reversible, and selective inhibitor of tubulin synthesis in *C. reinhardi*.

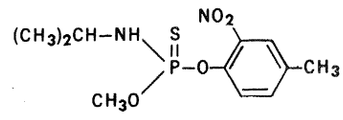

Fig. 1. Amiprophos methyl [APM; *O*-methyl-*O*-(4-methyl-6-nitrophenyl-*N*-isopropyl-phosphorothioamidate].

Gametic cells of strain 137c were obtained by overnight incubation of vegetative cells in a nitrogen- and sulfate-deficient medium (medium V) as described (*5*). For analysis of tubulin induction, cells were deflagellated by incubation at *p*H 4.7 for 1 minute (*8*) and labeled for 30-minute intervals with $H_2{}^{35}SO_4$. Labeling procedures were modifications (*5*) of those developed by Lefebvre and Rosenbaum (*9*); we used 100 μCi of carrier-free $H_2{}^{35}SO_4$ to label approximately 10^7 cells in 0.5 ml of medium V. After labeling, cells were precipitated with ten volumes of acetone and dried. The acetone powders were dissolved in 2 percent sodium dodecyl sulfate, 5 percent mercaptoethanol, 10 percent glycerol, 60 m*M* tris (*p*H 7.6), and a portion (containing 2×10^6 count/min) was subjected to electrophoresis (except where noted) on a 9 percent polyacrylamide gel containing sodium dodecyl sulfate. Electrophoretic conditions were as described (*4*, *5*).

When gametic cells of *C. reinhardi* were deflagellated, tubulin synthesis began within 15 minutes and continued at high levels (15 to 20 percent of total cellular protein synthesis) for approximately 1 1/2 hours (*4*, *5*) (Fig. 2A, tracks 1 to 4). If these cells were treated with 3 μ*M* APM 5 minutes prior to deflagellation, tubulin induction was inhibited (Fig. 2A, tracks 5 to 8). In this experiment only small quantities of tubulin synthesis were detected in the first (15 to 45 minutes) labeling period after deflagellation and only trace amounts in the next or subsequent periods. Thus, APM has a rapid and severe effect on tubulin induction after deflagellation.

In addition to blocking the initial induction of tubulin synthesis, APM also caused a rapid cessation of tubulin synthesis when applied to cells at any time during the inductive cycle. For instance, if induction was allowed to proceed for 15 minutes prior to administration of APM, tubulin synthesis in the succeeding 30-minute period (Fig. 2B, track 1) was greatly reduced compared to that in nontreated cells (Fig. 2A, track 1). In the following labeling period little or no tubulin synthesis is evident (Fig. 2B, track 2). Even more dramatic were the

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effects observed when APM was applied to fully induced cells 45 minutes after deflagellation (Fig. 2B, tracks 5 to 9). In this case, the shutdown of tubulin synthesis was very rapid. The meager amount of tubulin produced in the period immediately after the addition of APM suggests that in this experiment tubulin synthesis could not have persisted for more than 10 or 15 minutes after the APM treatment was initiated.

The inhibitory effects of APM on flagellar outgrowth and tubulin induction were reversible. For example, if cells in which flagellar outgrowth and tubulin induction had been blocked (Fig. 2A, tracks 5 to 8) were removed from medium containing APM at 105 minutes after deflagellation, an apparently normal round of tubulin induction was initiated within 15 minutes (Fig. 2A, tracks 9 to 11) (*10*). The rapidity with which the effects of APM could be reversed was the same regardless of whether APM was removed immediately after deflagellation or 3 hours later (data not shown). These results demonstrate that the ability to generate or respond (or both) to signals calling for tubulin synthesis is not destroyed even during lengthy treatments with APM.

At concentrations below 30 μM, APM had little or no effect on overall rates of protein synthesis (data not shown). A comparison of proteins produced in the presence and absence of APM (Fig. 2, A and B) revealed that APM was highly selective in its inhibition of tubulin synthesis. When more detailed analyses of cellular and flagellar proteins were made with higher resolution 7.5 percent polyacrylamide gels (Fig. 3), we routinely observed an effect of APM on only tubulin and one other cellular protein ($Mr \cong$ 157,000) (*11*). The synthesis of other major cellular proteins, including several presumptive flagellar proteins, appeared to be totally unaffected by APM treatment.

To determine whether the inhibitory action of APM was directed against inducible proteins in general or against tubulin induction in particular, we assayed the effects of APM on a special class of proteins whose synthesis is induced immediately after zygote formation in *C. reinhardi* (*12*). Addition of high levels of APM to gametes as early as 15 minutes before zygote formation had no effect on the subsequent induction and synthesis of early, zygote-specific proteins (Fig. 4, tracks 3 and 4). These data provide additional evidence of the specificity of APM's effect on the induction of tubulin synthesis.

Although the data (Fig. 2, A and B, and Fig. 3) suggest that APM causes a cessation of tubulin synthesis, two alternative possibilities exist. The first is that APM treatment could result in the rapid induction or activation of a tubulin-specific protease. The second is that such treatment could cause the directed secretion of tubulin molecules into the external medium (*13*) where they might be rapidly destroyed. Both possibilities were eliminated by the results of an experiment in which translation in vitro

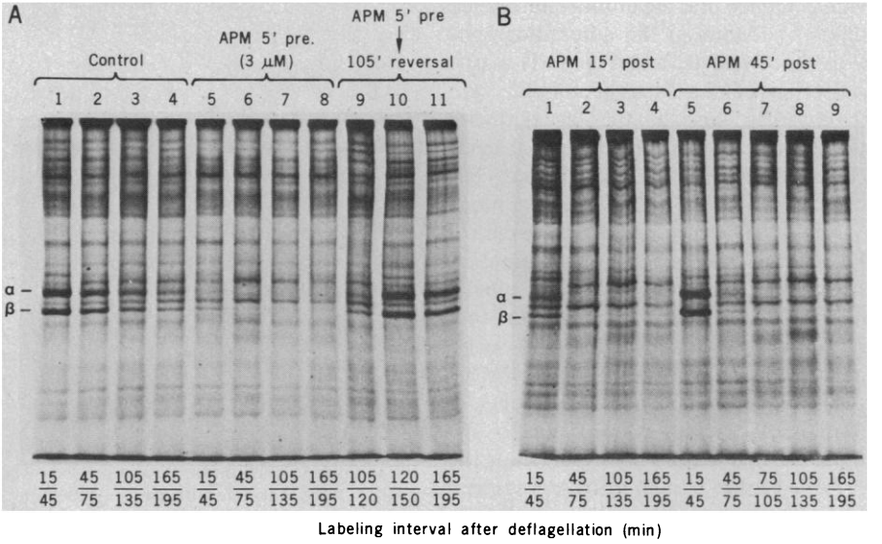

Fig. 2. (A) Autoradiograph of sodium dodecyl sulfate–polyacrylamide gels containing $H_2{}^{35}SO_4$-labeled proteins from gametic cells of *C. reinhardi* deflagellated in the presence (tracks 5 to 11) or absence (tracks 1 to 4) of 3 μM APM. Some of the cells exposed to APM (starting at 5 minutes prior to deflagellation) were centrifuged and resuspended in fresh medium lacking APM at 105 minutes after deflagellation (tracks 9 to 11). Cultures were deflagellated and labeled for 30-minute intervals after deflagellation, as indicated. The migration of the α (56,000 daltons) and β (53,000 daltons) subunits of flagellar tubulin are marked. (B) Effect of APM on tubulin synthesis when added 15 and 45 minutes after deflagellation. Deflagellated gametes were treated with 3 μM APM immediately before the first labeling period (that is, at 15 minutes after deflagellation) (tracks 1 to 4) or the second labeling period (at 45 minutes) (tracks 5 to 9).

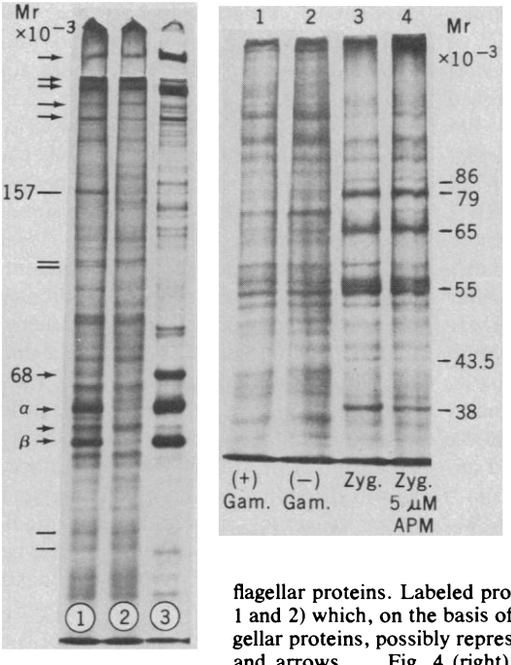

Fig. 3 (left). Selective effects of AMP on tubulin synthesis in deflagellated gametic cells. Proteins from cells labeled with $H_2{}^{35}SO_4$ from 30 to 60 minutes after deflagellation are displayed on a 7.5 percent polyacrylamide gel (with a 3.5 percent stacking gel) containing 0.1 percent sodium dodecyl sulfate (*4, 5*). Proteins from nontreated (track 1) and APM-treated (track 2) deflagellated cells were prepared as described in Fig. 2. In addition, flagella were isolated (*16*) from ^{35}S-labeled cells 60 minutes after deflagellation, resuspended in sodium dodecyl sulfate sample buffer and prepared for electrophoresis in a manner similar to that used for whole cells (*4, 5*). Autoradiographic exposure times were chosen for flagellar proteins (track 3), which resulted in substantial overexposure of the highly radioactive tubulin subunits but allowed resolution of several of the less abundant flagellar proteins. Labeled proteins in whole cell preparations (tracks 1 and 2) which, on the basis of apparent comigration with labeled flagellar proteins, possibly represent flagellar proteins are noted by lines and arrows. Fig. 4 (right). Effects of APM on the induction of zygote-specific proteins during the first hour of zygotic differentiation. Portions of parental gametes of (+) and (−) mating types were labeled with $H_2{}^{35}SO_4$ for 60 minutes just prior to mating (tracks 1 and 2, respectively). After 10 minutes of mating, freshly formed zygotes were labeled for 60 minutes in the absence (track 3) or presence (track 4) of 5 μM APM. Apparent molecular weights (Mr) of the major early zygote-specific proteins are marked.

was used to examine the messenger RNA (mRNA) content of polyribosomes isolated from control and APM-treated cells 45 minutes after deflagellation (Fig. 5A). This experiment shows a nearly complete absence of tubulin mRNA from the polyribosomes of cells treated with as little as 1 μM APM. Such data provide evidence that the absence of labeled tubulin subunits in the autoradiographs (Fig. 2, A and B, and Fig. 3) is a true reflection of the lack of tubulin synthesis. The lack of tubulin synthesis therefore was due either to the absence of tubulin mRNA in APM-treated cells or it was due to a highly selective block in tubulin mRNA translation. The data of Fig. 5B show that APM had no direct effect on the translation in vitro of tubulin mRNA. Additional experiments (not shown) in which total cell polyadenylated mRNA was translated in vitro gave no evidence for the presence of tubulin mRNA in APM-treated cells. Although these results do not rule out the possibility that tubulin mRNA is sequestered in a distinctive, nontranslatable form, it would appear most likely that APM does not have a direct effect on the translation of tubulin mRNA, but rather affects the quantity of tubulin mRNA available for translation.

In general, the quantity of a particular mRNA in the cell is determined by its rate of synthesis and processing and by its rate of decay. In the case of APM-treated cells, it is not clear from the data which of these mechanisms plays the predominant role in regulating cellular levels of tubulin mRNA. The kinetics of tubulin induction after reversal of APM inhibition are similar to those observed during induction in the absence of APM (Fig. 2A) and are therefore consistent with the possibility that removal of APM results in the renewal of tubulin mRNA transcription. In contrast, the exceptionally rapid rate at which tubulin synthesis is curtailed in fully induced cells after APM treatment (Fig. 2B) must be explained either by a high rate of tubulin mRNA turnover in *Chlamydomonas* or by a rapid enhancement in the vulnerability of tubulin mRNA to cellular nucleases in response to APM. A detailed understanding of how tubulin synthesis is regulated in both control and APM-treated cells will depend on the resolution of the relative contributions of selective gene transcription and selective mRNA destruction to tubulin mRNA availability. Progress in this direction will depend on the preparation and utilization of specific molecular probes for tubulin mRNA.

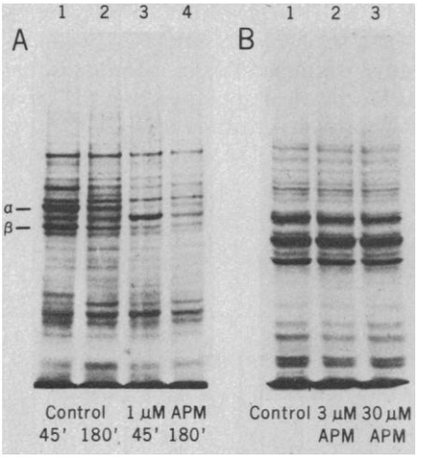

Fig. 5. (A) [^{35}S]Methionine-labeled products of the in vitro translation of polyribosomes isolated from deflagellated cells 45 and 180 minutes after deflagellation (tracks 1 and 2) and of polysomes isolated at like times from cells treated with 1 μM APM 5 minutes prior to deflagellation (tracks 3 and 4). Procedures for isolation of polyribosomes and their translation in the wheat germ system in vitro were as described (*4*). (B) Lack of effect of APM on the in vitro translation of tubulin mRNA. The mRNA was extracted from polyribosomes isolated from cells 45 minutes after deflagellation (at the height of tubulin induction). This mRNA was translated in the in vitro wheat germ system (*4*) in the absence (track 1) or presence of APM (3 μM, track 2; 30 μM, track 3). [^{35}S]Methionine-labeled polypeptide products were analyzed on 8 percent polyacrylamide gels containing 0.1 percent sodium dodecyl sulfate.

APM acts quickly and reversibly to inhibit both tubulin synthesis and flagellar outgrowth in *C. reinhardi*. The mechanism by which both processes are simultaneously affected is unknown (*14*). APM does not block the in vitro assembly of brain tubulin into microtubules (*15*). Thus the lack of flagellar regeneration in APM-treated cells may not be due to a direct interference with tubulin assembly. However, even if this were the case, it is not apparent why there should be an effect on tubulin induction since, as was discussed earlier, colchicine (and other reagents) can completely block flagellar outgrowth without interfering with the induction of tubulin synthesis (*5*). APM might act to suppress the generation or reception (or both) of a signal (or signals) that normally serves to control both flagellar regeneration and tubulin synthesis. In any event, it is evident that APM's properties make it a highly useful reagent for further studies of the control mechanisms involved in the regulation of tubulin mRNA production and destruction in *C. reinhardi*.

PHILIP S. COLLIS
DONALD P. WEEKS
Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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10. If tubulin synthesis is arrested by APM treatment part way through the inductive cycle (Fig. 2B), subsequent removal of APM results in a rapid resumption of tubulin synthesis and completion of the remainder of the normal cycle.
11. The apparent decreased synthesis of the 68,000-molecular-weight protein noted in this analysis (Fig. 3, track 2) has not been observed in any other of our analyses of cellular and flagellar proteins of APM-treated cells. A continued analysis is being made of certain *C. reinhardi* cultures in which a set of minor proteins have been noted whose migration on sodium dodecyl sulfate–polyacrylamide gels is similar to that of tubulin subunits, but whose synthesis prior to or after deflagellation is not affected by APM (Fig. 3, track 2).
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14. It has been shown earlier (*4*, *8*) that gametic and vegetative cells of *C. reinhardi* contain a cytoplasmic pool of flagellar proteins that is sufficiently large to allow the regeneration of flagella of one-third to one-half normal length in the absence of protein synthesis (that is, in the presence of cycloheximide). Thus, it is unlikely that the lack of flagellar regeneration in the presence of APM is due to the inhibition of tubulin synthesis or the synthesis of any other flagellar protein.
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