

CHARACTERIZATION OF THE COLCHICINE BINDING OF MEMBRANE FRACTIONS FROM RAT AND MOUSE LIVER

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INTRODUCTION

A variety of cytological studies have produced the concept that the antimitotic drugs colchicine and Colcemid bind to specific proteins, the "tubulins" (1, 3, 4, 20, 38, 42). Moreover it has been implied in most of these studies that these "colchicine-binding proteins" are recovered in a soluble form, i.e., in the 100,000 *g* supernate from a cellular homogenate. However, recently, colchicine-binding has been described for particulate cellular subfractions from various tissues,

especially in membrane fractions from mammalian brain and liver (6, 8, 25, 26, 36, 42). The colchicine-binding capability is different in the various membranes and seems to be especially high, for example, in nuclear membranes from mammalian liver and microsomes and synaptic membranes from mammalian brain. The occurrence of colchicine-binding proteins in such a particle-bound form has been alternatively explained as due to (a) the association of tubulin with such membranes (8, 36) or (b) that colchicine

and related drugs can also be bound by nontubulin membrane components. The present study extends our earlier investigations on the localization and distribution of colchicine-binding sites in liver and brain cell fractions and will present some differences in the binding characteristics of tubulins and membranes. It suggests that, in contrast to the association of tubulin with brain membrane fractions, much of the binding to liver membrane fractions is nonspecific.

MATERIALS AND METHODS

Brains of young albino rats (Wistar II, 150 g) and mice (N. M. R. I. BR 66, 16–20 g) were freed from meninges, surface blood capillaries etc., and were immediately homogenized by several strokes with a glass-Teflon Potter-Elvehjem homogenizer in the assay medium (3 ml per brain) containing 0.01 M MgCl₂, 10⁻⁴ M guanosine triphosphate (GTP) in 0.01 M potassium phosphate buffer, the whole adjusted to a final pH of 6.8. This homogenate was centrifuged at 100,000 *g* for 1 h at 4°C to separate the “100,000 *g* supernate” and the “brain particulate fraction.” From rat liver homogenate (in 0.4 M sucrose, 0.07 M KCl, 2% gum arabic, 0.01 M Tris, pH 7.2) recovery studies were performed in the crude fractionation indicated in Fig. 1. Purified nuclei, nuclear membranes, and microsomes were also prepared from rat and mouse liver homogenates (15, 21). Sonication of membrane proteins was performed with a Branson Sonifier (Branson Instruments Co., Stamford, Conn.) equipped with a microtip at low power for 2–6 × 30 s in 2 ml (cooled by a crushed ice water mixture). [³H]- or [¹⁴C]-colchicine (sp act 5.7 Ci/mmol and 15 mCi/mmol) and [³H]Colcemid (sp act 21 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.). Lumicolchicine was prepared by exposure of colchicine to UV light (350 nm, 8 watt) in 95% ethanol (43) for 20 min, separated by thin layer chromatography (silicagel G, CCl₄/MeOH 95:5, vol/vol), and identified by its maxima at 228 and 266 nm (compared to those at 247 and 350 nm in colchicine; 11, 43). Radiochemical purity and possible chemical alterations of the radioactive drugs were checked by scanning the thin-layer chromatograms with a gas-flow-counter and comparing the mobilities with that of unlabeled colchicine (35). After the assay the bound radioactivity was reisolated (3, 8) and identified in the same way. The incubation was carried out in the “assay medium” under gentle magnetic stirring. The following parameters were varied: (a) protein concentrations from 0.2 to 5 mg/ml; (b) colchicine concentrations from 10⁻⁹ to 10⁻³ M (by adding unlabeled colchicine; E. Merck AG, Darmstadt, Germany); (c) incubation time from 0 min to 6 h;

(d) incubation temperature from 0°C to 90°C, with or without preincubation at 90°C for 30 min. Bound colchicine was determined by the filter technique according to Weisenberg et al. (38; for details see also refs. 2 and 35). In some experiments designed to study the strength of the binding, we allowed the bound colchicine to equilibrate with the wash medium, in each of the six wash steps, for 10 min at room temperature. When membranous material was assayed, this method was routinely compared with that of the repeated washes by ultracentrifugation (36) and with the method of Feit and Barondes (8), which uses a centrifugation through 10% sucrose (in our experiments this step was followed by a centrifugation for 30 min at 160,000 *g*). If not otherwise indicated, the determinations of the bound drug represent the differences of the amounts bound in the specific probe and that in the corresponding zero time and 0°C “blank.” All wash steps were carried out at 4°C, except in determinations of the temperature dependence of the binding in which the washes were done at the incubation temperatures (20–60°C). Protein was determined by the method of Lowry et al. (27). The radioactivity bound on the filter papers was counted in 10 ml toluene based scintillation fluid, containing 1 ml NCS (Nuclear Chicago Solubilizer, Amersham/Searle Corp., Arlington Heights, Ill.) in a liquid scintillation counter at an efficiency of 40% for ³H- and 85% for ¹⁴C-labeled material.

RESULTS

Membrane and nuclear fractions from rat liver have a significant colchicine-binding activity as detected both by binding assays (36) and autoradiography (18). A recovery study of colchicine-binding capacity through crude fractions from homogenized rat liver is shown in Fig. 1, demonstrating the remarkable amount and the specific activity (per milligram protein) of drug binding, especially in the membrane fractions. When we compared the binding activities of nuclear and microsomal membranes prepared in parallel at different protein concentrations over a range of 0.3–3.0 mg/ml, using the “centrifugation method” (36), the nuclear membranes consistently bound more colchicine than the microsomes (Fig. 2), and, likewise, more than rat liver plasma membranes prepared according to Ray (32). When the membrane fractions were depleted and extracted of lipids according to Fleischer et al. (10), the efficiency of binding colchicine was not significantly diminished (35). This is in accord with the view that it is membrane protein which binds the drug and rules out any preferential partition into the membrane lipids as the explanation for the

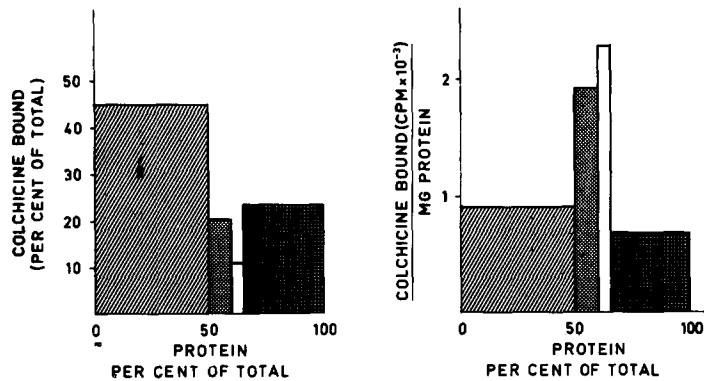


FIGURE 1 Recovery study of colchicine binding in crude fractions from the rat liver homogenate after centrifugation at 1,500 *g* (15 min), 10,000 *g* (10 min), and 100,000 *g* (1 h). The colchicine binding of the pellets sequentially obtained ("nuclei," hatched blocks; "mitochondria," dotted blocks; "microsomes," blank blocks) and the "100,000 *g* supernatant" (cross-hatched blocks) is demonstrated as total colchicine binding per fraction (left), and as specific binding activity per protein content (right). In vitro incubation was carried out at 37°C for 1 h with 5×10^{-7} M [³H]colchicine at a protein concentration of 5 mg/ml.

membrane binding of colchicine. The binding of colchicine to membranes from the endoplasmic reticulum and the nuclear envelope strongly increases with temperature (see below) but is different from the binding to brain supernatant protein or to purified tubulin by not reaching saturation in a range from 10^{-9} M to 10^{-2} M. Fig. 3 shows the binding curves in between 10^{-8} M and 10^{-4} M. When one plots the binding data of colchicine to brain supernatant protein and liver nuclear membranes and microsomes according to Scatchard (33; reviews in 22, 23, and 37), one sees that the brain supernatant protein exhibits a normal "tubulin-like" binding characteristic with ca. 6×10^{14} effective binding sites per mg protein (Fig. 4; compare, e.g., refs: 30, 38, 42) whereas the curves for the membrane fractions are significantly different. The microsomes did not show any specific binding, even at low concentrations. The nuclear membranes, however, revealed a complex curve suggesting either negative cooperativity or heterogeneity of binding sites (compare, e.g., 24). Due to the complicated situation a definite value for the number of specific binding sites per milligram of protein (n_M) cannot be obtained from the curve. The binding of colchicine to nuclear and microsomal membranes is relatively stable as demonstrated in repeated washes with assay medium, using both the filter assay and the centrifugation method. After removal of the unbound colchicine, i.e. from the third wash on, the loss of radioactivity from the membrane fractions was, in our hands, compa-

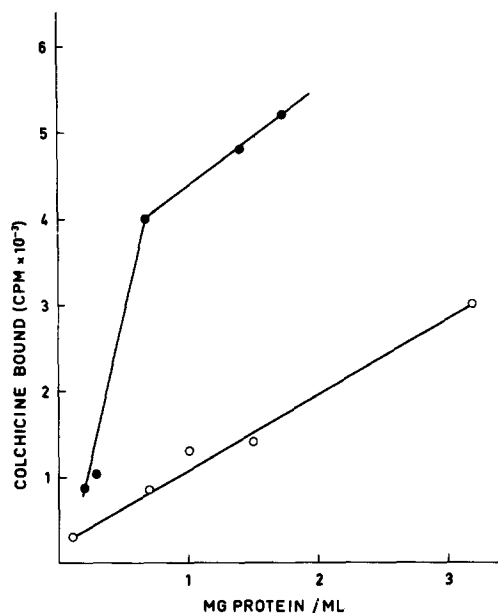


FIGURE 2 Binding of colchicine by rat liver microsomes (O) and nuclear membranes (●) at different protein concentrations after in vitro incubation in 10^{-4} M [¹⁴C]colchicine for 1 h at 37°C.

table to that of the brain supernatant tubulin: each additional wash resulted in a loss of ca. 5%.

The colchicine-binding activity of tubulin in the brain supernate is heat sensitive (total destruction at 60°C; cf. refs. 5 and 42). In contrast to this, the membranes studied are even enhanced in colchicine-binding activity when incubated at

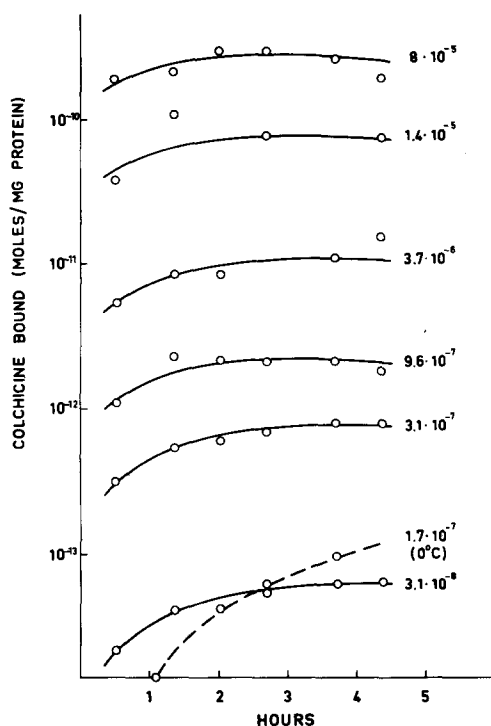


FIGURE 3 Colchicine binding to rat liver microsomes as a function of time for various molar concentrations of colchicine (indicated for each curve) at a constant protein concentration (10 mg/ml) after incubation at 37°C (—) and 0°C (- - -), using both [³H]- and [¹⁴C]-colchicine.

higher temperatures or after preheating at 90°C for 30 min (Fig. 5). However, the temperature during the wash procedures has also a marked effect on the amount of colchicine bound: higher temperatures render more colchicine released. Extensive sonication before the binding assay leads to a slight (approximate 1.5-fold) increase of colchicine binding (for details see 35). It is excluded, however, that the specific difference between the nuclear membranes and the other membranes studied, especially the microsomes, is merely due to an increased membrane surface ratio, since the nuclear membrane fragments, as we prepare them, are larger than the corresponding microsomes (see, e.g., refs. 15 and 21).

In order to characterize the binding to the membrane structures, we compared the colchicine binding with the capacity to bind lumicolchicine which is biologically inactive and not bound by tubulin (42). In our assays lumicolchicine was not bound by the brain 100,000 g supernatant proteins (35, see also ref. 8). All the brain fractions

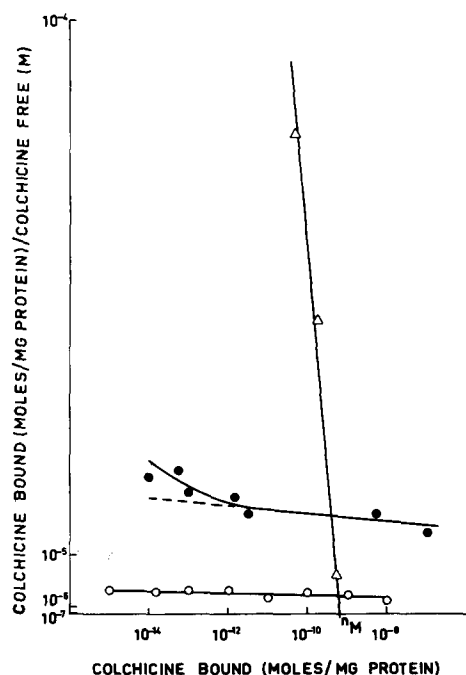


FIGURE 4 Scatchard plot for binding of colchicine to rat liver microsomes (○) and nuclear membranes (●) and rat brain 100,000 g supernatant (△). n_M indicates the maximal number of moles colchicine bound per milligram brain supernatant protein. Incubation was carried out at 37°C for 90 min in the "assay medium." Each value is an average from at least four experiments.

bound much more colchicine than lumicolchicine but the ratio of lumicolchicine to colchicine binding is significantly higher in the brain "particulate fraction" (Fig. 6). With the liver fractions, however, the microsomes bound nearly equal amounts of colchicine and lumicolchicine, and the nuclear membranes were consistently even slightly more effective in binding lumicolchicine. When one plots the differences of bound colchicine and lumicolchicine as a function of the time of incubation, one obtains curves characteristic of the various fractions assayed as shown in Fig. 7. While the relatively poor colchicine-binding activity contained in a rat liver 100,000 g supernatant which had been "directly" prepared from the homogenate by a single centrifugation (i.e., in a manner identical to that used for the brain supernatant protein) had a binding behaviour indicative of some tubulin-like specificity (Fig. 7), the microsomes and nuclear membranes showed neither specific binding nor did the difference of

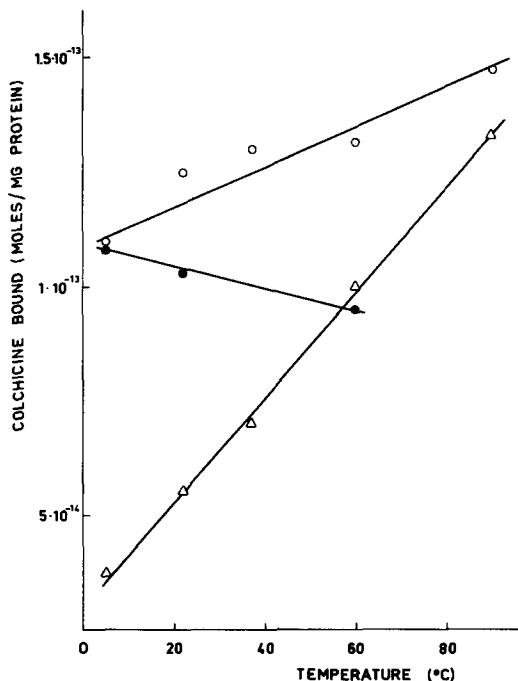


FIGURE 5 Colchicine binding to microsomes as function of the incubation temperature after preheating at 90°C during 30 min (○) or without preheating (△). Washes were carried out, as usual, in the cold (4°C). The third curve (●) shows the effect of washing at different incubation temperatures (at 4, 20, or 60°C) using the preheated microsomes.

colchicine and lumicolchicine binding reveal the pronounced "late" saturation typical for tubulin (compare 4, 5, 39). In some cell systems Colcemid is more effective in mitotic arrest as well as in binding to tubulin (e.g. 20). When we examined the binding of Colcemid to the liver nuclear membrane and microsomal fractions, we did not note a difference, in comparison with colchicine, in the Scatchard plot curves. While the binding (per milligram protein) of Colcemid was not significantly different from that of colchicine in the microsomes, the nuclear membranes were more effective in binding Colcemid by a factor varying from 1.5 to 2.0.

DISCUSSION

The results confirm and extend the earlier demonstrations that membrane material can bind colchicine and Colcemid in a relatively stable mode (6, 8, 20, 25, 26, 36, 42). The presence of such membranous binding sites is also indicated to us in the data of Gillespie (19; her "particulate bound

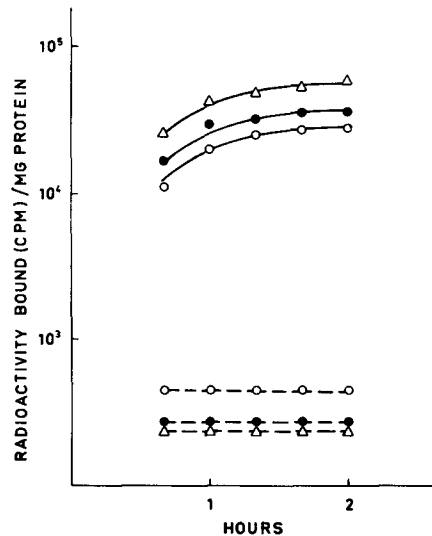


FIGURE 6 Specific binding activities of rat brain homogenate (●), 100,000 g pellet (○), and 100,000 g supernate (△) in binding colchicine (—) and lumicolchicine (---).

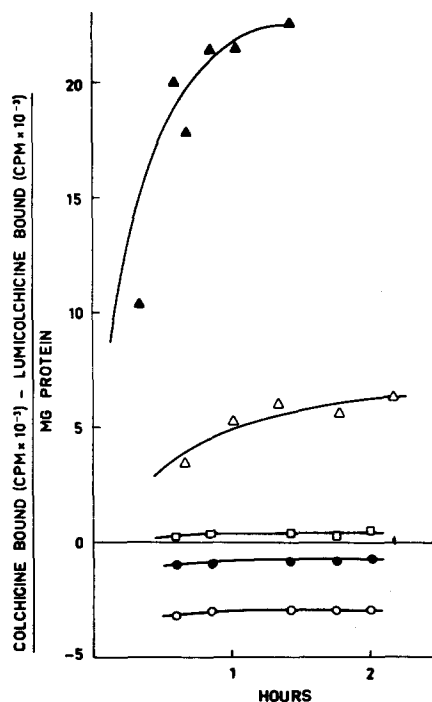


FIGURE 7 Comparison of the specificity of colchicine binding of rat brain 100,000 g supernate (▲) with 100,000 g supernate (△), microsomes (□), plasma membranes (●), and nuclear membranes (○) from rat liver plotted as the differences of colchicine and lumicolchicine bound per milligram protein in aliquots.

colchicine") who studied the colchicine binding using tissue slices. Our data further add to the indications that different types of membranes vary in their binding activity, particularly high activity being associated with nuclear membranes from mammalian liver and with nerve ending and synaptosomal fractions (see the references mentioned above and ref. 9). The existence of some colchicine-binding sites in membranes might also be expected from the existence of enzymes in the microsomal membranes of liver, which are capable of hydroxylation and degradation of colchicine (34), and from the inhibitory effects of colchicine (but also of lumicolchicine) on the transport of nucleosides across the plasma membranes of various cultured cells (28). However, in contrast to the colchicine binding in membrane-containing fractions from brain tissue (8), the binding to the liver membranes differs in a series of characteristics from its binding to tubulin. Such differences are the heat stability, its dependence on drug concentration over a range of 10^{-8} – 10^{-2} M, and its nonspecificity as indicated by the extremely low ratios of colchicine binding compared to the binding of lumicolchicine. Some of the properties of such a membranous colchicine binding could suggest a contribution by (probably hydrophobic) membrane surface adsorption, for example, its increase after sonication and after membrane disintegration through preheating (see also ref. 22). However, increase of binding activity by heating of membranes should not be considered a priori as indication of a nonspecific adsorption as has been shown for the high affinity binding sites of cytochrome to mitochondrial membranes (40). The complex Scatchard plot of the nuclear membranes might indicate the presence of one component with higher affinity superimposed on a low affinity and nonspecific binding (Fig. 4).

In a diversity of cellular situations microtubules exhibit a close relationship with membranes, especially the nuclear membranes, and sometimes even seem to have their origin where membrane breakdowns occur (e.g. 7, 14, 16, 17, 31, 41). This had led to concepts of membrane-associated foci nucleating assemblies of microtubules (12, 13, 29, 31, 36). Although our data demonstrate that the vast part of the membrane colchicine binding cannot be explained by the existence of tubulin in these membranes, they do, on the other hand, not rule out in general the occurrence of mem-

brane-associated tubulin. From a crude estimation, however, assuming that all colchicine-binding sites of membrane tubulin would be accessible, it is clear that only a minute amount of the membrane proteins in rat liver could be constituted by such a proposed microtubular membrane component (for different situation in brain membrane fractions see ref. 9).

We are indebted for helpful discussions to Drs. E. D. Jarasch, P. M. M. Rae (Max-Planck-Institute for Biology, Tübingen, Germany), and G. Siebert (Institute of Biological Chemistry, University of Stuttgart-Hohenheim, Germany) and to Dr. R. Kay for reading and correcting the manuscript.

The authors gratefully acknowledge the financial support from the Deutsche Forschungsgemeinschaft (grant no. Fr 308/6).

Received for publication 8 May 1973, and in revised form 18 September 1973.

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