COLCHICINE BINDING OF A HIGH-SPEED SUPERNATANT OF *CHLAMYDOMONAS REINHARDI*

Dorothy FLANAGAN and J. R. WARR
Biology Department, University of York, Heslington, York, Y01 5DD England

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1. Introduction

The unicellular eucaryotic alga, *Chlamydomonas reinhardi* has been extensively used for studies on the genetic control of microtubule assembly [1],[2], and on the synthesis of tubulin, the principal protein in microtubules [3–5]. One of the most widely used techniques for studying tubulin from animal sources is colchicine binding [6] and it is therefore unfortunate that colchicine binding to tubulin or tubulin-like proteins from plant sources is in general very low or non-existent [7]. This problem is particularly acute in the study of colchicine resistant mutants of *Chlamydomonas* [8–10] since one possible mechanism for colchicine resistance is that there could be altered interaction between tubulin or a tubulin-like protein and colchicine in these resistant mutants.

We now report the existence of a low level of colchicine binding activity in high speed supernatants of *Chlamydomonas* and describe some properties of this binding activity.

2. Materials and methods

Wild type, mating type plus cells (strain 32C, Cambridge Collection of Algae) were grown in modified Sager and Granick medium [11]. Between 2 and 5 litres were grown for each experiment for 5 days to mid log phase in 200 ml volumes at 25°C illuminated at 500 foot candles. Cells were harvested by centrifugation at 4000 × *g* at 4°C in an MSE High Speed 18 centrifuge and resuspended in cold PMG buffer containing 5 mM sodium phosphate, 0.25 M sucrose, 0.1 mM GTP (Sigma Grade II), 10 mM $MgCl_2$ and 20 mM sodium glutamate at pH 6.8. The cells were disrupted by an Aminco French pressure cell and the resulting homogenate was centrifuged at 100 000 × *g* for 1 h at 0°C in a Beckman L2-65B ultracentrifuge using an SW 56 rotor to obtain the high-speed supernatant.

Colchicine binding assays were carried out by adding 20 μl [^{3}H]colchicine (Radiochemicals Centre, Amersham; 10 μCi/ml) at a concentration of 1.67 μM to 200 μl of the high-speed supernatant containing at least 1 mg of protein in a thin-walled glass tube. Samples were incubated for 45 min at 25°C in a water bath before the reaction was terminated by transferring the tubes to an ice bath and adding 2 ml of cold 2% TEAE-23 cellulose suspension in PMG buffer. After 5 min each sample was collected on two 1.8 mm circles of Whatman GF/C glass fibre filter paper and each filter was washed three times with 5 ml of cold PMG buffer. The filters were transferred to scintillation vials to which 8 ml of Biofluor (New England Nuclear) were added before counting in a Beckman LS 230 counter. Quench correction was by the external standards/channels ratio method. In order to correct for loss of binding activity during the 45-min incubation period, samples of supernatant were preincubated at 25°C for periods of up to 4 h before incubating with [^{3}H]colchicine, as discussed more fully in the results section. Samples were set up in triplicate for all time points. Blank runs contained 200 μl PMG buffer in place of the supernatant. Thin-layer chromatography of our [^{3}H]colchicine using chloroform/acetone/diethylamine (5:4:1) as a solvent revealed an impurity containing about 34% of the radioactivity close to the solvent front. This impurity gave no detectable binding to the *Chlamydomonas* extract.

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Lumicolchicine was prepared by irradiating a solution of colchicine (British Drug Houses) in 95% ethanol with long wave (3560 nm) ultraviolet light [12]. The conversion of colchicine to lumicolchicines was followed by measuring the decrease in absorbancy at 350 nm. Podophyllotoxin (Aldrich Chemical Company) was dissolved in 10% ethanol (1 mM) or 50% ethanol (5 mM and 10 mM) before adding to the incubation mixture. Controls contained the same ethanol concentration. Vinblastine sulphate was obtained from Eli Lilly and Co.

The protein concentration was determined by the Lowry method [13] with bovine serum albumin as a standard.

3. Results

Preliminary experiments indicated the presence of a low level of colchicine binding activity in 100 000 × *g* supernatants of *Chlamydomonas* in PMG buffer, with optimal binding around 25°C. This temperature was employed for all subsequent experiments. When the period of incubation was varied, the total amount of colchicine bound increased to a peak around 45 min and then declined markedly (fig.1). A 45-min incubation period was therefore adopted for all subsequent experiments.

Colchicine binding activity to tubulin from animal sources decays with first order kinetics [14,15]. To allow for the decay of colchicine binding activity which occurs during the period of incubation, it is preferable to determine the *initial* binding capacity by *pre-incubating* aliquots for differing times in the absence of colchicine and then determining the colchicine bound during a fixed incubation period. When bound colchicine is plotted against preincubation time, the first order decay line may be extrapolated back to the start of the incubation period to determine the initial capacity (cf. fig.2 in [15]). We therefore preincubated the 100 000 × *g Chlamydomonas* supernatant at 25°C for varying periods before measuring the colchicine binding activity (fig.2). Although the decay has first order kinetics between about 60 and 200 min preincubation, there is a distinct shoulder during the first 60 min which may represent a balance between activation and decay of colchicine binding material. This phenomenon has now been consistently observed in cell extracts prepared on twenty five different occasions. Simple extrapolation back to determine the initial binding capacity is therefore not possible with this material.

Colchicine binding to samples containing less than 1 mg of protein was too low to be reproducible. In some experiments a slight increase in the colchicine bound per unit protein was observed with increasing

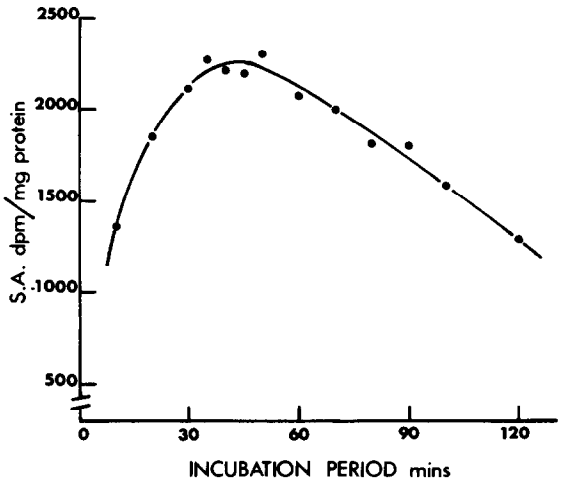

Fig.1. The effect of increasing length of incubation period on the specific activity of colchicine binding in *Chlamydomonas* extract. 200 μl of supernatant containing 2.25 mg of protein were incubated with 20 μl of [^{3}H]colchicine for varying lengths of time.

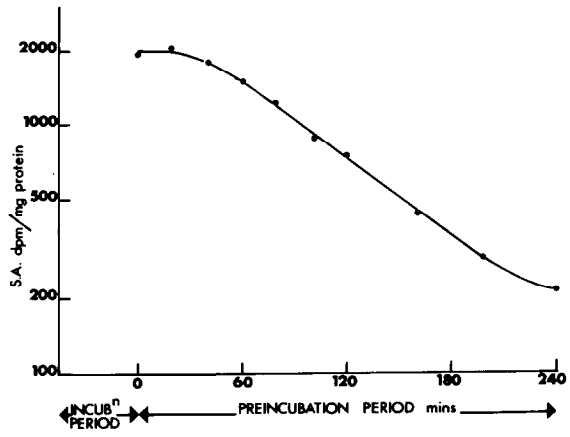

Fig.2. Decay of binding activity during preincubation of supernatant at 25°C prior to incubation with [^{3}H]colchicine.

protein concentrations. This increase was around 20% per doubling of the protein concentration.

Colchicine binding activity of animal tubulin can be influenced by the presence of GTP, vinblastine, sucrose and glycerol (see [16] for refs) and therefore we investigated the effects of these substances on colchicine binding in our material. 0.1 mM GTP was routinely present in the buffers used during disruption of the cells and colchicine binding; omission of the substance did not markedly affect the results which were obtained. Addition of 0.1 mM vinblastine to the buffers also did not significantly affect colchicine binding. 0.25 M sucrose is normally present in the buffers; absence of sucrose reduced the level of binding observed and increasing the sucrose concentration to 1.00 M increased the observed colchicine binding (fig.3).

Gillespie [17] has reported that dithiothreitol partially stabilises colchicine binding activity from rat brain tubulin. Inclusion of 1.0 mM DTT in isolation and incubation buffers raised the binding activity of samples which had not been preincubated but did not raise the binding activity of those which had been preincubated for 1 h or more (fig.4). If the shoulder normally observed between 0 and 60 minutes preincubation does represent a balance between the activation and the decay of colchicine binding activity, it may be that DTT brings about the activation of binding activity almost immediately but leaves the

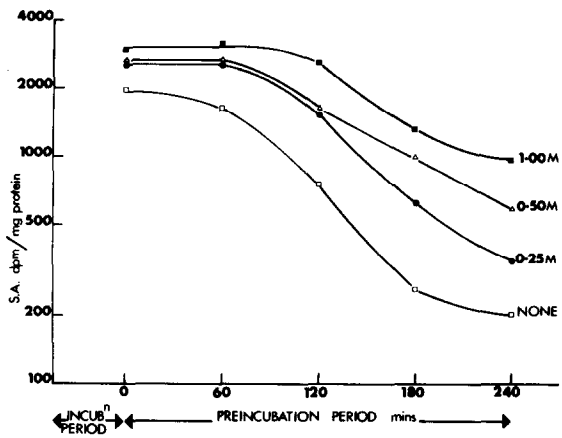

Fig.3. The effect of varying sucrose concentrations on colchicine binding activity (0.25 M was routinely used in other experiments).

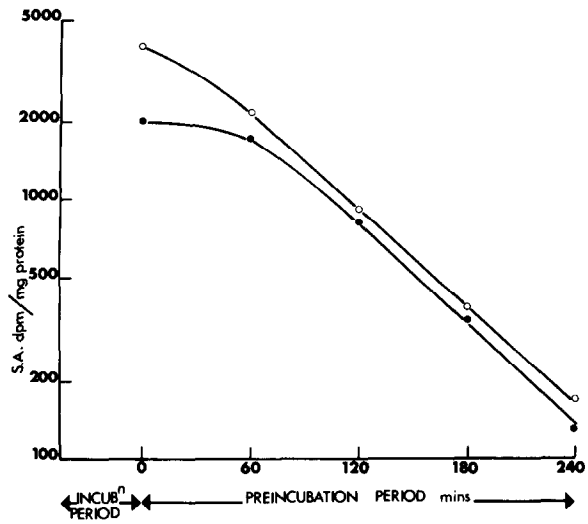

Fig.4. The effect of dithiothreitol on colchicine binding activity. (●——●) control. (○——○) 1.0 mM DTT.

decay rate unchanged. This could explain why the decay kinetics approximate much more closely to simple first order decay in the presence of DTT.

There have been several reports of colchicine interacting with proteins other than tubulins (reviewed in [6,7]). Lumicolchicines, U.V.-induced products of colchicine [18], do not compete with colchicine during specific binding to microtubular proteins but they do compete during other non-specific interactions. We have found that lumicolchicines have no effect on colchicine binding of the 100 000 × *g Chlamydomonas* extracts even at very high concentrations, although cold colchicine at the same concentration reduces the binding to about 13% of controls (fig.5). Podophyllotoxin, an alkaloid which interacts with specific colchicine binding to animal tubulin [6], competes with binding of colchicine to *Chlamydomonas* extracts but only at concentrations of 1 mM and above which are considerably higher than are normally used in other systems.

4. Discussion

The nature of the colchicine binding material observed during these experiments has not been established with any certainty and the possibility that binding

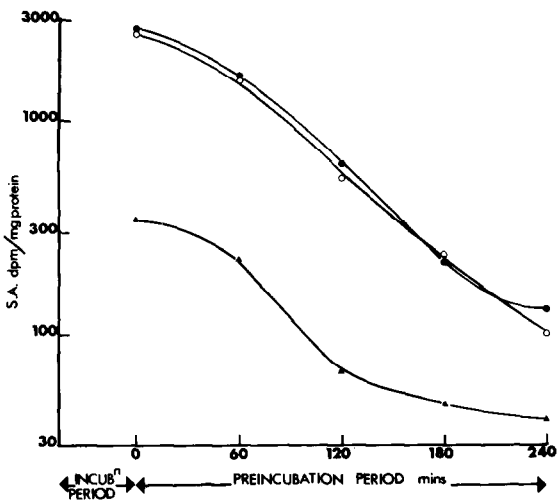

Fig.5. Effect of lumicolchicines on colchicine binding activity. Samples of *Chlamydomonas* supernatant were incubated in the presence of 5.0 mM lumicolchicines (○———○), 5.0 mM cold colchicine (▲———▲) and in buffer alone (●———●).

could be to non-tubulin proteins is very real. Colchicine binding activity in preparations of the water mould *Allomyces* is associated with a component of mol. wt 30 000 [19] in contrast with tubulin, which is a dimer of mol. wt of around 120 000.

We have observed that lumicolchicines do not interfere with colchicine binding although unlabelled colchicine at the same concentration does so (fig.5). Although such a result provides some evidence that the observed binding is to tubulin we should recall that the general view that lumicolchicine provides a discriminator between binding to tubulin and non-specific binding is in itself based on experiments with animal material. There are major and largely unexplained differences between colchicine interactions with animal as opposed to plant proteins [7]. Consequently, the lumicolchicine experiment cannot be so confidently interpreted with material from *Chlamydomonas* as it would be with material from a mammal.

Burns [20] reported no colchicine binding activity in high speed supernatants of *Chlamydomonas*. Sucrose was absent from his isolation medium and normally present in ours, but this seems to be an unlikely explanation for the discrepancy since omission of sucrose in one experiment of ours still resulted in some binding. Burns's technique was most strikingly different from ours in that he loaded his experimental material onto a DEAE cellulose column and then eluted with a NaCl gradient. He did in fact observe a small amount of [^{3}H]colchicine eluting at the beginning of the gradient (fig.2c in [20]) which he attributed to the tail of the unbound colchicine resulting from insufficient washing. Strictly, however, one is not able to eliminate the possibility that this small peak could have arisen from very labile binding to DEAE bound proteins on the column (Burns, personal communication), which might correspond to the colchicine binding which we have observed.

The non-linear decay of colchicine binding activity during the early period of pre-incubation could represent activation or temporary stabilisation of colchicine binding activity. It is of particular interest that dithiothreitol raised the binding level of samples which had not been pre-incubated (fig.4). This phenomenon merits further study.

Acknowledgement

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