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The binding of colchicine by sarcoma 180 cells

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THE METAPHASE arrest of dividing cell populations that is produced by colchicine was first described more than 30 yr ago,[1] but the biochemical mechanism of this action remains unknown. Anu mber of biochemical changes produced by colchicine have been described.[2–6] They include inhibition of the synthesis of DNA and of RNA and protein, a reduction in the level of ATP in regenerating liver, and increased formation of cytosine nucleotides. None of these effects, however, has been related directly to mitotic arrest, which involves some structural deformation.[7] Another biochemical approach to this problem is to study the intracellular disposition of colchicine itself. Taylor[8] has described the kinetics of the uptake and loss of colchicine-^{3}H in cultures of human KB cells, and suggested that the drug may be bound to intracellular components. Further evidence of such binding in grasshopper embryos has been obtained by Wilson and Friedkin.[9] We have studied the uptake of colchicine-^{3}H by sarcoma 180 (S180) cells and its intracellular distribution in order to determine if some type of binding does indeed occur in mammalian cells.

S180 in the ascites form was carried in Swiss mice (CD-1, 25–30 g). The ascitic fluid was withdrawn, contaminating erythrocytes were lysed by exposure to hypotonic saline (0·2%), and the cells finally resuspended in Eagle's medium. Colchicine was custom tritiated by Schwarz BioResearch, Inc. Extensive purification was carried out on this material, first by chromatography on columns of silicic acid that were eluted with mixtures of chloroform and methanol, and then by thin-layer chromatography on 0·5 mm layers of silica gel HF_{254} (Merck). The plates were eluted with three solvents: methanol, chloroform–isopropanol (3 : 1, v/v), and *n*-butanol–95% ethanol–concentrated ammonium hydroxide–water (4 : 1 : 2 9 1, v/v). The colchicine-^{3}H obtained has a sp. act. of 112·9 mc/ m-mole.

Tumor cells (2·1 to 4·4 × 10^8 cells) in 16 ml of Eagle's medium were incubated at 37° with 0·04 μmole of colchicine-^{3}H for up to 120 min. The cells were separated from the medium by centrifuging through sucrose solution (0·25 m) in Shevky-Stafford and McNaught centrifuge tubes.[10] Drug entered the cells rapidly, reaching maximum intracellular levels in about 20 min. After extraction of the cell with cold perchloric acid (0·5 M), a fraction of the radioactivity remained in the residue, but could be released in acid-soluble form by heating with sodium hydroxide (0·2 M) for 30 min at 90°. The amount of this bound fraction increased during the 120-min incubation to about 7 per cent of the total intracellular radioactivity. Similar findings have been reported with another antimitotic alkaloid, vinblastine.[11] Although alkaline hydrolysis decomposes RNA, the bound radioactivity that is released by such treatment does not appear to be associated with this nucleic acid. When the residues obtained by washing cells exposed to colchicine-^{3}H with cold acid were neutralized and incubated with ribonuclease or deoxyribonuclease, so as to produde almost complete hydrolysis of RNA or DNA, the

amount of tritium released in acid-soluble form was not significantly greater than when the enzymes were omitted from the incubation mixture. Paper and TLC of the acid-soluble extracts from the colchicine-^{3}H-treated cells revealed only one component that behaved like colchicine. In the alkali-labile fraction, a significant amount of a radioactive material that migrated like desacetylcolchicine was detected. Control experiments suggested that this was an artifact of alkaline hydrolysis.

In further examination of the acid-soluble radioactivity, S180 cells exposed to colchicine-^{3}H for 1 hr were washed free of medium as before, suspended in 0·25 M sucrose solution, and sonified. Between 93 and 99 per cent of the intracellular radioactivity remained in the supernatant fraction obtained by centrifuging the homogenate for 60 min at 100,000 g. During dialysis against 0·25 M sucrose solution at 14°, the initial rate of loss of tritium from the labeled supernatant fraction was significantly lower than for a mixture of colchicine-^{3}H and bovine serum albumin fraction V of the same protein content as the supernatant (Fig. 1). Half-times for this process varied from 4·2 to 6·8 hr

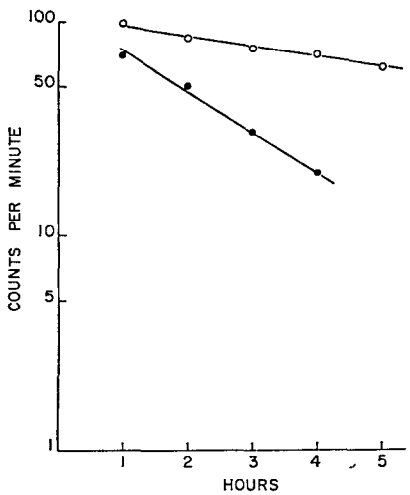

FIG. 1. The initial rate of loss of colchicine-^{3}H during dialysis. Supernatant fraction from cells exposed to colchicine-^{3}H for 1 hr (○——○); a mixture of colchicine-^{3}H and bovine serum albumin of the same protein content (5·1 mg/ml) and similar radioactivity to the labeled supernatant (●——●) Values represent the difference between the radioactivity in the bag (volume, 2 ml) and that in the dialysate (volume, 300 ml) per ml.

for the supernatant and from 1·4 to 1·9 hr for the mixture in four separate experiments. Rates for loss of tritium from the mixture did not differ significantly from that obtained with a solution of colchicine-^{3}H without protein. These data may best be interpreted as a retardation of diffusion rates resulting from reversible association of the drug with a nondialyzable component of the high-speed supernatant fraction. Further evidence for such binding was provided by gel filtration on 20 × 2 cm columns of Sephadex G-25 (Fig. 2). A significant percentage (20–35) of the radioactivity eluted with the protein fraction and before free colchicine-^{3}H.

The effects of different conditions upon the stability of the slowly diffusing fraction were determined by dialysis for periods of 24 hr (Table 1). Dialysis against solutions of urea caused essentially complete dissociation, as with the complex of actinomycin D with DNA;[12] the sensitivity to high ionic strength in the form of solutions of sodium chloride, was less marked. Glutamic acid, which prevents some biochemical effects of colchicine in Ehrlich ascites cells,[10] did not affect binding of the labeled drug. On the other hand, heating the labeled supernatant or carrying out the dialysis at 37° favored dissociation. Surprisingly, vinblastine and vincristine, which resemble colchicine in their biological and biochemical action, inhibited dissociation of the bound drug. Retention of radioactivity was dependent upon the pH of the medium and exhibited a sharp maximum at around pH 6.

Association of colchicine-^{3}H with cellular protein was not dependent upon the integrity of the cell, since it occurred during equilibrium dialysis of the compound with isolated high-speed supernatant fraction from untreated cells (Table 2). A number of compounds were tested in this system. Vinblastine and vincristine stimulated the binding of colchicine; griseofulvin and *p*-fluorophenylalanine inhibited this process; and glutamic, aspartic and fumaric acids, and tryptophan had no effect.

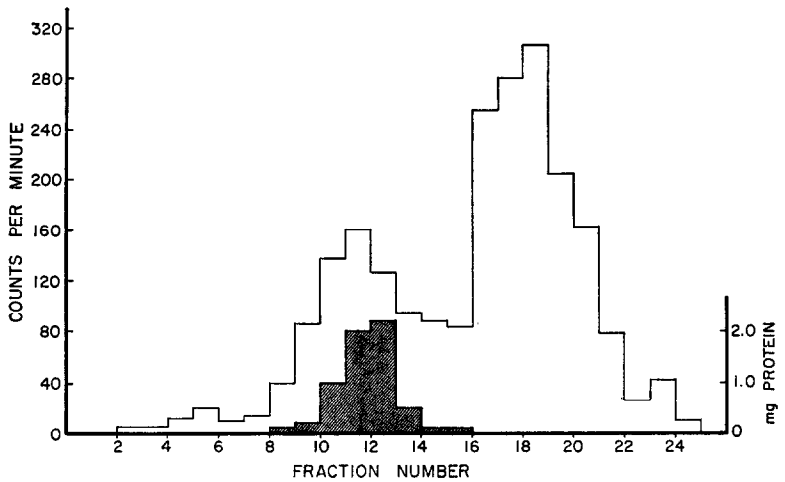

FIG. 2. Gel filtration of the high-speed supernatant fraction from cells exposed to colchicine-^{3}H as described in Table 1. A 2 × 20 cm column of Sephadex G-25 was employed. Elution was carried out with 5-ml fractions of 0·05 M sodium chloride solution. □ ≡ Radioactivity; ▨ ≡ protein content.

TABLE 1. EFFECT OF DIFFERENT CONDITIONS ON THE RETENTION OF COLCHICINE-^{3}H BY THE HIGH-SPEED SUPERNATANT FROM S180 CELLS EXPOSED TO LABELED DRUG

Treatment of supernatant*	Dialysis conditions†	Temperature (°C)	Bound colchicine (μμmole/mg protein)
None	Sucrose	14	0·87
None	Sucrose	25	0·55
None	Sucrose	37	0·16
60°, 10 min	Sucrose	25	0·02
None	Urea, 1 M	25	0·08
None	Urea, 2 M	25	0·05
None	Urea, 3 M	25	0·03
None	NaCl, 1 M	25	0·62
None	NaCl, 2 M	25	0·37
None	NaCl, 3 M	25	0·10
Glutamic acid (10 μmole /ml)	Sucrose	25	0·54
Vinblastine (1 μmole/ml)	Sucrose	25	0·76
Vincristine (1 μmole/ml)	Sucrose	25	0·94

* Cells (2·1 to 4·4 × 10^8) were incubated for 60 min with 0·04 μmole colchicine-^{3}H at 37°. After removal of medium, the cells were lysed and the homogenate was centrifuged (100,000*g*) for 30 min. This supernatant fraction, in 0·25 M sucrose, was adjusted to pH 6 with 0·05 M phosphate buffer; all added compounds were also brought to this pH.

† 100 vol. 0·25 M sucrose solution, or the indicated solutions, at pH 6 were used; dialysis was continued for 24 hr.

TABLE 2. EFFECT OF DIFFERENT COMPOUNDS ON THE BINDING OF COLCHICINE-^{3}H BY ISOLATED HIGH-SPEED SUPERNATANT FRACTION

Compounds*	Concentration† (μmole/ml)	Bound colchicine (μμmole/mg protein
Control		0·28
Aspartic acid	10	0·25
Fumaric acid	10	0·26
Glutamic acid	10	0·28
Tryptophan	10	0·30
p-Fluorophenylalanine	1	0·17
Griseofulvin	1	0·09
Vinblastine	1	0·58
Vincristine	1	0·61

* All solutions were brought to pH 6. Test samples (2 ml final volume) were dialyzed for 24 hr against 20 ml of external medium, with colchicine-^{3}H at the same level (20 μμmole/ml) on both sides of the membrane.

† This refers to initial concentration in the dialysis bag; compounds were not added to the external medium.

It has been reported, on the basis of measurements of optical rotation, that colchicine associates with DNA.[13] The present data suggest that colchicine also undergoes binding to proteins in the high-speed supernatant fraction of S180 cells. Since the total amount of bound alkaloid is very small, it is not unreasonable to conclude that binding is specific for relatively few protein molecules. Such binding may well represent the mechanism by which colchicine exerts its antimitotic action and some of its inhibitory effects on synthetic processes. Studies designed to identify and purify the protein fractions with which colchicine associates, as well as to clarify the role of such proteins in cell division, are in progress.

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