

FLUORESCEIN COLCHICINE

Synthesis, Purification, and Biological Activity

JOHN I. CLARK and DONITA GARLAND

From the Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115, and the Department of Biochemistry, University of Washington, School of Medicine, Seattle, Washington 98195. Dr. Clark's present address is the Department of Physics, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Dr. Garland's present address is the Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20014.

ABSTRACT

The synthesis of a fluorescent colchicine derivative permits the localization of colchicine-binding receptors in cells. Fluorescein colchicine (FC) was prepared by the addition of fluorescein isothiocyanate to deacetyl colchicine. The product, FC, was separated from the reactants by thin-layer chromatography (TLC). The purity of FC was demonstrated by TLC, UV spectral analysis, and analysis of the kinetics of photodecomposition. FC inhibited [³H]colchicine binding to purified brain tubulin. The biological activity of FC was compared to the activity of unlabeled colchicine on mitosis, motility, secretion, and myogenesis. The effects of FC were identical to those of unlabeled colchicine in all biological systems tested. The results demonstrate that FC may be substituted for colchicine in biological experiments without significant loss in specificity or effectiveness.

KEY WORDS colchicine · fluorescein-labeled colchicine

In addition to its medical history, dating back to 1550 B. C. (12), colchicine has two important biological characteristics: (a) a highly specific association with microtubule proteins (40, 41) and (b) remarkable effects on several basic cell functions including mitosis (16), secretion (43), cell morphology (33), motility (37), intracellular transport of macromolecules (23), microtubule assembly (3, 13), and mitogenic activation (21, 38). Previous investigations have utilized the specificity of colchicine in studying colchicine-binding microtubule proteins and their role in cell functions dependent upon microtubule assembly (8, 20, 25, 33, 39). However, microtubule assembly is not clearly involved in all colchicine-sensitive

cell processes: nucleoside transport (22), cholinergic activation (35), albumin secretion (24), axonal transport (5), stimulation of ciliogenesis (10, 31), stimulation of antibody production (32), and modulation of surface receptor movements (36, 44). The presence of colchicine-binding microtubule proteins in the membranes of mitochondria (1), nuclei (30), and synaptosomal endings (26) suggests a cellular function for colchicine-binding proteins in addition to microtubule assembly. The present paper is a report of the synthesis, purification, and biological testing of fluorescein thiocarbonyl colchicine (FC) (Fig. 1), a highly fluorescent derivative of colchicine that maintains the specificity and biological activity of unlabeled colchicine. FC provides a new cytological method for studying the relationships between colchicine-binding sites and cell functions.

MATERIALS AND METHODS

The synthesis of fluorescent colchicine from fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, Mo.) and deacetyl colchicine will be described in detail in Results. It should be noted that the remethylation step in the production of deacetyl colchicine included the generation of diazomethane, a rather toxic and volatile compound requiring special precautions, such as the use of smooth glass joints sealed with Teflon tape instead of ground glass joints (2). Deacetylation of colchicine by the method of Wilson and Friedkin (42) yielded a mixture of deacetyl colchicine and its isomer iso-deacetyl colchicine. The separation and purification of the colchicine derivatives were especially difficult because of their similarities in molecular weight and charge. Maximal separation was obtained by thin-layer chromatography (TLC) using precoated aluminum-backed, 0.25-mm-thickness silica gel (Sil Gel F-254) sheets from EM Laboratories, Inc. (Elmsford, N. Y.). The plates were developed with an acetone/methanol (9.5:0.5) solvent system in glass tanks lined by Whatman chromatography paper. The products were detected on the plates by use of illumination with UV light, Blak Ray UVL-21 or UVS-21 sources (Black Light Corp. of America, San Gabriel, Calif.) for visible and short wavelength UV irradiation. After TLC separation, the bands were scraped from the plates, eluted from the silica gel with 95% ethanol, evaporated to dryness with a stream of nitrogen gas, redissolved in 95% ethanol to a concentration of 1 μ M, and prepared for UV analysis in a Cary 15 spectrophotometer (Varian Associates, Instrument Div., Palo Alto, Calif.). Colchicine concentrations were calculated from the optical density at 350 nm ($\epsilon = 16,500$) (42). The leading band, $R_f = 0.28$, gave a UV absorption maximum at 345 nm and was identified as iso-deacetyl colchicine. The slower band, $R_f = 0.14$, had an absorption maximum at 351 nm, characteristic of deacetyl colchicine and was used in the synthesis of FC. All salts and solvents used in the synthesis were reagent grade obtained from J. T. Baker Chemical Co. (Phillipsburg, N. J.) or Merck & Co., Inc. except where noted.

Cell Preparations

SEA URCHINS: *Strongylocentrotus franciscanus* were collected at the University of Washington Friday Harbor Laboratory (Friday Harbor, Wash.). Spawning was induced by the injection of 5–8 ml of 0.55 M KCl solution through the oral membrane into the perivisceral cavity (7). Eggs were obtained fresh for each experiment just before fertilization and washed three times in filtered seawater before use, while sperm were stored in a refrigerator (-4°C) for as long as 48 h before use. To achieve fertilization, 0.5 ml of sperm were diluted in 50 ml of filtered seawater and ~ 2 ml of this diluted sperm suspension was added to 50 ml of the suspended eggs in a 250-ml beaker. After 10 min, the eggs were observed with a compound light microscope. If a fertilization

membrane was present indicating that fertilization had occurred, the zygotes were washed three times with filtered seawater to remove the excess sperm and maintained on a seawater table at 12° – 14°C . Solutions of colchicine derivatives in seawater were diluted into aliquots of the zygotes ~ 30 min after fertilization, and the effects on the first mitosis were observed.

MACROPHAGES: Macrophages were obtained by peritoneal lavage. The cells were seeded on glass cover slips and maintained in Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) with 2% L-glutamine, 150 U/ml of penicillin-streptomycin, and 10–20% fetal calf serum. Monolayer cultures of peritoneal macrophages were exposed to test compounds by the following method: Samples of the compound were dissolved in culture medium without serum at 37°C . The complete medium was poured off of the cell culture, and 5 ml of the test medium was added in its place. Serum was avoided because colchicine and other colchicine derivatives may have affinity for components in the serum (9, 22). The macrophages in the study cultures were monitored with an inverted phase microscope or a Zeiss Universal microscope equipped with Nomarski differential interference contrast (DIC) optics.

The effects of FC, colchicine, and other derivatives on living preparations of macrophages were studied by Nomarski (DIC) and fluorescence microscopy. Living macrophages were grown on cover slips in medium containing drugs or in control medium without drugs. Cover slips removed from the culture dish were placed in a pool of medium (with or without drugs) on a microscope slide. A second, larger cover slip was added over the culture cover slip and sealed with Vaseline (Chesebrough-Ponds, Inc., Greenwich, Conn.) to create a microchamber having a clean, dry upper surface. The effects of FC, fluorescein, colchicine, and fluorescein lumi-colchicine on the macrophages were observed and recorded on Tri-X or Plus-X Pan film (Eastman Kodak Co., Rochester, N. Y.).

Dr. H. P. Ehrlich (Department of Biochemistry, University of Washington) observed the effects of FC on collagen synthesis in *in vitro* cultures of chick cranial bones (11).

Chick embryo myogenic cells were generously provided by Dr. M. Nameroff (Department of Biological Structure, University of Washington). The effects of FC were compared to the effects of colchicine and fluorescein on mitosis and myotube formation in cultures of chick embryo myogenic cells.

RESULTS

A flow chart for the preparation of fluorescent colchicine derivatives is shown in Fig. 1. The FC used in biological tests was synthesized by the addition of 1 ml of 1.0 mM deacetyl colchicine in 95% ethanol to 1 ml of an ethanol solution of 7

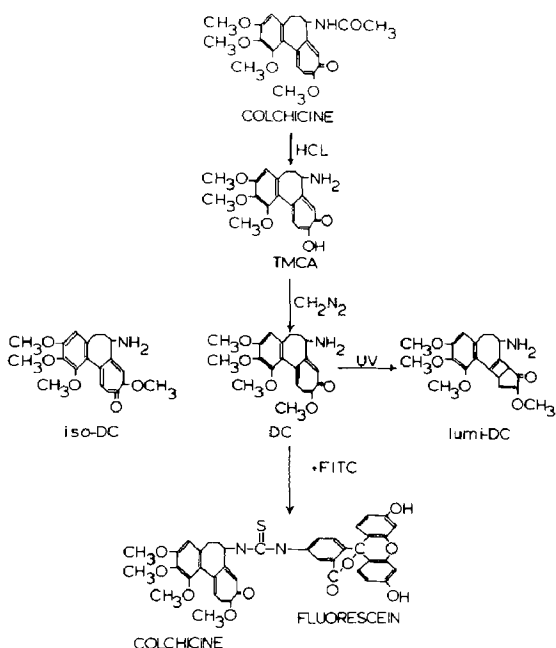


FIGURE 1 This flow chart diagrams the synthesis of FC, iso-colchicine, and lumi-colchicine. Colchicine was deacetylated to trimethyl colchicinnic acid (TMCA), remethylated to deacetyl colchicine (DC) and its isomer iso-deacetyl colchicine (iso-DC). Iso-DC and DC can be combined with fluorescein isothiocyanate (FITC) to yield the fluorescent products. The proposed chemical structure of FC is shown. Ultraviolet irradiation converts deacetyl colchicine to lumi-deacetyl colchicine (lumi-DC) which can be labeled with FITC.

mM fluorescein isothiocyanate I (FITC) (Sigma Chemical Co.). After 15 min at room temperature, the reaction mixture was separated by TLC. The product, FC ($R_f = 0.38$), migrated between the unreacted FITC ($R_f = 0.59$) and unreacted deacetyl colchicine ($R_f = 0.14$). The FC which migrated as a single band was eluted from the silica gel with absolute ethanol. The spectral characteristics of FC were compared with those of unreacted fluorescein and unlabeled colchicine in Fig. 2. The purified product was evaporated to dryness, redissolved in absolute ethanol to a concentration of ~ 0.1 mM, centrifuged to remove any remaining silica gel, and refrigerated at 4°C . The synthesis yields ~ 1.5 ml of 0.1 mM FC which can be stored for 2 mo without significant loss of biological activity. Fresh preparations not more than 2 wk old were used for biological experiments. The thin-layer chromatographic separation provided pure FC, uncontaminated by other colchicine derivatives that might affect the biological activity of FC.

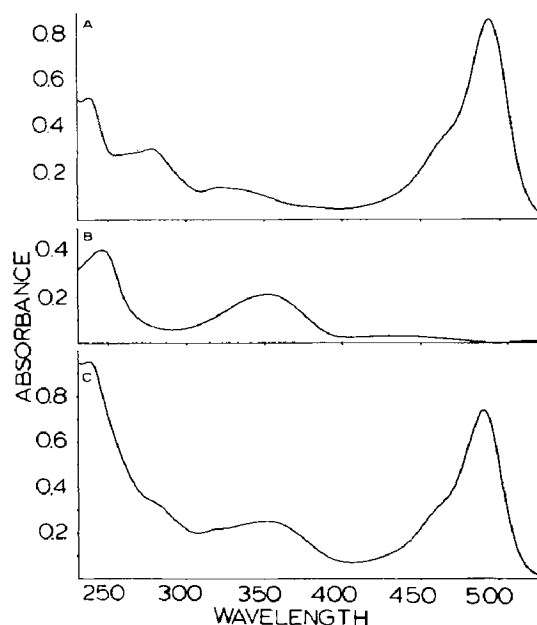


FIGURE 2 A comparison of the UV spectra for fluorescein isothiocyanate (A), deacetyl colchicine (B) and fluorescent colchicine (C) demonstrates that the purified fluorescent colchicine has characteristic absorbance of fluorescein at 490 nm and the tropolone colchicine absorbance at 350 nm. Each compound was diluted to $10 \mu\text{M}$ concentration in 0.14 M phosphate buffer at pH 7.0. FITC shows a slight absorbance at 350 nm.

The purity of FC solutions was tested by two methods. Ethanol solutions of colchicine decompose to lumi-colchicine when exposed to intense UV light, and the purity of a colchicine solution can be determined on the basis of the kinetics of UV-induced decomposition. The kinetics of colchicine decomposition are first order if the solution is pure, but impure mixtures of colchicine derivatives do not show first-order kinetics (42). The rate of the reaction is so sensitive to the composition of a colchicine solution that isomers can be distinguished by this kinetic method (42). The reaction was monitored spectrophotometrically by observing the change in optical density at 350 nm with the time of exposure to UV light.

Fig. 3 shows the kinetic plots obtained for the photodecomposition of FC, fluorescein iso-colchicine, and a mixture of the two compounds. The mixture is clearly nonlinear while the separated purified isomers are linear. The nonlinearity of the FC decomposition, after 2.5 min of exposure to UV, is due to the overlapping 350-nm absorbance from fluorescein (see Fig. 2). Linearity of the kinetics of photodecomposition suggests that

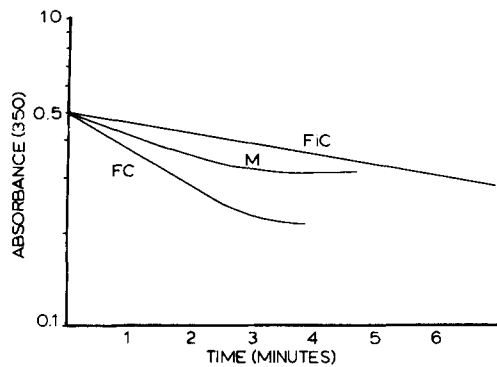


FIGURE 3 The purity of colchicine derivatives can be demonstrated by the kinetics of the UV-induced decay of the 350-nm absorbance. The linear decay curve for fluorescein iso-colchicine (FiC) is shown in the top graph. The presence of contaminating compounds results in a nonlinear graph, demonstrated by a mixture of fluorescein iso-colchicine and FC (*M*). The purified FC is degraded linearly until 2.5 min when the plot becomes nonlinear due to interference from the 350-nm absorbance of the fluorescein moiety (see Fig. 2).

the separation by TLC yields only one colchicine derivative and that it is not contaminated with other derivatives. The second method for testing the purity of FC product used radioactive deacetyl colchicine (a generous gift of Dr. J. Bryan, Department of Biology, University of Pennsylvania) in the synthesis of FC. It was observed that the location of the single fluorescent spot on the thin-layer chromatogram coincided with the location of high radioactivity due to the [^3H]colchicine, indicating that a chromatographically pure fluorescent [^3H]colchicine had been obtained. The only other peak of radioactivity corresponded to the unreacted [^3H]deacetyl colchicine.

The excitation and emission spectra of fluorescein in physiological solutions were not changed significantly by the reaction with deacetyl colchicine. The excitation spectrum recorded at 520-nm emission wavelength gave a maximum at 490 nm, characteristic of FITC. The emission spectrum, recorded using 480-nm excitation wavelength, had a maximum at 521 nm characteristic of fluorescein (Fig. 4).

Although the thin-layer chromatographic purification of the derivatives was only practical for small amounts of product, it was much more successful than column chromatography attempted with silica gel, alumina, bio-gel, or Sephadex.

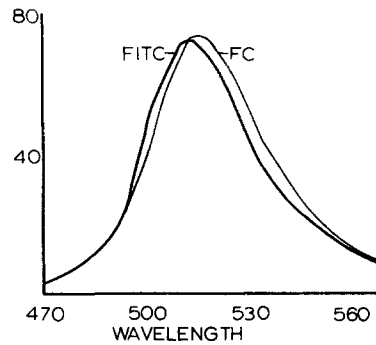


FIGURE 4 The fluorescence emission spectrum for fluorescent colchicine (maximum at 521 nm) varies only slightly from that for fluorescein isothiocyanate (maximum at 519 nm). 10^{-7} M solutions of FITC or FC in 0.14 M phosphate buffer were excited at 480 nm, and the emission spectrum was recorded on a Perkin-Elmer MPF3 spectrofluorometer (Perkin-Elmer Corp., Boller & Chivens Div., South Pasadena, Calif.). The emission intensities are in arbitrary units.

Effects of FC on Biological Systems

FC competitively inhibited the binding of colchicine to tubulin. This was determined using porcine brain tubulin prepared by two cycles of polymerization-depolymerization (27). The polymerized tubulin was then dissolved in 10 mM sodium phosphate buffer, pH 6.5, 0.8 M sucrose, 10 mM MgCl_2 , 0.1 mM GTP. Samples of either FC or [^3H]colchicine were evaporated to dryness with a stream of nitrogen and then dissolved in the above-described buffer. Approx. 0.5 mg of tubulin was incubated for 6 h at 37°C with [^3H]colchicine concentrations varying from 0.7 to 40 μM in the presence of fixed FC concentrations. Separate experiments used FC concentrations in the range of 10 μM –50 μM . Protein-bound colchicine was measured by absorption of the labeled colchicine-protein complex (sample size 0.1 ml) to a 0.5 ml column of diethylaminoethyl (DEAE)-cellulose (Whatman DE23) pre-equilibrated with 10 column volumes of 10 mM sodium phosphate buffer, pH 6.5, with 10 mM MgCl_2 . The columns were washed twice with 1 ml of the equilibration buffer. The DEAE-cellulose was transferred to the scintillant (80 g of naphthalene, 5 g of Omnifluor [New England Nuclear, Pilot Chemicals Div., Watertown, Mass.], 1 liter of dioxane). Protein-bound [^3H]colchicine was released into the scintillant while the DEAE-cellulose settled to the bottom of the vial. Examination of Line-

weaver-Burke plots revealed that FC competitively inhibited the binding of [^3H]colchicine ($K_a = 2 \times 10^6 \text{ M}^{-1}$) with an association constant of $2 \times 10^5 \text{ M}^{-1}$.

The effect of FC on mitosis in sea urchin zygotes is shown in Fig. 5. 30 min after fertilization, batches of zygotes in seawater were exposed to varying concentrations of FC or unlabeled colchicine. Untreated controls were observed and, when the first cleavage was completed in ~99% of the zygotes in the untreated sample, the number of completed mitoses in colchicine- and FC-treated samples was counted, with a Nikon compound microscope at $\times 40$. At least three random fields of ~50 zygotes were counted from each preparation. Counts were within 5% of each other for each sample. The percentage of completed cleavages was recorded at each concentration of FC or colchicine. The minimum effective concentration of FC resulting in mitotic arrest was equivalent to the minimum effective concentration of unlabeled colchicine. The reversibility of the FC effect was also comparable to the reversibility of the colchicine effect. Treatments in which concentrations above 1 mM were used produced generally irreversible blockage of mitosis. Lower concentrations could be washed out with fresh seawater and mitosis reinitiated in ~50% of the cells, providing the cells had not been exposed to the treatment for more than 30 min. FC was extracted from the metaphase-arrested zygotes by washing the cells with warm ethanol. The recovered fluorescent compound was analyzed by TLC and UV spectroscopy and appeared unchanged after binding in the metaphase-arrested cells.

FC did not bind to sea urchin sperm tails.

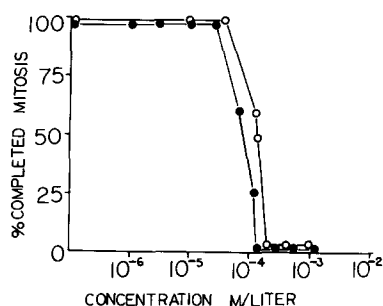


FIGURE 5 Dose-response plots for the inhibition of mitosis in sea urchin zygotes show that colchicine (open circles) and FC (closed circles) are equally effective in the inhibition of mitosis.

Living sea urchin sperm suspended in $1 \mu\text{M}$ FC in seawater showed no apparent fluorescence in the tails after unbound FC was washed from the preparation and the sperm were observed by fluorescence microscopy. Sperm tails which had been detached from the heads by repeated passage through a 26-gauge needle and collected by centrifugation at maximum speed in a clinical centrifuge also lacked fluorescence after incubation in FC and washing to remove unbound FC. In addition, demembrated sperm tails, prepared by the technique of Gibbons and Gibbons (14), showed no binding after incubation with FC. From these results, it was concluded that the FC does not bind to intact microtubules of the stable class present in sea urchin sperm tails.

The effect of FC on collagen synthesis was compared with that of unlabeled colchicine in chick cranial bones grown in vitro (11). Concentrations of colchicine or FC as low as $1 \mu\text{M}$ decreased the rate of procollagen conversion to collagen with comparable effectiveness.

FC inhibited mitosis in cell cultures of chick embryo cells prepared from 8-day-old embryos. Cell cultures of chick embryo myogenic cells were treated with $1 \mu\text{M}$ colchicine, $1 \mu\text{M}$ FC, or $1 \mu\text{M}$ unlabeled fluorescein in the culture medium. In another set of control cultures, the medium was changed but no compounds were added. After incubation at 37°C for 24 h, numerous cells arrested in metaphase had accumulated in both the FC- and colchicine-treated cultures. Colchicine-treated cultures could not be distinguished from FC-treated cultures on the basis of the number of metaphase-arrested cells or the general appearance of the cultures. The cultures treated with fluorescein and the control cultures were identical to one another, indicating that fluorescein alone had no effect on normal mitosis of myogenic cells while both colchicine and FC inhibited mitosis.

Similar results were obtained when the effect of FC was compared with the effect of colchicine on myotube fusion in cell cultures from 11-12-day-old chick embryo myogenic cells. The colchicine and FC at concentrations of $5 \mu\text{M}$ inhibited the fusion process and caused the disruption of the previously formed myotubes into multinucleated "myosacs." Untreated and fluorescein-treated cultures of myogenic cells began to fuse and form multinucleated myotubes. The fluorescein label apparently does not alter the biological

activity of colchicine in these cell systems.

Cell cultures of macrophages responded rapidly to colchicine treatment and were used in a series of experiments to compare colchicine, FC, fluorescein iso-colchicine, fluorescein lumi-colchicine, and fluorescein alone (Table I). Treatment of cultured macrophages with concentrations of colchicine or FC above 0.5 μM caused the cells to round up and inhibited their mobility. The colchicine effect was reversible if the cells had been incubated with doses of colchicine or FC lower than 5 μM for periods of time <1 h. Cells treated with fluorescein alone for 30 min at concentrations as high as 0.1 mM were unaffected. When the free fluorescein was washed out of the cultures and replaced with fresh medium, no fluorescence was observed in the cells, indicating that fluorescein was probably unable to penetrate the intact plasma membrane. Cells incubated for periods of time >45 min showed some fluorescence in discrete spots apparently due to the uptake of fluorescein by pinocytosis.

To test the possibility that the morphological effects of FC may have been due to the synergistic effect of colchicine and fluorescein, cells were treated with equal concentrations of unbound fluorescein and unlabeled colchicine in the same medium. The cells rounded up and microspike attachments could be seen, but no fluorescence was observed in the cells after the free fluorescein was washed out of the cultures. The dose-response was identical to that for colchicine alone (minimum effective dose = 0.5 μM). This result suggested that there was no cooperative action between fluorescein and colchicine. The morphological changes in cells exposed to FC were due to the colchicine and the intracellular fluorescence was due to the fluorescein moiety on the FC.

Fluorescein lumi-colchicine, a fluorescent but inactive derivative of colchicine, was synthesized by irradiation of FC. Concentrations of the fluorescent lumi-colchicine up to 0.1 mM produced no morphological response in the macrophage cell culture even after 2-h treatment. After the fluorescent lumi-colchicine was washed out with fresh medium, a low level of fluorescence was observed in the cells, which may be due to the very low affinity of lumi-colchicine for tubulin (4) or to incomplete conversion of FC to lumi-FC during preparation of the inactive derivative.

DISCUSSION

These results suggest that the addition of FITC to deacetyl colchicine does not impair the specificity or biological activity of colchicine. Fluorescein-labeled colchicine, FC, the product of FITC and deacetyl colchicine, was analyzed by TLC, UV spectroscopy, and chemical kinetics, and was shown to be a single, pure compound. The combination of colchicine (mol wt 399) with the fluorescent label, FITC (mol wt 499), provides a specific cytological tracer for studying the cellular role of colchicine-binding microtubule proteins in cells.

Fluorescent-labeled colchicine was shown to be comparable to unlabeled colchicine in several cell systems, and the results are summarized in Table II. FC inhibited mitosis (Fig. 5) and caused morphological changes in macrophages at concentrations identical to those of unlabeled colchicine. Myotube formation (18) and collagen synthesis (11) were as sensitive to FC as to unlabeled colchicine. FC competitively inhibited [^3H]colchicine binding to purified brain tubulin, suggesting that the receptor site for FC on tubulin is the same as for unlabeled colchicine. The association con-

TABLE I
FC Effects on Macrophages

| Concn | Colchicine | FC | Fluorescein alone | Fluorescent lumi-colchicine | Control |
|------------------|------------|-----------|-------------------|-----------------------------|--|
| <i>M</i> | | | | | |
| 10 ⁻⁴ | Rounding | Rounding | No effect | No effect | No effect |
| 10 ⁻⁵ | " | " | " | " | Medium changed without adding test compounds |
| 10 ⁻⁶ | " | " | " | " | " |
| 10 ⁻⁷ | No effect | No effect | " | " | " |
| 10 ⁻⁸ | " | " | " | " | " |

This table compares the effects of FC with the effects of other colchicine derivatives on macrophages. Only the FC and colchicine altered the morphology of the macrophages, and the minimum effective concentration for both was ~0.5 μM .

TABLE II
FC and Colchicine

| Biological system | Effects | Fluorescence distribution |
|--|--|---|
| Macrophages in cell culture | Cell rounding and inhibition of motility | General cytoplasmic fluorescence |
| Chick embryo myogenic cells in culture | Inhibition of fusion | Inhibited cells showed general cytoplasmic fluorescence |
| Chick fibroblasts in culture | Metaphase arrest | Arrested cells showed general cytoplasmic fluorescence |
| Collagen secretion in chick calvaria culture | Inhibition of secretion | Not observed |
| Sea urchin embryos | Mitotic arrest | General cytoplasmic fluorescence |
| Capped ovarian granulosa cells in culture | None observed | Bright fluorescence in the caps |
| Rat erythrocytes | None observed | No fluorescence |
| Sea urchin sperm tails | None observed | No fluorescence |

This table summarizes the effects of FC and colchicine on the biological systems used in the comparison of FC and colchicine. The distribution of fluorescence after the cells were treated with FC is also noted. "Not observed" means that the distribution of FC binding was not checked with a fluorescence microscope. "None observed" means that the cells were checked for changes in their morphology by light microscopy but that none were seen. The biological effects of FC were indistinguishable from the effects of colchicine on these systems.

stant of FC for tubulin, $2 \times 10^5 \text{ M}^{-1}$, was within the range of reported values for unlabeled colchicine (10^5 – 10^7) (29). The similarity between the biological action of FC and the effect of colchicine permits the use of FC in the investigation of colchicine-sensitive cell systems whether or not microtubules are involved.

It is significant that some colchicine-sensitive systems show no clear microtubule involvement. The disruption of microtubules in vitro (3) and in vivo (28), the inhibition of the organization of the mitotic apparatus (17) and other morphological changes (33, 34) correlate well with colchicine inhibition of microtubule assembly. However, the absence of convincing evidence that microtubule assembly is associated with the effects of colchicine on albumin secretion (24), collagenase production (15), axonal transport (5), surface receptor mobility (44), cholinergic stimulation (35), and other cell functions (8) suggests that colchicine may act on a process involving microtubule protein but not necessarily involving microtubule assembly. Our approach to this problem was to develop a fluorescent derivative for in vivo and in vitro studies of colchicine effects. Colchicine distributions in animal tissues, cells, and subcellular components may now be investigated with confidence by fluorescence microscopy, spectrofluorometry, and other analytical methods in which fluorescent colchicine is used. The application of fluorescent colchicine to problems of cell division,

motility, and differentiation will assist in understanding colchicine sensitivity and its relation to microtubule proteins.

The specificity of colchicine for its receptor proteins permits the cytological localization of colchicine receptors in fixed cells, and the method should eventually permit quantitative studies of cellular tubulin pools. However, it should be emphasized that fluorescence microscopy is subject to the limitations of all morphological methods requiring histological processing. The variable nature of cells and tissues can influence fluorescence emission. For example, mammalian sperm cells have significant intrinsic fluorescence at the fluorescein wavelength (19), discouraging their use with FC. In some cells, such as *Chlamydomonas reinhardtii* and *Hemaphysalis katharinae*, nonspecific FC binding has been observed. In other cases, the fixatives or organic solvents that are used in dehydration extract or enhance the intrinsic fluorescence (6). Photographic processing can also enhance or eliminate problematic fluorescence. Fluorochromes are often sensitive to their chemical environment, and problems of amplification and quenching of the fluorescent signals are difficult to evaluate by existing morphological methods. Living cells vary in their ability to take up fluorochromes. Although fluorescence labeling techniques are useful in demonstrating the morphological distributions of cellular components, the application of fluorescence techniques to

quantitative, experimental cytology requires that many problems must be considered in addition to the specificity of a labeled molecule for its cellular receptor.

We are grateful to Dr. David Teller, Dr. E. M. Eddy, and members of the Departments of Biochemistry and Biological Structure at the University of Washington for their support, and to Dr. J. Bryan and L. Wilson for advice and assistance. We thank Dr. A. O. D. Willows for the opportunity to carry out portions of the work at the University of Washington Friday Harbor Laboratory.

Portions of the work were completed at the Harvard Medical School under the sponsorship of Dr. D. W. Fawcett and the Department of Anatomy. The research was supported by a postdoctoral fellowship from the Pharmaceutical Manufacturers Association Foundation to Dr. Clark and National Institutes of Health grants HD 02344 and GM 00136.

Received for publication 21 June 1976, and in revised form 31 October 1977.

REFERENCES

1. BHATTACHARYA, B., and J. WOLFF. 1975. Membrane-bound tubulin in brain and thyroid tissue. *J. Biol. Chem.* **250**:7639-7646.
2. BLATT, A. H. 1956. *Organic Synthesis*. Vol. 3. John Wiley & Sons, Inc., New York. 165.
3. BORISY, G. G., J. B. OLMSTED, J. M. MARCUM, and C. ALLEN. 1974. Microtubule assembly *in vitro*. *Fed. Proc.* **33**:167-174.
4. BRYAN, J. 1972. Definition of 3 classes of binding sites in isolated microtubule crystals. *Biochemistry*. **11**:2611-2616.
5. BYERS, M. R. 1974. Structural correlates of rapid axonal transport: Evidence that microtubules may not be directly involved. *Brain Res.* **75**:97-113.
6. COONS, A. H., H. J. CREECH, and R. N. JONES. 1941. The fluorescein labeling of antibodies. *Proc. Soc. Exp. Biol. Med.* **47**:200-202.
7. COSTELLO, D. P., M. E. DAVIDSON, A. EGGERS, M. H. FOX, and C. HENLEY. 1957. *Methods for Obtaining and Handling Marine Eggs and Embryos*. Marine Biological Laboratories, Woods Hole, Mass.
8. DEYSSON, G. 1968. Antimitotic substances. *Int. Rev. Cytol.* **24**:99-148.
9. DONIGAN, D. W., and R. J. OWELLEN. 1973. Interaction of vinblastine, vincristine and colchicine with serum proteins. *Biochem. Pharmacol.* **22**:2113-2119.
10. DUSTIN, P., J. P. HUBERT, and J. FLAMENT-DURAND. 1975. Action of colchicine on axonal flow and pituitary cells in the hypothalamo-pituitary system of the rat. *Ann. N. Y. Acad. Sci.* **253**:670-684.
11. EHRLICH, H. P., R. ROSS, and P. BORNSTEIN. 1974. Effects of anti-microtubular agents on the secretion of collagen. *J. Cell Biol.* **62**:390-405.
12. EIGSTI, O. J., and P. DUSTIN. 1955. *Colchicine*. Iowa State College Press, Ames, Iowa.
13. GASKIN, F., C. R. CANTOR, and M. L. SHELANSKI. 1975. Biochemical Studies on the *in vitro* assembly and disassembly of microtubules. *Ann. N. Y. Acad. Sci.* **253**:133-146.
14. GIBBONS, B. H., and I. R. GIBBONS. 1972. Flagellar movement and adenosine triphosphatase activity in sea urchin sperm extracted with Triton X-100. *J. Cell Biol.* **54**:75-97.
15. HARRIS, E. D., and S. M. KRANE. 1971. The effects of colchicine on collagenase in cultures of *Rheumatoid Synovium*. *Arthritis Rheum.* **14**:669-684.
16. INOUÉ, S. 1952. The effects of colchicine on the microscopic and submicroscopic structure of the mitotic spindle. *Exp. Cell Res.* **2**:305-318. (Suppl.)
17. INOUÉ, S., and H. SATO. 1967. Cell motility by labile association of molecules. *J. Gen. Physiol.* **50**:259-292. (Suppl.)
18. ISHIKAWA, H., R. BISCHOFF, and H. HOLTZER. 1968. Mitosis and intermediate-sized filaments in developing skeletal muscle. *J. Cell Biol.* **38**:538-555.
19. KORMANO, M. 1967. Dye permeability and alkaline phosphatase activity of testicular capillaries in the postnatal rat. *Histochemistry*. **9**:327-338.
20. MARGULIS, L. 1973. Colchicine sensitive microtubules. *Int. Rev. Cytol.* **34**:333-361.
21. MEDRANO, E., R. PIRAS, and J. MURDOH. 1974. Effect of colchicine, vinblastine and cytochalasin B on human lymphocyte transformation by phytohemagglutinin. *Exp. Cell Res.* **86**:295-300.
22. MIZEL, S. B., and L. WILSON. 1972. Nucleoside transport in mammalian cells: Inhibition by colchicine. *Biochemistry*. **11**:2573-2578.
23. PORTER, K. R. 1973. Microtubules in intracellular locomotion. *Ciba Found. Symp.* **14**:149-168.
24. REDMAN, C. M., D. BANERJEE, K. HOWELL, and G. E. PALADE. 1975. Colchicine inhibition of plasma protein release from rat hepatocytes. *J. Cell Biol.* **66**:42-69.
25. ROSENBAUM, J. L., and K. CARLSON. 1969. Cilia regeneration in *Tetrahymena* and its inhibition by colchicine. *J. Cell Biol.* **40**:415-425.
26. SHELANSKI, M. L., H. FEIT, R. W. BERRY, and M. P. DANIELS. 1972. Some Biochemical Aspects of Neurotubule and Neurofilament Proteins. In *Functional and Structural Proteins of the Nervous System*. A. N. Davison, P. Mandel, and I. G. Morgan, editors. Plenum Press, New York. 55-68.
27. SHELANSKI, M. L., F. GASKIN, and C. R. CANTOR. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **70**:765-768.

28. SHELANSKI, M. L., and E. W. TAYLOR. 1967. Isolation of a protein subunit from microtubules. *J. Cell Biol.* **34**:549-554.
29. SHERLINE, P., J. T. LEUNG, and D. M. KIPNID. 1975. Binding of colchicine to purified microtubule protein. *J. Biol. Chem.* **250**:5481-5486.
30. STADLER, J., and W. W. FRANKE. 1974. Characterization of the colchicine binding of membrane fractions from rat and mouse liver. *J. Cell Biol.* **60**:297-303.
31. STUBBLEFIELD, E., and B. R. BRINKLEY. 1966. Cilia formation in Chinese hamster fibroblasts in response to colcemid treatment. *J. Cell Biol.* **30**:645-652.
32. TANAKA, N., and A. H. COONS. 1956. Effects of colchicine on antibody production. *Bull. N. Y. Acad. Med.* **32**:171-172.
33. TILNEY, L. G. 1971. Origin and Continuity of Microtubules. In *Origin and Continuity of Cells and Organelles*. J. Reinert and H. Ursprung. Springer Verlag, New York. 222-260.
34. TILNEY, L. G., and J. R. GIBBINS. 1969. Microtubules in the formation and development of the primary mesenchyme in *Arbacia Punctulata*. *J. Cell Biol.* **41**:227-250.
35. TRIFARO, J. M., B. COLLIER, D. LASTOWECKA, and D. STERN. Inhibition by colchicine and by vinblastine of acetylcholine induced catecholamine release from the adrenal gland. *Mol. Pharmacol.* **8**:264-267.
36. UKENA, T. E., J. Z. BORYSENKO, M. J. KARNOVSKY, and R. D. BERLIN. 1974. Effects of colchicine, cytochalasin B, and 2-deoxyglucose on the topographical organization of surface bound concanavalin-A in normal and transformed fibroblasts. *J. Cell Biol.* **61**:70-82.
37. VASILIEV, J. M., I. M. GELFAND, L. V. DOMNINA, O. Y. IVANOVA, S. G. KOMM, and L. V. OLSHEVSKAJA. 1970. Effects of colcemid on the locomotory behavior of fibroblasts. *J. Embryol. Exp. Morphol.* **24**:625-640.
38. WANG, J. L., G. R. GUNTHER, and G. M. EDELMAN. 1975. Inhibition by colchicine of the mitogenic stimulation of lymphocytes prior to the S phase. *J. Cell Biol.* **66**:128-144.
39. WEISENBERG, R. C. 1972. Changes in the Organization of tubulin during meiosis in the eggs of the surf clam, *Spisula Solidissima*. *J. Cell Biol.* **54**:266-278.
40. WEISENBERG, R. D., G. G. BORISY, and E. W. TAYLOR. 1968. The colchicine binding protein of mammalian brain and its relation to microtubules. *Biochemistry.* **7**:4466-4479.
41. WILSON, L., and J. BRYAN. 1974. Biochemical and pharmacological properties of microtubules. *Adv. Cell Mol. Biol.* **3**:21-71.
42. WILSON, L., and M. FRIEDKIN. 1966. Biochemical events of mitosis. I. Synthesis and properties of colchicine labeled with tritium in its acetyl moiety. *Biochemistry.* **5**:2463-2471.
43. WOLFF, J., and J. A. WILLIAMS. 1973. The role of microtubules and microfilaments in thyroid secretion. *Rec. Prog. Horm. Res.* **29**:229-285.
44. YAHARA, I., and G. M. EDELMAN. 1975. Electron microscopic analysis of the modulation of lymphocyte receptor mobility. *Exp. Cell Res.* **91**:125-142.