THE MECHANISM OF COLCHICINE INHIBITION OF MITOSIS

I. Kinetics of Inhibition and the Binding of H^3-Colchicine

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ABSTRACT

H^3-colchicine of high specific activity (2.5 curies per mM) was prepared in order to study the mechanism of colchicine inhibition of mitosis in cultures of human cells, strain K.B. No direct effects on the duration of the cell cycle or macromolecular synthesis were demonstrable at a concentration of colchicine which completely inhibited mitosis. The radioactive compound was bound to the cells at a rate proportional to colchicine concentration. The binding appeared to be reversible since the radioactivity of the cells reached a maximum value for a given concentration and was slowly lost after resuspension of the cells in fresh medium. A suitable exposure to colchicine produced accumulation of metaphase-blocked mitoses after the colchicine was removed from the medium. An exposure of 6 to 8 hours at 10^{-7} M was sufficient to block essentially all the cells in metaphase, thus indicating that colchicine is bound to the majority of interphase cells. The data are in quantitative agreement with a mechanism involving reversible binding of colchicine to a set of cellular sites. Based on the correlation between the time of first appearance of blocked mitoses and the radioactivity per cell, it is suggested that if a critical fraction (3 to 5 per cent) of the sites are complexed, the cell is unable to form a functional mitotic spindle.

INTRODUCTION

Colchicine is believed to interfere with cell division through its disruptive action on the mitotic spindle (1). Direct evidence for this has been obtained from polarized light microscopy (2) and from isolation of the remnants of the mitotic apparatus of colchicine-treated sea urchin eggs (3).

This inhibition by colchicine presumably occurs either through a direct or indirect effect of the compound on the spindle. An example of indirect action would be the activation of an enzyme which attacks the spindle while a direct action might involve binding of colchicine to spindle fibers causing them to dissociate into protein subunits. Since colchicine in concentrations less than 10^{-7} M completely inhibits mitosis in our cultures, the association constant of an assumed colchicine-protein complex should be sufficiently large to enable the complex to be isolated. These considerations led us to prepare tritium-labeled colchicine of high specific activity to facilitate a study of the mechanism of action.

In this report, experiments are described on the effects of colchicine on cellular metabolism, on the kinetics of inhibition of mitosis, and on the uptake and binding of radioactive colchicine. It will be shown that the results are consistent with reversible

binding of colchicine to a set of sites within the cell and lead to the conclusion that mitosis will be inhibited if a critical fraction of these sites are occupied by colchicine.

A second paper will deal with the binding of colchicine to cellular protein.

MATERIALS AND METHODS

Cell Cultures

All experiments were performed with suspension cultures of human cells, strain K.B. (4). The cultures were maintained in logarithmic growth with a doubling time of 24 hours. The culture conditions and the methods for determining the rates of macromolecular synthesis, cell number, and mitotic index have recently been described in detail (5) and will be briefly stated here.

The rates of synthesis of DNA, RNA, and protein were determined colorimetrically and by measurement of incorporation of C^{14}-thymidine, C^{14}-adenine, and C^{14}-valine, respectively. Mitotic index measurements were made on aliquots of the culture fixed in alcohol-acetic acid 3:1, and stained with methyl-green pyronin. In some experiments it was necessary to measure the number of cells in the stages of mitosis after short periods of time in the presence of colchicine. Cells were classified as normal metaphases if a metaphase plate was present. Blocked metaphases were distinguished by the presence of short, thick, and in some cases fused chromosomes, and the absence of a metaphase plate. Cells were definitely assigned to prophase if they possessed relatively long, slender chromosomes. Cells in late prophase, in which chromosome coiling was far advanced were difficult to distinguish from cells which had been blocked in metaphase for a short time. Since the values which we obtained for the prophase index in colchicine-treated cultures did not differ significantly from the controls we shall assume that the prophase index remained constant.

Mitotic spindles were examined in thin slide preparations by polarized light with a modified Leitz petrographic microscope (6). Cell counts were obtained with a Coulter Counter.

Colony forming ability was determined by plating aliquots diluted to contain 100 to 200 cells, in Petri dishes following the method of Ham and Puck (7).

Puromycin was a gift of the American Cyanamid Company, Pearl River, New York, and fluorodeoxyuridine was provided by the Cancer Chemotherapy National Service Center of the National Cancer Institute, Bethesda, Maryland.

Preparation of Tritium-Labeled Colchicine

Colchicine was prepared by methylation of colchiceine (obtained from K and K Laboratories, Jamaica, New York) with diazomethane in tritiated water (8). The reaction was performed by the New England Nuclear Corporation, Boston. The reaction products were colchicine and isocolchicine, the positional isomer obtained by interchanging the methyl ether and carbonyl groups of the tropolone ring.

Approximately 10 mg of the reaction mixture dissolved in chloroform was adsorbed to a 1 x 10 cm column of benzene-saturated alumina (Alcoa, Chromatographic Grade) and eluted with 0.5 per cent methanol in chloroform (8). The alcohol concentration was critical and it was necessary to remove the ethanol present in reagent grade chloroform by repeated freezing (9). A yellow impurity, also found in U.S.P. colchicine, remained adsorbed to the column. The strong colchicine absorption band centered at 350 mμ (molar extinction coefficient 1.8×10^4) was used to monitor the column effluent.

The radioactivity was separated into two main components by alumina column chromatography. The first was identified as colchicine by its absorption at 350 mμ, its specific biological activity (the minimum concentration necessary to completely inhibit mitosis of K.B. cells in 1 hour) and its chromatographic behavior on silica gel.

Colchicine, isocolchicine, and colchiceine can be resolved by thin layer chromatography (TLC) on silica gel using the solvent system butanol-ammonia-water, 2:1:2. 2.5 μg of the first component from the alumina column was chromatographed with 50 μg of colchicine. About 90 per cent of the radioactivity migrated with colchicine but a second radioactive substance was present (Fig. 1 *a*). This substance was rechromatographed with colchicine carrier and was found as a separate peak (Fig. 1 *b*). By combining material eluted from a number of chromatography plates, a sufficient amount of the unknown was obtained to test its action on the cells. Since the concentration of the material is unknown, comparison was made on the basis of radioactivity. When used at eight times the minimum radioactivity of colchicine that blocks mitosis, this material had no antimitotic effect. Also the compound was not bound to the cells. At the concentration present in the colchicine preparation this compound would account for less than 0.5 per cent of the radioactive uptake. Therefore, in most experiments it was not necessary to remove this material. However, the various kinds of labeling experiments reported here, involving measurements of the rate of incorporation, the maximum incorporation, and retention of activity after resuspension were checked with at least one experiment using the TLC-purified colchicine, and no differences were found.

The second component eluted from the alumina column had less than one twentieth the specific biological activity of colchicine and migrated behind colchicine in TLC chromatography. Since the reaction produces nearly equal amounts of colchicine

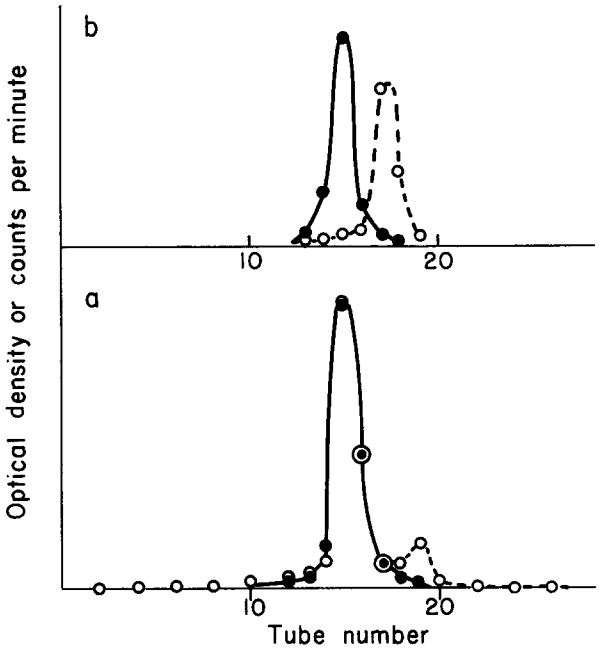

FIGURE 1 Thin layer chromatography of colchicine preparation obtained from alumina column purification step. (*a*) 2.5 μg of H^3-colchicine preparation plus 50 μg of U.S.P. colchicine in methanol were applied to silica gel plate and run with butanol-ammonia-water 2:1:2 as solvent. The maximum radioactivity was adjusted to coincide with the maximum optical density at 350 mμ. ●, optical density. ○, counts per minute. (*b*) Radioactive material from tube 19 was rechromatographed in the presence of U.S.P. colchicine. The chromatograms were cut into strips and eluted with 3 ml methanol for determinations of optical density and radioactivity.

and isocolchicine (8), the second component was presumed to be isocolchicine, although an authentic sample of this compound was not available for comparison.

The colchicine solution from the column was evaporated to dryness in a vacuum desiccator in the dark, redissolved in methanol and stored at 4°C. The specific activity of the product was approximately 2.5 curies/mM.

Counting Methods

To determine incorporation into intact cells, aliquots of the culture were washed three times by centrifugation in 10 ml of phosphate-buffered isotonic saline solution pH 7.4 (5). The precipitated cells were dissolved in 1 ml of 10X Hyamine by allowing them to stand overnight at 37°. The solution was transferred to a glass counting bottle by rinsing with a total of 10 ml of diotol (10) and counted in a Tri-Carb Scintillation Spectrometer. When necessary, corrections were made for quenching by addition of known amounts of a tritiated toluene standard.

The thin-layer chromatograms were cut into strips, eluted with methanol and an aliquot of the methanolic solution was counted in the diotol scintillation fluid.

EXPERIMENTAL RESULTS

Effect of Colchicine on Cell Growth and Metabolism

Since preliminary experiments showed that a colchicine concentration of 5×10^{-8} M was sufficient to arrest mitosis completely, a concentration of 10^{-7} M was used to determine the effects of colchicine on growth rate and macromolecular synthesis.

A typical curve for the accumulation of cells in mitosis is shown in Fig. 2. Since the culture was in logarithmic growth at the time of addition of colchicine, the accumulation of cells in mitosis can be calculated from the doubling time (T_0) of the uninhibited culture. If all cells which enter mitosis are blocked there, the relative number in mitosis at time t is given by

$$\int_{T_o - t - \Delta T_M}^{T_o} dN_T/N_o = e^{\lambda(t+\Delta T_M)} - 1 \qquad (1)$$

where dN_T is the number of cells of age T to $T + dT$, and ΔT_M is the duration of mitosis and $\lambda = \log_e 2/T_0$. The derivation of equation 1 has been given in a previous paper (5). T_0 was 24 hours and ΔT_M was 1.25 hours.

The curve of Fig. 2 is a plot of $e^{\lambda(t+\Delta T_M)} - 1$, allowing for a 1 hour delay in the appearance of blocked cells. The experimental points follow the theoretical curve for at least 11 hours, thus indicating no effect of colchicine during this period on any aspect of cell metabolism which significantly determines the progress of cells around the mitotic cycle. The number of mitoses then begins to fall below the expected number, probably through

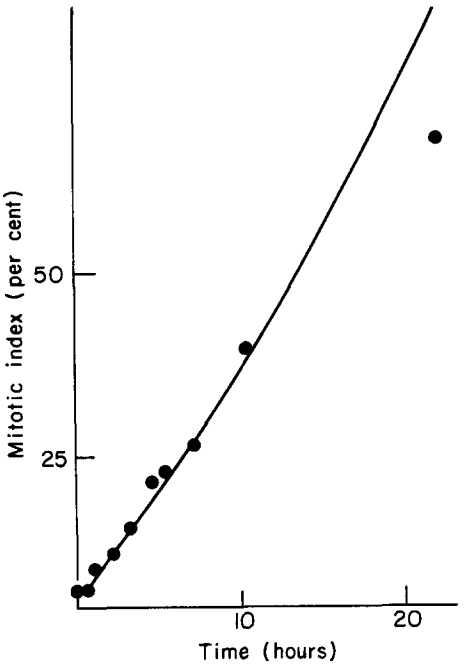

FIGURE 2 Accumulation of mitoses in the presence of 10^{-7} M colchicine. The solid line is theoretical and based on Equation 1. For details see text.

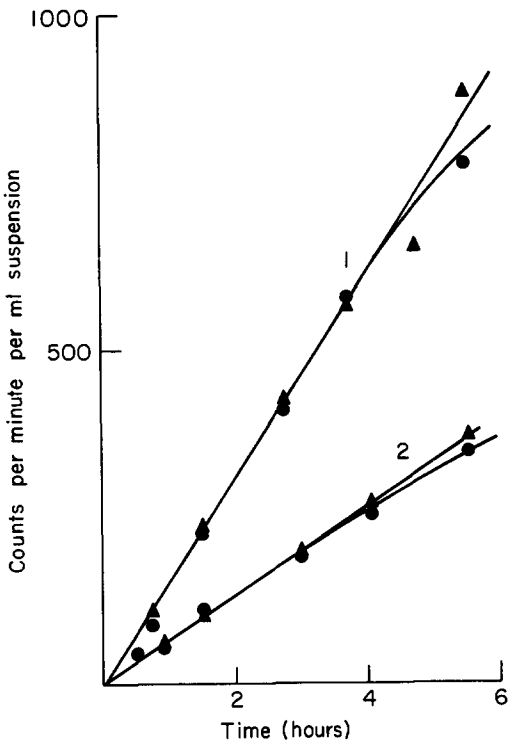

FIGURE 3 Incorporation of C^{14}-adenine (1) and C^{14}-valine (2) into cellular nucleic acid and protein. ▲, control; ●, 10^{-7} M colchicine added at zero time.

degeneration of blocked cells or regression of some of the blocked cells into an interphase-like configuration.

Measurements were made of the rates of protein, RNA, and DNA syntheses after addition of colchicine. Plots of the incorporation of C^{14}-valine into cell protein and of C^{14}-adenine into nucleic acid are shown in Fig. 3. (As in the latter experiments 85 to 90 per cent of the radioactivity was found in RNA, it is convenient to refer to the C^{14}-adenine incorporation as RNA synthesis.) The rates did not differ significantly from those in the control over a 5 to 6 hour period, but they did decrease after this time. Similar results were obtained for DNA synthesis by colorimetric analysis and by incorporation of C^{14}-thymidine.

Thus, at a concentration of 10^{-7} M, cell division was completely blocked in about 1 hour while the rates of DNA, RNA, and protein synthesis were not affected for several hours. Since the rates of macromolecular synthesis in cells in mitosis are less than in interphase cells, the decreasing rates at longer times are probably secondary effects.

It has been reported (11, 12) that mesoinositol or ATP could reduce the antimitotic effect of colchicine. Mesoinositol was said to prevent accumulation of blocked mitoses after about 12 hours while ATP reduced the rate of accumulation of mitoses from the start of inhibition. Since total cell number was not determined in these experiments, it is not clear whether the effect was a result of reduced growth rate, increased regression of blocked cells, or direct action on the mechanism of colchicine inhibition. As these experiments could indicate an effect of colchicine on some general aspect of cell metabolism, it was necessary to repeat them on our system.

The effects of inositol (6×10^{-4} M) and ATP (5×10^{-3} M) were tested at the minimum concentration of colchicine that produced complete mitotic inhibition (4×10^{-8} M). As will be shown below, lower concentrations lead to an increased delay in the appearance of blocked mitoses and a decreased rate of accumulation. Therefore any reduction in the effectiveness of colchicine should be readily detectable. The effects of the same concentrations of inositol and ATP were also measured on the controls; the duration of the delay and the accumulation of blocked cells did not differ

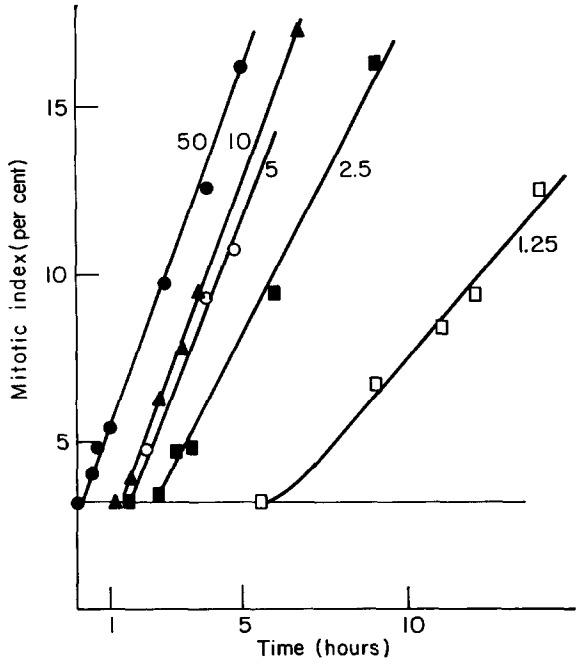

FIGURE 4 Accumulation of mitoses in cultures treated with colchicine at zero time. The numbers beside the curves refer to colchicine concentrations of 50, 10, 5, 2.5, and 1.25 × 10^{-8} M.

significantly from that obtained by colchicine alone, while the controls grew normally.

Kinetics of Inhibition of Mitosis

In attempting to elucidate the mechanism of action of colchicine it is necessary to investigate the kinetics of inhibition of mitosis and to correlate the results with the uptake and binding of radioactive colchicine. We will first consider mitotic index experiments.

The rate of accumulation of mitoses was measured over a range of colchicine concentrations from 10^{-8} to 5 × 10^{-7} M. A plot of typical experimental results is given in Fig. 4. At concentrations greater than 5 × 10^{-8} M mitoses accumulated at the maximum rate; the only effect of increasing concentration was to decrease the interval before accumulation began. This interval will be referred to as the lag time, T_L. As the concentration was reduced below 5 × 10^{-8} M, T_L increased further, and the slope of the mitotic index curve decreased.

A number of determinations of T_L were made over a wide concentration range and the data are shown in Fig. 5, in which T_L is plotted against $1/C$. The choice of $1/C$ rather than C for the abscissa was made partly to enable extrapolation to high concentrations but also because a linear relation between T_L and $1/C$ would have a simple interpretation. However, the T_L plot was non-linear

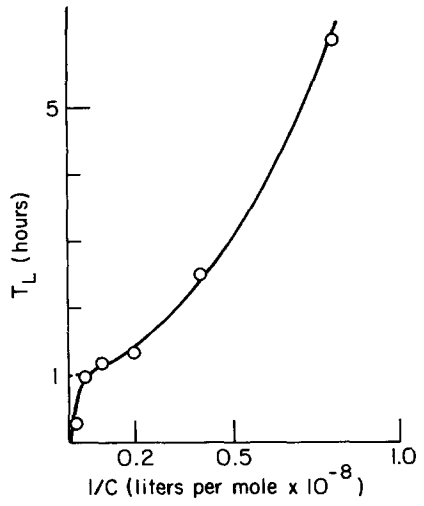

FIGURE 5 The time lag before increase in mitotic index T_L versus the reciprocal of the colchicine concentration. Values of T_L were obtained by linear extrapolation of curves of the type shown in Fig. 4 to the initial value of the mitotic index.

and in fact showed a complex dependence on concentration. Up to a concentration of 2 × 10^{-7} M, T_L values fitted a curve of decreasing slope which extrapolated to 0.9 hours, while at higher concentrations the curve showed a point of inflexion and approached a limiting value of zero.

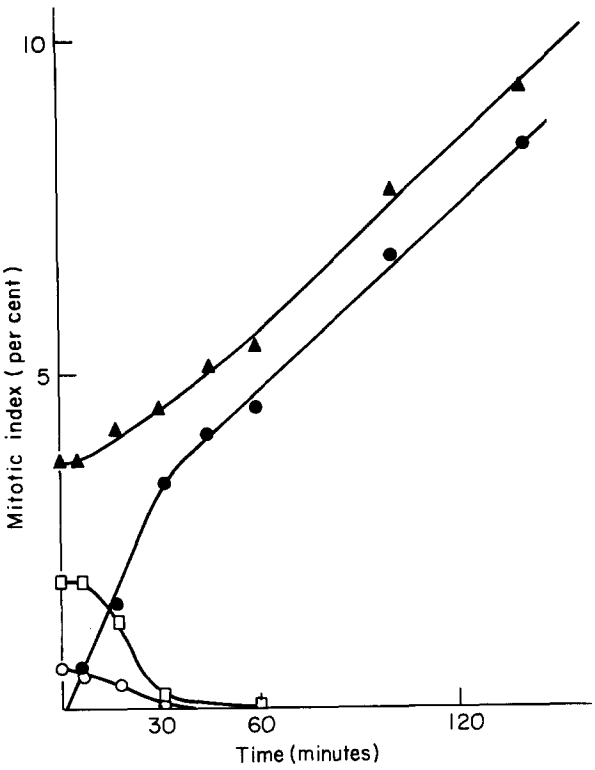

FIGURE 6 The total mitotic index (▲), blocked metaphase index (●), metaphase index (□), and anaphase index (○) after the addition of colchicine at time zero to a concentration of 5×10^{-7} M.

The fact that the curve for concentrations less than 2×10^{-7} M extrapolates to a value of 0.9 hours, which is equal to the duration of metaphase plus anaphase, suggests that cells in different stages of mitosis are differentially sensitive to inhibition.

Therefore measurements were made of the number of metaphase, blocked metaphase and anaphase cells during the first few hours after addition of colchicine. In Fig. 6 and Fig. 7 the various quantities are plotted for a high colchicine concentration (5×10^{-7} M) and for that concentration (2×10^{-7} M) which corresponds to the change of slope of the T_L plot (Fig. 5). The mitotic index was determined by counting 2000 to 4000 cells. Then to obtain sufficiently accurate values for metaphase and anaphase indices, the slides were scanned again and only cells in mitosis were counted and categorized. The anaphase or metaphase index was obtained by multiplying the previously obtained mitotic index by the percentage of anaphase or metaphase cells in the second mitotic cell count. This procedure reduced the labor, since a count of even 200 mitotic cells corresponds to counting about 6000 cells.

In order to interpret the curves of Fig. 6 and Fig. 7 we note first that for large values of t the slopes of the total mitotic index or blocked metaphase index curves correspond to the theoretical values calculated for complete inhibition of mitosis. Since this slope is a measure of the rate of arrival of cells at mitosis the slope of the blocked metaphase curve at early times cannot exceed this value unless cells present in metaphase at t = 0 remain blocked there.

As shown in Fig. 6 ($C = 5 \times 10^{-7}$ M), at early times the slope of the blocked metaphase curve was higher than at later times indicating that some cells in metaphase at $t = 0$ were being blocked. The durations of metaphase and anaphase are about 40 minutes and 15 minutes. The first appearance of blocked cells occurred at about 3 to 5 minutes and during the next 40 minutes the slope exceeded the value for complete inhibition. In addition extrapolation of the curve from times greater than 40 minutes gave a value at the beginning of the block of about 2 per cent which corresponds to the metaphase index. Thus, essentially all the cells in metaphase 5 minutes after addition of colchicine were blocked.

At a lower colchicine concentration (2×10^{-7}

M) the slope of the blocked metaphase curve (Fig. 7) was only slightly higher at early times than at later times. By extrapolating the curve at later times to the time of the first appearance of blocked metaphases, (10 to 15 minutes) we obtain a value of about 1 per cent. Therefore about half the cells present in metaphase 15 minutes after adding colchicine were blocked there, which corresponds to about 40 per cent of the metaphases present at $t = 0$. The metaphase index decreased to zero between 15 and 75 minutes while the anaphase index reached zero at 50 minutes. The decrease in metaphases from 50 to 75 minutes was caused entirely by blocking since there were no anaphases present during this interval. This decrease corresponds to about 35 per cent of the original number, which gives a second estimate of the number of original metaphases which were blocked. Up to 50 minutes the total mitotic index did not increase because the increase in blocked metaphases was balanced by the completion of mitosis by cells already in metaphase or anaphase.

At a still lower concentration (5×10^{-8} M) the early slope of the blocked metaphase curve (not shown) was less than the complete inhibition value. Thus a fraction of the cells entered metaphase and completed mitosis but this fraction decreased with time until a complete block was achieved.

The results of this set of experiments provide an explanation for the shape of the T_L versus $1/C$ curve (Fig. 5). At colchicine concentrations below about 2×10^{-7} M there is a lag of at least 0.9 hours owing to completion of mitosis by metaphases and anaphases present at $t = 0$. As the concentration is raised the lag is reduced by the blocking of an increasing fraction of the metaphases present at zero time.

Therefore, to follow the time course of inhibition, it is necessary to measure the time of first appearance of blocked metaphases rather than the time at which the total mitotic index increases. This parameter can be obtained from blocked metaphase index curves, such as those shown in Figs. 6 and 7, by extrapolating to zero index.

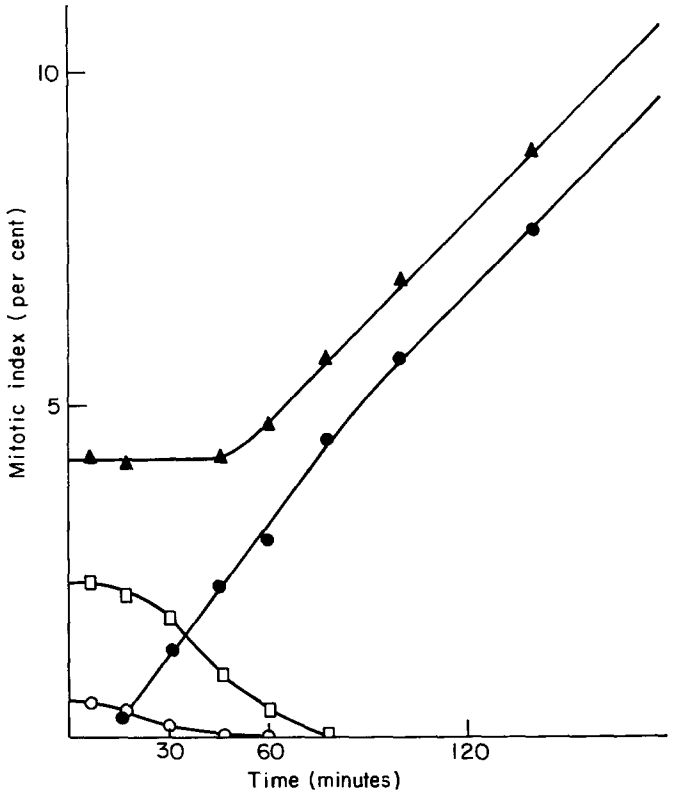

FIGURE 7 The total mitotic index (▲), blocked metaphase index (●), metaphase index (□), and anaphase index (○) after the addition of colchicine at time zero to a concentration of 2×10^{-7} M.

When such values were plotted against $1/C$ over the concentration range from 5 to 50 $\times$ 10^{-8} M, they fell on a straight line through the origin.

Recovery After Colchicine Inhibition

The effect of time of exposure to colchicine on the accumulation of mitoses was investigated by stopping colchicine uptake by diluting or resuspending the cells. The cell density was increased to about 5 $\times$ 10^6 cells/ml by centrifuging one liter of culture and resuspending the cells in 50 ml of growth medium. A period of 30 minutes was allowed for the cells to return to steady state growth. Colchicine was then added to a concentration of 10^{-7} M. At various times 5 ml aliquots were withdrawn and pipetted into 250 ml of growth medium. The mitotic index curves shown in Fig. 8 are a composite of two such experiments.

The experiments described in the last section showed that blocked metaphases first appeared at about 0.6 hours at 10^{-7} M. When the uptake was stopped before 0.6 hours no increase in mitotic index occurred. For longer exposure times there was an accumulation of mitoses after dilution of the culture and the period of this further accumulation increased with the time of exposure to colchicine. Thus dilution at 0.63, 1.5, 2.25, and 2.75 hours allowed cells to accumulate in mitosis for further periods of 0.5, 1.5, 2.0, and more than 4 hours, respectively. An exposure of 6 to 8 hours was sufficient to completely block the culture. In this case the mitotic index at 24 hours was nearly as high as the value obtained by continuous exposure to colchicine. Apparently the block was largely irreversible, as the majority of the cells blocked in metaphase did not regenerate a spindle when the external colchicine was removed.

Similar results were obtained at a higher concentration except that the time of exposure required to produce a given accumulation of mitoses was reduced.

The plating efficiency of the cells decreased with increasing time of exposure to colchicine and was less than 1 per cent after an exposure of 36 hours. The decrease in plating efficiency was faster than the rate of arrival of cells at mitosis indicating that some cells still in interphase at the time of plating were still irreversibly blocked on arrival at mitosis.

A simple explanation can be given for these results if we assume (*a*) that colchicine is bound reversibly by the majority of the cells in the population, but at such a slow rate that several hours are required to saturate the cells, and (*b*) that colchicine is slowly lost from the cells after dilution of the culture but cells continue to be blocked at

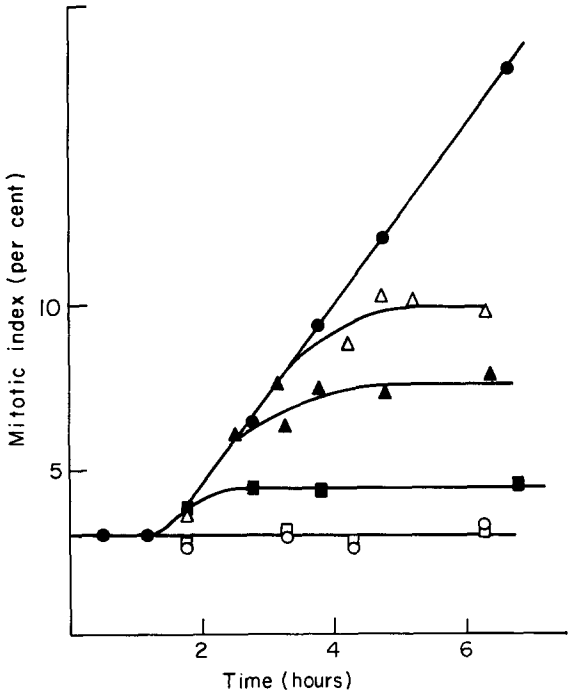

FIGURE 8 Accumulation of mitoses after exposure to 10^{-7} M colchicine for varying periods of time. Colchicine was added at zero time and an aliquot of the culture was diluted 50-fold at 0.2 (○), 0.4 (□), 0.63 (■), 1.5 (▲), 2.25 (△), and 2.75 (●) hours.

metaphase as long as the bound colchicine per cell exceeds a critical value. This explanation is consistent with the results on the binding of H^3-colchicine.

Uptake of Radioactive Colchicine

Two steps must be distinguished in the process of inhibition by colchicine, first, the penetration of the cell membrane, and secondly, the conversion of internal colchicine to a form which is not readily diffusible, which we shall refer to as bound colchicine. As the binding is not likely to be covalent we must first determine how much radioactivity is lost when the cells are washed free of labelled medium.

H^3-colchicine was added to a culture at a concentration of 10^{-7} M and after 1 hour a 10 ml aliquot was withdrawn, centrifuged 2 minutes in a clinical centrifuge, and washed three times by resuspension and sedimentation in phosphate-buffered isotonic saline. By determining at each step the total radioactivity per milliliter of the suspension and of the supernatant after sedimentation, the radioactivity of the cells can be obtained by difference. For the first step the radioactivity of the suspension and supernatant are both large numbers, so that the total activity in the cells could not be determined accurately, but in a number of experiments the total loss of activity was about 40 per cent. However, the loss between the second and third wash was less than 5 per cent. We therefore define bound colchicine as the radioactivity present after three washes. The results given later (see p. 158) indicate that the loss of bound radioactivity in the washing procedure was about 5 per cent.

Since the two processes, entry and binding, may proceed at quite different rates, the relative amount of activity removed by washing may depend on the time of exposure to colchicine. Experiments were therefore carried out at concentrations of 50, 10, and 5×10^{-8} M over a 2 hour period to distinguish between bound and free colchicine uptake. An 800 ml culture was harvested and resuspended in 50 ml of fresh growth medium. After allowing 30 minutes for the clumps to be dispersed, colchicine was added, and 5 ml and 2 ml samples were taken at various times. The 2 ml sample was washed three times to obtain the bound radioactivity. The 5 ml sample was centrifuged for 2 minutes in a Constable centrifuge tube calibrated in units of 0.01 ml. The supernate was removed by a fine pipette leaving a constant volume of 0.08 ml of which about 0.06 ml was packed cells. The radioactivity of the supernate was determined several times during the experiment and the sample radioactivity was corrected for supernatant volume to give the total uptake.

The results of an experiment at 50×10^{-8} M are shown in Fig. 9. The radioactivity in the packed cells became equal to the radioactivity in an equal volume of medium in less than 3 minutes. (The latter value is shown in Fig. 9 by the horizontal dashed line.) Although up to about 40 per cent of the packed cell volume may be occupied by radioactive medium, this will introduce a small but constant error which does not affect the shape of the curve. The total count rose rapidly during the first 10 minutes and then increased at a lower but nearly constant rate. The rate of incorporation into a bound form increased slowly to a maxiumum value during the interval of rapid uptake of total radioactivity. For times greater than 10 or 15 minutes the difference between total count and bound count was approximately constant. Similar results were obtained at 10 and 5×10^{-8} M.

The curves therefore have a simple interpretation. The period of rapid increase in total count is determined by the time for colchicine to penetrate the cell and come to equilibrium with external colchicine. During this phase the rate of binding increases in proportion to increasing internal colchicine concentration. After this initial rapid uptake, further increase in radioactivity is due to the bound form. The difference between the two curves is a measure of free internal colchicine and should be roughly equal to the activity of an equal volume of medium. At concentration of 5 to 50×10^{-8} M the ratios of free internal colchicine to external colchicine were in the range from 1.5 to 2.0. Since these ratios depend on the accuracy of several measurements, we can only conclude that the concentration of the internal colchicine pool is not markedly different from the concentration in the medium.

The initial penetration phase was completed in 10 to 15 minutes over the range of concentration studied. Since this phase is short, and is not very dependent on concentration, the rate limiting step in colchicine inhibition is not the rate of penetration of colchicine into the cell, but is determined by a process in which colchicine is bound to some cellular component(s).

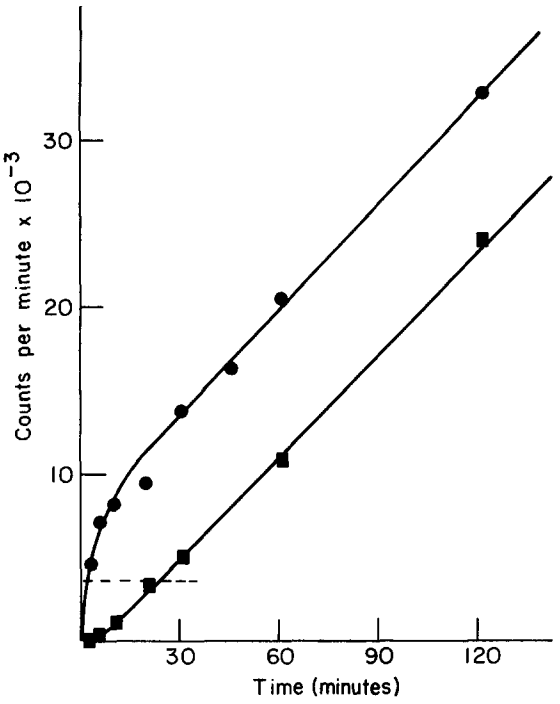

FIGURE 9 Uptake of H^3-colchicine, 50×10^{-8} M by unwashed cells (●) and cells washed three times by sedimentation in phosphate-buffered isotonic saline (■). The horizontal dashed line is the radioactivity in a volume of medium equal to the volume of packed cells in the samples. For details see text.

Concentration Dependence of Colchicine Uptake

The accumulation of bound colchicine over a range of concentrations is shown in Fig. 10. In this figure and in all the results to follow the uptake is expressed per 3×10^6 cells. At a concentration of 50×10^{-8} M or higher a plateau was reached in about 4 to 5 hours, while at lower concentrations a longer time was required. At 2.5×10^{-8} M a plateau was not obtained and at this concentration the slope of the mitotic index versus time curve is less than the theoretical value (Fig. 4) indicating an incomplete mitotic block.

Because the total cell count decreases after 12 hours exposure to colchicine, the statement that a plateau is reached must be qualified since the radioactivity is expressed on a per cell basis. If the binding by a cell depends on its position in the mitotic cycle, degeneration of cells with more than the average radioactivity could mask further uptake. Although a plateau was obtained in several experiments at different concentrations, a further slow uptake of about 20 per cent of the plateau value spread over the remainder of the generation time (24 hours) could go undetected.

If we assume that the cell contains a set of colchicine binding sites, the activity at the plateau

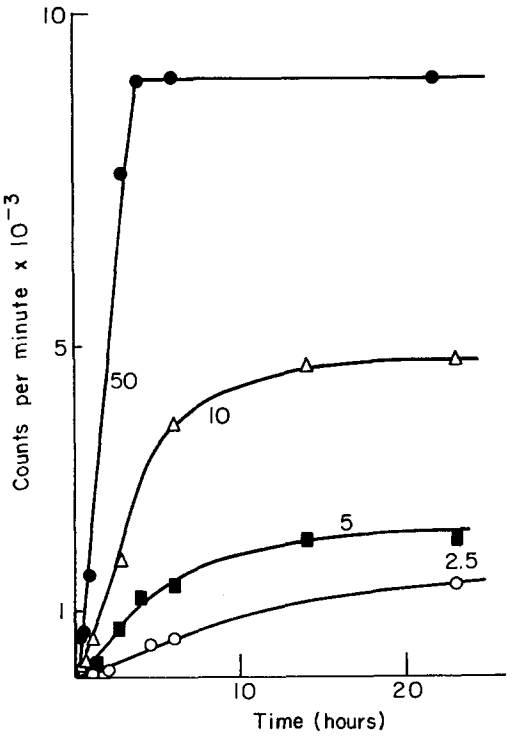

FIGURE 10 Incorporation of H^3-colchicine *versus* time for colchicine concentrations of 50, 10, 5, and 2.5 $\times$ 10^{-8} M. All data are expressed per 3×10^6 cells.

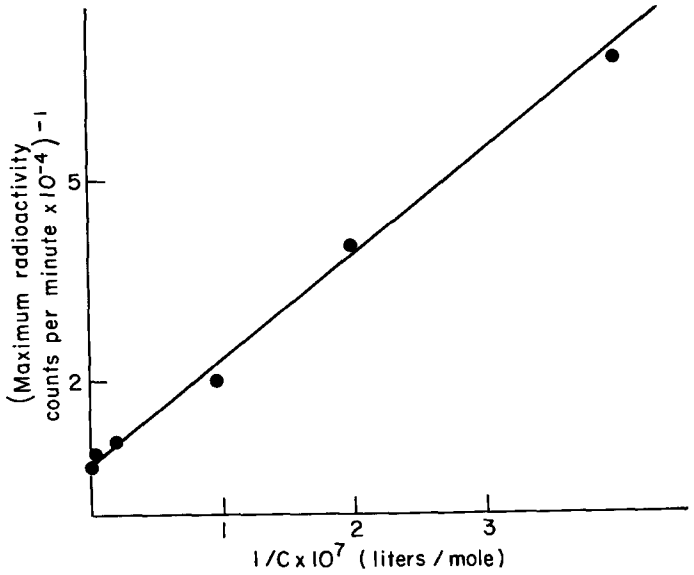

FIGURE 11 Reciprocal of the maximum radioactivity incorporated per 3×10^6 cells *versus* reciprocal of the colchicine concentration.

will be proportional to the number of binding sites occupied by colchicine. If the system is in equilibrium, it can be shown that a plot of $(\text{cpm})^{-1}$ versus C^{-1} should be a straight line. The data are plotted in Fig. 11 over the concentration range from 2.5 to 1000×10^{-8} M. At the higher concentrations the radioactive sample was diluted with non-radioactive colchicine and the data were corrected for the difference in specific activity. In view of the errors involved, the data fit reasonably well to a straight line.

Factors Determining the Time to Reach Maximum Incorporation

Two possible explanations will be considered for the relatively long time required to reach saturation (T_s). In the first the rate of the binding reaction is relatively high and the saturation time is determined by increase in the number of binding sites. At $t = 0$ the culture is in logarithmic growth but as time passes the cells accumulate at metaphase while macromolecular synthesis is unaffected in the cells still in interphase. The radioactivity will continue to increase until all the cells in the population have completed synthesis of the sites. In the second explanation we assume that the saturation time is the time necessary for the binding reaction to reach equilibrium.

1. The first explanation is unlikely to be correct over the entire concentration range since the saturation time decreases with concentration. However, for concentrations from 5×10^{-7} to 100×10^{-7} M, T_s decreases by perhaps 1 hour, which is within the experimental error, so that this 4 to 5 hour period could reflect site accumulation. To test this hypothesis, the growth state was perturbed by inhibiting protein synthesis and by partially synchronizing the culture with 5′-fluorodeoxyuridine.

It will be shown in a later report that the binding sites appear to be protein(s). Therefore if T_s measures a process of synthesis of binding sites we would expect a smaller saturation time and a lower plateau radioactivity when protein synthesis is inhibited. The rate of protein synthesis was reduced to about 20 per cent of the control by puromycin, 4×10^{-6} M. The results of a study of the effects of puromycin on protein synthesis and mitosis in K.B. cells have been reported previously (5). After ½ hour in the presence of puromycin, colchicine was added to a concentration of 5×10^{-7} M. The colchicine uptakes per unit protein content of the control and the puromycin inhibited culture were the same within experimental error, each reaching the same plateau radioactivity at 5.5 hours.

To test whether uptake was dependent on the age distribution of the cells in the culture, partial synchrony was obtained by pretreating with 5′-fluorodeoxyuridine, 5×10^{-7} M, for 16 hours. An aliquot of the culture was kept as a control and

radioactive colchicine, 5×10^{-7} M, was added to the remainder, and 5 hours were allowed for the activity to reach a plateau. The colchicine treated culture was divided into two parts and thymidine was added to one of them and the uptake was followed for another 5 hours. Thymidine was also added to the control culture which had not received colchicine. This culture showed a burst of mitoses at 7 hours. There was a small increase in radioactivity of the colchicine treated culture after thymidine addition but the increase in count over the plateau value was only 2 per cent and 5 per cent in two separate experiments, which is scarcely outside the limits of error. Also, the saturation time was still equal to 6 hours. FUDR treatment shifts most of the population into the G_1 period without changing the saturation time which provides further evidence that synthesis of binding sites is not the primary factor in determining the rate of colchicine uptake. Furthermore, any synthetic processes initiated with resumption of DNA synthesis do not raise the colchicine binding appreciably.

2. Direct evidence for the second explanation that T_s is determined by the rate of the binding reaction can best be obtained in a cell-free system and this aspect of the problem will be treated in a subsequent report. However if the rate of uptake by cells is determined by a chemical reaction which comes to equilibrium in a time T_s, the rate of binding at early times will depend on colchicine concentration. Therefore, if the reaction is first order in colchicine concentration and if the internal and external colchicine concentrations are equal, a plot of the rate of uptake versus concentration should be a straight line. To test this possibility a culture was divided into five aliquots and the binding was measured from 0.75 to 2.25 hours over the range from 5 to 150 $\times 10^{-8}$ M. In Fig. 12 the slopes of the incorporation curves are plotted and it can be seen that the data are fitted by a straight line.

Reversibility of Colchicine Uptake

We have shown (see p. 152) that exposure to colchicine could lead to accumulation of blocked mitoses after colchicine was removed from the medium and that the period of further accumulation increased with the time of exposure to colchicine. These results suggest that colchicine remains bound in the cells after its removal from the medium. To test this possibility cultures were exposed to H^3-colchicine, and after varying periods of time the uptake was stopped by diluting the culture 50-fold. In Fig. 13 we have plotted the uptake and loss of radioactivity for cultures

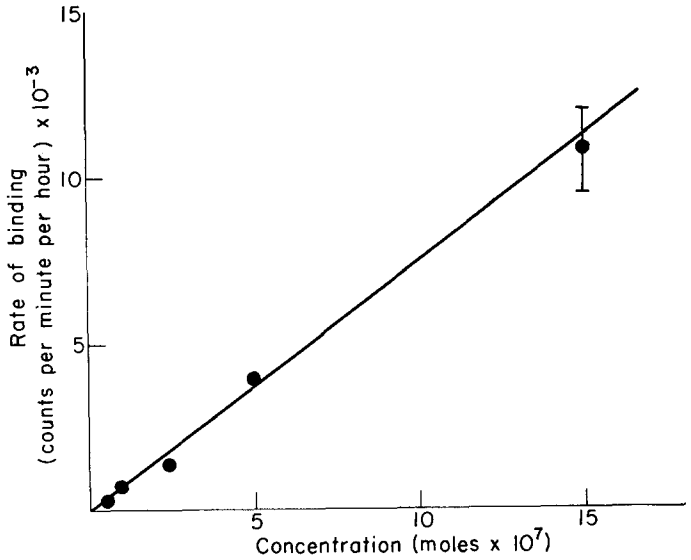

FIGURE 12 Rate of incorporation of H^3-colchicine *versus* colchicine concentration. Rates were obtained from the slope of incorporation *versus* time curves (as in Fig. 10) from 0.75 to 2.25 hours. At the highest concentration the incorporation rate was not constant over this time interval and the slope at zero time was estimated by eye.

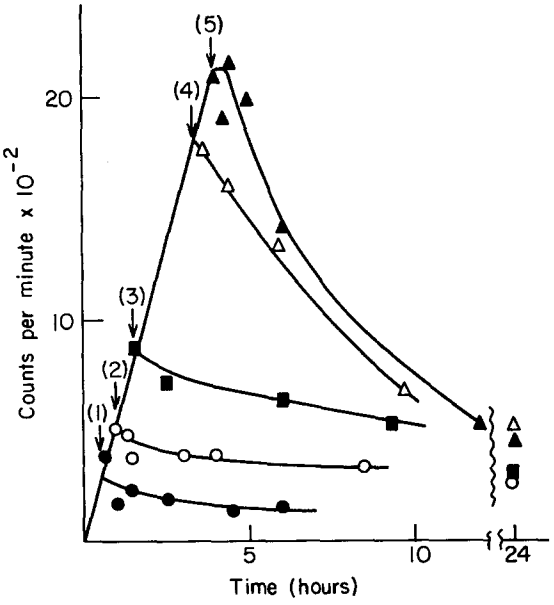

FIGURE 13 Retention of radioactivity after 50-fold dilution of the culture. H^3-colchicine, 10^{-7} M, was added at zero time and aliquots of the culture were diluted 50-fold at the times indicated by the arrows. (Data for dilutions *2* and *4* were obtained in one experiment and the rest in another. The uptake curve for *2* and *4* was adjusted to congruence with that for *1*, *3*, and *5*.)

exposed to 10^{-7} M colchicine for periods of 0.5, 1.0, 1.5, 3.3, and 3.9 hours.

Dilution of the culture stopped the uptake in less than 0.5 hours and radioactivity was slowly lost by the cells. Values for the maximum mitotic index and for the radioactivity retained at the end of the index accumulation period are given in Table I. Dilution at 0.5 hours prevented index increase, while for longer exposures there was a period of accumulation of mitoses which continued until the radioactivity declined to about 400–800 CPM. Values of the maximum index for a given exposure time were not reproducible to better than 5 per cent, and the corresponding accumulation times were uncertain by 1 to 2 hours. However the results do suggest that there is a critical value for the colchicine binding and that if this value is exceeded the cell is unable to form a functional mitotic spindle.

DISCUSSION AND CONCLUSIONS

At this stage it is important to distinguish between the experimental results and our interpretation of them.

The experimental findings are as follows:—

1. At a colchicine concentration of 10^{-7} M there was no measurable effect on the rates of DNA, RNA, and protein synthesis nor on the rate of progress of cells around the mitotic cycle, for some hours after mitosis was completely inhibited.

2. A plot of T_L, the time lag before increase in the mitotic index, against $1/C$ had an inflection point at about 2×10^{-7} M.

3. At concentrations greater than 2×10^{-7} M, cells in metaphase at $t = 0$ were blocked. At somewhat lower concentrations the original metaphases and anaphases completed mitosis while prophase cells were blocked.

4. Exposure of the culture to colchicine for increasing periods of time led to increasing periods of further accumulation of mitoses after the inhibitor was removed from the medium. An exposure of 6 to 8 hours at 10^{-7} M was sufficient to block essentially all the cells in the culture as they reached metaphase.

5. Colchicine penetrated the cells rapidly and equilibrated with external colchicine in less than 15 minutes.

6. Colchicine was present in the cells in a bound form which was only slowly lost when cells were resuspended on colchicine-free medium.

7. The rate of binding of colchicine was proportional to concentration.

8. Bound radioactivity reached a maximum value per cell for a given concentration of colchicine.

9. The time to attain the maximum radioactivity depended on concentration and could be reduced to 4 to 5 hours at very high concentrations (10^{-5} M).

The main features of the experimental results can be interpreted on the basis of a simple model. Since colchicine does not interfere with cellular metabolism or macromolecular synthesis, its action is on a target unique to mitosis. We therefore assume that the cell contains a set of sites capable of binding colchicine and that a normal mitotic spindle can not be formed if a critical fraction of the sites is occupied by colchicine.

Quantitative agreement with the experimental data can be obtained by expressing the binding in terms of a first order chemical reaction. Since the rate of penetration of the cell by colchicine was shown to be much faster than the rate of binding (Fig. 9) and the internal colchicine concentration was roughly equal to the external concentration, it is reasonable to ignore the penetration step in our model. Let S_0 be the average molar concentration of binding sites. The rate of colchicine uptake at early times was found to be proportional to colchicine concentration (Fig. 12). Therefore the rate can be expressed as $R_1 = k_1 (C) (S)$ where (C) and (S) are the concentration of colchicine and of free binding sites and k_1 is the specific rate constant. The binding reaches a maximum value which indicates that equilibrium is established and therefore we have

$$K = k_1/k_{-1} = (CS)/(C)(S) \qquad (2)$$

where (CS) is the concentration of occupied sites, k_{-1} is the specific rate constant for dissociation, and K is the equilibrium constant. The rate of dissociation is given by $R_{-1} = k_{-1} (CS)$. Since $(S) = S_0 - (CS)$, we may write equation 2 in the form

$$\frac{1}{(CS)} = \frac{1}{S_0} + \frac{1}{KS_0}\frac{1}{(C)}$$

Therefore, since (CS) is proportional to the radioactivity at saturation, and (C) can be taken as approximately equal to the external colchicine concentration, a plot of (maximum counts per minute)$^{-1}$ *versus* C^{-1} should be a straight line. The data are plotted in this form in Fig. 11 and can be seen to lie approximately on a straight line.

From the slope and intercept of the line we calculate the values of S_0 and K to be 1.6×10^4 CPM per 3×10^6 cells and 4×10^6 liters moles^{-1}. k_1 can be evaluated from Fig. 12, since at early times $(S) \cong S_0$ and $k_1 = R_1/[(C)S_0]$. $k_1 = 0.5 \times 10^6$ liters moles^{-1} hours^{-1} and $k_{-1} = k_1/K = 0.13$ hours^{-1}.

Using the values of these parameters some conclusions can be drawn. First, from the value of k_{-1} we may calculate the rate of loss of radioactivity when the cells are resuspended on fresh medium. When the uptake has reached 1000 CPM/3×10^6 cells the initial rate of loss should be about 150 CPM per hour which agrees reasonably well with the value of 200 CPM per hour obtained from the data plotted in Fig. 13. It also follows that the loss of bound radioactivity in the washing procedure did not exceed 5 per cent.

An estimate of the concentration of binding sites can be obtained from S_0. Assuming a molecular weight per site of 100,000, then from the specific activity of colchicine and the protein content per cell we calculate that the sites make up to 4 to 8 per cent of the total protein. This figure is probably too large for an enzyme but is reasonable enough for a structural protein.

Since an exposure to colchicine of 6 to 8 hours is sufficient to block essentially all the cells in the culture (doubling time, 24 hours), colchicine must be bound to interphase cells. The binding sites are probably present in all the cells although the amount of colchicine bound per cell may depend on its position in the mitotic cycle. If we ignore this possible heterogeneity in binding (which may be only a factor of two) it can be inferred from the uptake of radioactivity (Fig. 10) at the time of first appearance of blocked metaphases that an uptake of about 500 CPM per 3×10^6 cells was sufficient to block mitosis. A similar value (400 to

TABLE I

Retention of Radioactivity at the End of the Mitotic Index Accumulation Period

Time of dilution	Maximum mitotic index	Radioactivity at maximum mitotic index
(*hours*)	(*per cent*)	(*cpm per* 3×10^6 *cells*)
0.5	3.2 (normal)	250
1.0	15	400
1.5	13	600
3.3	24	750
3.9	50	700
7.0*	75	600

At the times indicated in column 1, an aliquot of a culture at an approximate density of 5×10^6 cells/ml was diluted 50-fold with fresh medium. Colchicine, 10^{-7} M, was added to all cultures at $t = 0$.

* In this case a culture at the density of 2×10 cells/ml was resuspended on fresh medium.

800 CPM per 3×10^6 cells) was obtained from measurements of the accumulation of mitoses and retention of radioactivity after dilution of the culture (Fig. 13 and Table I). By comparing these estimates with S_0 the uptake required for saturation of the sites (1.6×10^4 CPM per 3×10^6 cells) we estimate that inhibition will occur if about 3 to 5 per cent of the sites are complexed with colchicine.

The time of first appearance of blocked mitoses was approximately proportional to $(\text{concentration})^{-1}$. Since the rate of incorporation is proportional to concentration the time to block a critical fraction of the sites would also be proportional to $(\text{concentration})^{-1}$. It is therefore probable that the rate limiting step in inhibition of mitosis is the formation of the colchicine-site complex. If other steps are involved, such as enzymatic activation of colchicine they are presumably much faster than the binding step. It should be noted that the kinetic laws obeyed by most enzyme reactions (Michelis-Menten kinetics) require that the $(\text{rate})^{-1}$ be proportional to $(\text{substrate concentration})^{-1}$. The colchicine binding does not follow this prediction but rather the rate is proportional to concentration which is the result expected for a first order reaction.

The inflection in the plot of the lag time for increase in mitotic index *versus* $1/C$ (Fig. 5) suggests that the different stages of mitosis differ in sensitivity to inhibition. For concentrations greater than 2×10^{-7} M, metaphases present at $t = 0$ are blocked while at lower concentrations metaphases complete mitosis while prophase cells are blocked. This finding is in agreement with the observations of Gaulden and Carlson (13) on grasshopper neuroblasts who reported blockage of prophases at 2×10^{-6} M while a tenfold increase in concentration was required to block metaphases.

While this work was in preparation, Puck and Sheffer (14) reported similar mitotic index accumulation curves for HeLa cells inhibited with colcemid. However it was stated that the lag was not reduced below 1 hour for a 20-fold increase in concentration. Since in their case the lag corresponds to the duration of mitosis, the cells in prophase at $t = 0$ were not affected, and the authors suggest that the result may be caused by a decrease in membrane permeability at the beginning of prophase. The differences between their experimental results and our own are probably more apparent than real. In our case the lag time also changes very little over a 4- to 5-fold concentration range from 5 to 20×10^{-8} M but does decrease at higher concentrations. Since there are numerous reports on the destruction of metaphase spindles by colchicine (1, 13) the experimental discrepancy is largely a matter of how great an increase in concentration is necessary to shorten the lag to the point where existing metaphases are blocked.

Our results do not provide convincing evidence against the permeability hypothesis but they do require the decrease in permeability to occur at the end of prophase rather than the beginning. Although there is no evidence for such a change, an important change does occur in the cell at this time, namely assembly of the spindle from its precursors. It is easy to imagine mechanisms in which a higher concentration of colchicine would be required to disrupt the mitotic spindle than to prevent its assembly.

Can the colchicine binding sites deduced from the kinetic data be identified with spindle protein or a precursor protein of the spindle? Since this point cannot be settled until the sites have been isolated and characterized it is not fruitful to devote much discussion to it at this time. The kinetics of inhibition of mitosis and of uptake of colchicine are consistent with binding to a structural protein of the spindle, but saturation of the target at times short compared to the generation time may present a difficulty. If spindle precursor protein is the target site we are forced to conclude that the amount present in a culture in exponential growth is a large fraction of the total amount in a culture in which most of the cells are blocked at metaphase.

Since the fate of spindle protein after division and the period in the mitotic cycle of new spindle protein synthesis is unknown this conclusion need not be inconsistent with the hypothesis that the target site is a spindle component. It is also possible that colchicine may bind to microtubule elements of the spindle and this component is present at all stages of the mitotic cycle since it has other functions.

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