Biochem. J. (1970) **117**, 773–777
Printed in Great Britain

The Binding *in vitro* of Colchicine to Axoplasmic Proteins from Chicken Sciatic Nerve

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(*Received* 9 *December* 1969)

1. Axoplasmic proteins were fractionated by means of Sephadex G-200 chromatography followed by isoelectric focusing. Nine groups of proteins were separated. 2. The binding of colchicine to these groups of proteins was examined and it appeared to associate most strongly with one protein group, of p*I* value 4.9–5.0, which is the major ^{14}C-labelled component of slow-transport protein. 3. Other fractions also bind colchicine. It is not clear whether these are separate proteins or subunits of the major colchicine-binding fraction.

Several models have been advanced to explain the mechanism of axonal flow. Weiss & Hiscoe (1948) proposed a bulk flow of axoplasm, due to continual growth, whereas Lubińska (1964) suggested a model in which a bidirectional movement of axoplasmic components produces an apparent overall flow away from the cell body. In an attempt to reconcile these conflicting views and to account for recent findings that protein flows at two distinct rates, it has been proposed that the slow transport of protein is a manifestation of the growth of longitudinally oriented molecules such as neurotubules and neurofilaments whereas the rapid transport is a reflection of the movement of organelles (McEwen & Grafstein, 1968; Bray & Austin, 1969). This hypothesis is supported by the observations of Droz (1966), with radioautographic electron-microscopy, which suggest that slow-transport protein is associated with neurofilaments, and by direct observations of neurons in tissue culture, which demonstrate the rapid movement of subcellular organelles (Geiger, 1963; Burdwood, 1965; Pomerat, Hendelman, Raiborn & Massey, 1967). Further, in sympathetic nerves there is only a rapid transport of catecholamine granules (Dahlström & Häggendal, 1967) but both rapid and slow transport of protein (Livett, Geffen & Austin, 1968).

Colchicine and a number of other mitotic inhibitors are known to interfere with cellular processes dependent on cytoplasmic microtubules (Tilney, 1968; Rosenbaum & Carlson, 1969). In the central nervous system, colchicine produces characteristic deformations of neurotubular and neurofilamentous structures (Wisniewski, Shelanski and Terry, 1968) and reversibly inhibits the growth of nerve processes of chicken dorsal-root ganglion cells in tissue culture (Daniels, 1968), possibly as a result of its action on neurotubules.

Weisenberg, Borisy & Taylor (1968) have isolated a colchicine-binding protein from mammalian brain that they suggest is neurotubular protein. The microtubules appear to be unstable during isolation and this protein appears in the soluble fraction, accounting for 5–10% of the total protein of that fraction.

Several authors have proposed that colchicine preferentially blocks rapid transport. High concentrations of colchicine cause an accumulation of catecholamine-storage granules both above and at the site of injection in sympathetic nerves (Dahlström, 1968) and also block the rapid phase of protein flow in the optic nerve (Karlsson & Sjöstrand, 1969). Local application of colchicine appears to interfere with the movement of certain enzymes in peripheral nerve (Kreutzberg, 1969). The accompanying paper (James, Bray, Morgan & Austin, 1970) shows that colchicine inhibits both rapid and slow axonal flow of proteins in the axons of the chicken sciatic nerve but that the slow transport is more profoundly affected.

The affinity of colchicine for both the slow and the rapidly flowing proteins in axons has been investigated in an attempt to correlate colchicine binding with the proteins involved in both these types of axonal flow.

MATERIALS AND METHODS

Chemicals. L-[U-^{14}C]Leucine (specific radioactivity 311 mCi/mmol) and [^{3}H]colchicine (^{3}H-labelled in the ring methoxyl; specific radioactivity 104 or 1720 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

GTP was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; Sephadex G-25 and G-200 were purchased from Pharmacia, Uppsala, Sweden. Ampholine [8% (w/w), pH range 3–10] was obtained from LKB-Producter AB, Bromma, Sweden.

Animals. Female chickens (White Leghorn × Australorp strain) 17–18 weeks old and weighing approx. 1.5kg were used.

Colchicine binding to soluble axonal protein. Chickens were killed and their sciatic nerves removed. Axoplasm was extruded by squeezing out the contents of the nerve by mechanical compression. Preliminary experiments showed that more than 90% of the proteins labelled with ^{14}C were extracted from the nerve by this procedure. The extruded material was first homogenized in 10 vol. of ice-cold 0.32M-sucrose containing 100mM-Na_2HPO_4–NaH_2PO_4 buffer, pH7.1, and 10mM-$MgCl_2$ in an all-glass homogenizer and secondly in a homogenizer fitted with a Teflon pestle with a clearance of 0.25mm. The homogenate was centrifuged at 100000*g* for 60min to isolate the soluble protein fraction and a portion was incubated at 37°C for 1.5h with 0.1ml (9.1μCi) of [^{3}H]colchicine (104mCi/mmol, 11.1μg/ml). Protein in a volume of 3ml was then passed through a Sephadex G-25 column (19.5cm × 0.8cm; equilibrated with 100mM-Na_2HPO_4–NaH_2PO_4 buffer, pH7.1, containing 10mM-$MgCl_2$; flow rate 20ml/h) to remove most of the unbound [^{3}H]colchicine. Protein eluted in the void volume on Sephadex G-25 was passed, in a volume of 5ml, through a Sephadex G-200 column (33.6cm × 1.8cm; equilibrated with the phosphate buffer described above, pH7.1; flow rate 15ml/h). Fractions (2.2ml) were collected by using an LKB fraction collector. Portions were taken for determination of radioactivity and protein.

Correlation of colchicine binding with transported protein by isoelectric focusing. Two groups, each of three chickens, were injected in the lumbar–sacral region with 30μCi (short-term experiment) and 12μCi (long-term experiment) of [^{14}C]leucine as described by Bray & Austin (1968). After 5h (short-term experiment) and 14 days (long-term experiment) the sciatic nerves were removed. For the short-term experiments the first 2cm of nerve proximal to the spinal cord was discarded and the extruded material from the following 8cm of nerve was homogenized in 0.32M-sucrose containing 50mM-Na_2HPO_4–NaH_2PO_4 buffer, pH6.5, 0.1mM-GTP and 10mM-$MgCl_2$. For long-term experiments the first 1cm of nerve was discarded and the extruded material from the following 5cm of nerve was homogenized in the same buffer. GTP was included in the medium to stabilize the colchicine-binding site of the protein (Adelman, Borisy, Shelanski, Weisenberg & Taylor, 1968). Soluble protein was obtained after centrifugation of the homogenate at 100000*g* for 60min and was then fractionated on a Sephadex G-200 column (90cm × 1.8cm; equilibrated with the above buffer from which sucrose was omitted; flow rate 15ml/h). Two major peaks of protein from each experiment were eluted and the fractions comprising each peak were pooled and concentrated to 3.9ml in a Diaflo ultrafiltration apparatus with a membrane with an exclusion limit of mol.wt. 10000.

The following abbreviations have been used: S-HMW and R-HMW, non-retarded proteins off Sephadex G-200 from slow- and rapid-transport experiments respectively; S-LMW and R-LMW, retarded proteins off Sephadex G-200 from slow- and rapid-transport experiments respectively.

All four protein concentrates, except the R-HMW protein, were incubated with 0.1ml (9.1μCi) of [^{3}H]colchicine (1720mCi/mmol, 8.63μg/ml) for 60min at 37°C and passed through, in a volume of 4ml, separate Sephadex G-25 columns (47cm × 0.9cm; equilibrated with the above sodium phosphate buffer; flow rate 20ml/h).

Isoelectric focusing. R-LMW, S-LMW and S-HMW protein were fractionated separately in an LKB isoelectric-focusing column containing 5.25ml of Ampholine (8%, w/w), pH range 3–10, in a discontinuous sucrose gradient containing 24 concentrations ranging from 0% to 46% sucrose in increments of 2%. The lower electrode (anode) was immersed in 20ml of a solution containing 60% sucrose and 0.1M-H_3PO_4 and the upper (cathode) in 10ml of 2% ethylene diamine. A voltage of 300V was applied for 20h. Preliminary experiments with bovine serum albumin indicated that equilibrium was essentially complete after 20h. Water at 1–2°C was pumped through the system of cooling jackets. After equilibration, 1.2ml or 2.0ml fractions were collected and the pH and E_{280} of each fraction determined.

Determination of radioactivity. Portions of each fraction were added directly to 10ml of 'aqueous' scintillation fluid (Bruno & Christian, 1961) and the radioactivity was measured in a Picker Nuclear Liquimat 220 liquid-scintillation spectrometer. The channels were set to completely eliminate ^{3}H radioactivity from the ^{14}C channel by using ^{14}C- and ^{3}H-labelled standards. Efficiencies of counting in the various samples were determined after quench checking by the addition of [^{3}H]- or [^{14}C]-toluene standards. These efficiencies were 8–13% for ^{3}H and 25–38% for ^{14}C.

RESULTS

The protein-fractionation experiments performed were: (*a*) total soluble protein incubated with [^{3}H]colchicine and fractionated on Sephadex G-200; (*b*) R-LMW protein incubated with [^{3}H]-colchicine and fractionated by isoelectric focusing; (*c*) S-LMW and S-HMW protein incubated with [^{3}H]colchicine and fractionated by isoelectric focusing.

Sephadex G-200 chromatography. Soluble protein from chicken sciatic nerve was incubated with [^{3}H]colchicine and most of the unbound colchicine removed with Sephadex G-25 as described in the Materials and Methods section. The results of the fractionation on Sephadex G-200 are presented in Fig. 1. There was partial resolution into two peaks of protein. The first peak contained those proteins eluted in the void volume and accounted for approx. 18% of the total protein. The second peak of retarded protein showed a shoulder, suggesting incomplete resolution of at least two separate components. There were three peaks of radioactivity: two of these represented colchicine associated with protein, and the third, major, peak was due to free

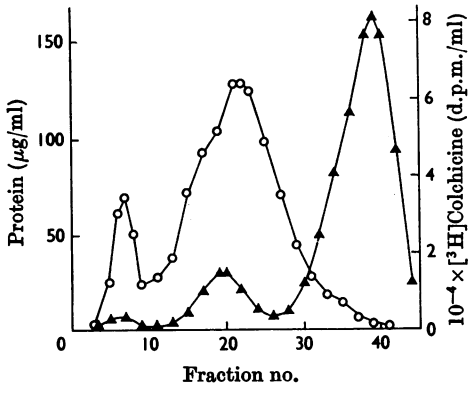

Fig. 1. Binding of colchicine to soluble axoplasmic proteins. Soluble protein of extruded axoplasm was incubated with 9.1 μCi of [^{3}H]colchicine for 1.5 h at 37°C in 100 mM-Na_2HPO_4–NaH_2PO_4 buffer, pH 7.1, containing 10 mM-$MgCl_2$. Most of the unbound [^{3}H]colchicine was removed with Sephadex G-25 as described in the Materials and Methods section. The incubation mixture was fractionated on a Sephadex G-200 column (33.6 cm × 1.8 cm). The fraction volume was 2.2 ml. ○, Protein; ▲, [^{3}H]-colchicine.

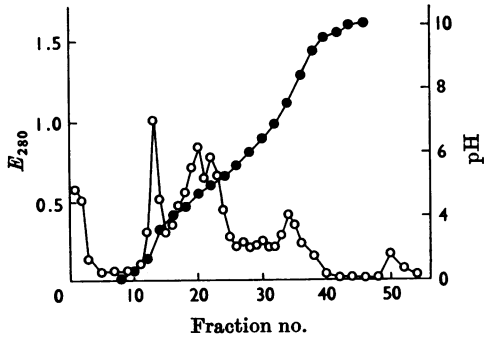

Fig. 2. Fractionation of R-LMW proteins by isoelectric focusing. Soluble axoplasmic protein was fractionated on a Sephadex G-200 column (90 cm × 1.8 cm) and the proteins in the retarded peak (R-LMW) were concentrated and further fractionated by isoelectric focusing. The fraction volume was 2.0 ml. ○, E_{280}; ●, pH gradient.

colchicine, not removed with Sephadex G-25 or to colchicine dissociated from the protein–colchicine complex.

Isoelectric focusing: *R-LMW protein.* R-LMW protein was fractionated by isoelectric focusing (Fig. 2). Protein was monitored in each fraction by measuring the extinction at 280 nm. This fraction was resolved into four major components with p*I* values of 3.4, 4.7, 5.0 and 7.55. In addition two minor components of p*I* 5.85 and 6.45 were found.

When ^{14}C-labelled R-LMW protein was fractionated by isoelectric focusing (Fig. 3), only two peaks of radioactivity, clearly due to protein, were resolved. These correspond to proteins with p*I* values of 4.7 and 5.0 and both bound [^{3}H]colchicine. The large ^{3}H peak of p*I* value 9.0 was due to free colchicine. It should be noted that the specific radioactivity of R-LMW protein is low, and other minor components that appear in Fig. 3 are probably without significance. However, protein peaks of p*I* values of 4.7 and 5.0 were consistently obtained.

Isoelectric focusing: *S-LMW and S-HMW protein.* A single peak of radioactive protein was found after fractionation of labelled S-LMW protein, corresponding to a p*I* value of 4.9 (Fig. 4). Again, colchicine was bound to this protein.

The distribution of radioactivity and proteins of the S-HMW fraction was more complex (Fig. 5). Three components with p*I* values of 3.8, 4.4 and 5.5 separated, but colchicine bound to only one of these (p*I* 3.8) and then only weakly. The specific radioactivities of R-HMW proteins were very low and it was not possible to compare the distribution of radioactivity in this fraction with that of S-HMW.

DISCUSSION

More than 75% of the rapid-transport proteins were confined to a group that is retarded by Sephadex G-200. A similar distribution in cat ventral nerve roots was found 3 h after administration of [^{3}H]leucine by Kidwai & Ochs (1969) who fractionated the soluble protein on Sephadex G-100. In our experiments the retarded fraction (R-LMW), when separated further by isoelectric focusing, resolved into two components with p*I* values of 4.7 and 5.0. A comparison with the equivalent fraction from slow-transport protein (S-LMW) shows that only one component, of p*I* value 4.9, is labelled, possibly corresponding to the p*I* 5.0 fraction of R-LMW. Nevertheless there is about six times as much labelled protein in the S-LMW peak as in the R-LMW peak, a result that is not unexpected since the specific radioactivity of the slow-transport protein is five to six times that of the rapid-transport protein (Bray & Austin, 1969).

The non-retarded fraction of slow-transport protein was resolved into three components, p*I* values 3.8, 4.4 and 5.5. Kidwai & Ochs (1969) found two labelled components, p*I* values 3.5 and 5.0, but a direct comparison may not be possible because of the different grades of Sephadex used for the initial fractionation.

Bound colchicine (Fig. 4) moved in an electric field to a position corresponding to a p*I* value of

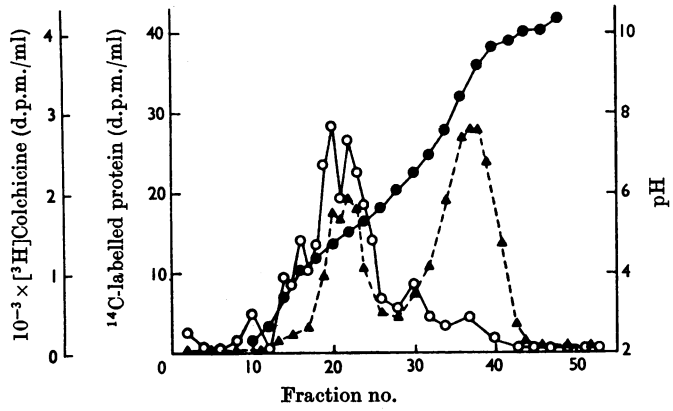

Fig. 3. Correlation of colchicine binding with R-LMW proteins. ^{14}C-labelled R-LMW was prepared as described in the Materials and Methods section and incubated with 9.1 μCi of [^{3}H]colchicine in 50mM-Na_2HPO_4–NaH_2PO_4 buffer, pH 6.5, containing 0.1mM-GTP and 10mM-$MgCl_2$. The reaction mixture was fractionated by isoelectric focusing and the ^{14}C-labelled protein and [^{3}H]colchicine were determined in each fraction (vol. 2.0ml). ○, ^{14}C-labelled protein; ▲, [^{3}H]colchicine; ●, pH gradient.

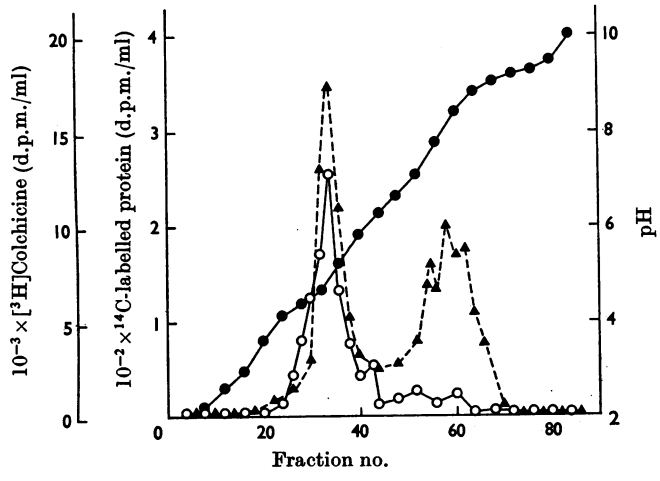

Fig. 4. Correlation of colchicine binding with S-LMW proteins. Slow-transport proteins were labelled with ^{14}C *in vivo* as described in the Materials and Methods section. After fractionation on Sephadex G-200 the retarded peak (S-LMW) was concentrated and incubated with [^{3}H]colchicine. The reaction mixture was then fractionated further by isoelectric focusing. The fraction volume was 1.2ml. ○, ^{14}C-labelled protein; ▲, [^{3}H]colchicine; ●, pH gradient.

4.9, the same position occupied by a peak of ^{14}C radioactivity. This was the major peak of ^{14}C radioactivity found and this result suggests that colchicine binds to a protein of p*I* value 4.9, a component that incorporates the greatest amount of radioactivity from [^{14}C]leucine. The amount of ^{14}C radioactivity in the p*I* 4.9 peak from the S-LMW fraction was about ten times that in the corresponding peak from the R-LMW fraction. Two other fractions that show some colchicine binding (p*I* 4.7 of R-LMW and p*I* 3.8 of S-HMW) also incorporate ^{14}C in much smaller amounts. It would appear then that the p*I* 4.9 peak of S-LMW may correspond to the protein isolated from brain (Weisenberg *et al.* 1968) that also binds colchicine, particularly since neurotubule and microtubule

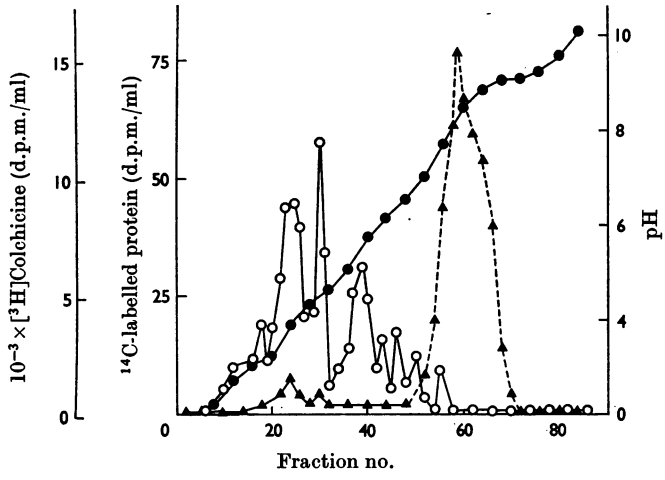

Fig. 5. Correlation of colchicine binding with S-HMW proteins. Slow-transport protein was labelled with ^{14}C (see Fig. 4). The non-retarded peak from Sephadex G-200 chromatography was incubated with [^{3}H]-colchicine and fractionated by isoelectric focusing. The fraction volume was 1.2ml. ○, ^{14}C-labelled protein; ▲, [^{3}H]colchicine; ●, pH gradient.

protein seems to be readily solubilized on homogenization of tissue (Maxfield & Hartley, 1957; Weisenberg *et al.* 1968).

It is clear that colchicine preferentially blocks slow-protein transport (James *et al.* 1970). Although colchicine binding is not confined to one component it is associated to a greater degree with the p*I* 4.9 peak of S-LMW, and it is possible that this peak contains proteins or protein subunits from neurotubules. The other colchicine-binding components may, however, also play a role in the effect of colchicine on axonal flow.

If this conclusion is correct, it supports the view that the growth of neurotubules is closely associated with slow transport of protein. However, the finding that rapid-transport protein also binds colchicine, although to a smaller extent, may mean that colchicine blocks axonal flow by interfering with more than one process.

A more definitive conclusion cannot be formed until further resolution of both slow-transport and rapid-transport proteins are achieved.

This work was supported by the Australian Research Grants Committee.

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