α-amanitin by displacing it from binding sites on the plasma albumin molecule (*4*, *11*).

The most effective antidote to α-amanitin, however, is cytochrome c (Table 1). It was tested against α-amanitin at a dose of 0.8 mg/kg, which invariably killed the control mice between 50 and 100 hours later. At this dose, the activity of penicillin and of the combined treatment with chloramphenicol and sulfamethoxazole were barely discernable, whereas cytochrome c, at 50 or 150 mg/kg, provided a survival rate of about 30 percent if applied 8 hours after the α-amanitin. If given only after 30 minutes, approximately 50 percent of the mice survived. The cytochrome was not consistently effective at either a lower dose (15 mg/kg), or a higher dose (450 mg/kg). The highest survival rate (62 percent) observed with any treatment following a dose of 0.8 mg/kg by 8 hours occurred if cytochrome c was combined with penicillin. Whether the combined regimen provides statistically significant advantages is unknown.

The cytochrome c preparation (*12*) was extracted from horse heart (*13*) and purified chromatographically (*14*).

The toxicity of malachite green in dogs and rats is antagonized by cytochrome c (*15*), but I have not found conclusive reports on its efficacy in other experimental intoxications. Clinical applications of the enzyme, mostly in the therapy of anoxic states, have been generally abandoned after a temporary surge of positive reports (*16*). The divergencies may be related to the variable activity displayed by different enzyme preparations. Moreover, cytochrome c has been reported to penetrate only into cells that have previously suffered toxic damage (*17*). This may also explain both the inconsistency of the clinical results and the effectiveness of the enzyme in α-amanitin poisoning. My finding also suggests a search for biochemical targets of α-amanitin other than RNA polymerase. The myocardial damage encountered in death cap poisoning and the reversibility by cytochrome c of electrocardiographic changes caused by malachite green (*15*) may represent a hint. Phalloidin has been reported to interfere in rat liver mitochondria with oxidative phosphorylation at the cytochrome c stage of the respiratory chain (*18*). Whether this applies for α-amanitin too has not yet been studied.

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Dibutyryl Cyclic Adenosine Monophosphate Stimulation of Colcemid-Inhibited Axonal Elongation

Abstract. *Adenosine 3′,5′-monophosphate (cyclic AMP) and its dibutyryl derivative induce a variety of morphological changes, including those associated with in vitro axonal maturation. Established sensory ganglia treated with dibutyryl cyclic AMP show significant increases in average axonal length and number in comparison with controls; those treated with maintenance doses of Colcemid show no increases in either parameter; simultaneous treatment with both agents results in growth statistically similar to that produced by dibutyryl cyclic AMP alone. The data are consistent with our hypothesis that cyclic AMP promotes axonal elongation by stimulating microtubule assembly from a preexisting subunit pool.*

Adenosine 3′,5′-monophosphate (cyclic AMP) and its dibutyryl derivative have recently been reported to induce a variety of effects on neoplastic tissue, including morphological transformation of various tumor cell lines to a more normal appearance (*1*) as well as induction of differentiation in neuroblastomal cells (*2*). These morphological alterations suggest the possibility that cyclic AMP and dibutyryl cyclic AMP act specifically on some component of the cytoskeletal system. Recently we reported that both nucleotides are capable of significantly enhancing neurite maturation in a nonneoplastic neuronal system (*3*). The elongating axonal processes in this system contain two prominent cytoskeletal elements, microfilaments and microtubules. Treatment of these neurites with Colcemid or colchicine inhibits further elongation and at higher concentrations induces retraction (*4*). These effects can reasonably be attributed to the specific disruptive action of these agents on the microtubular components of the axonal cytoskeletal system (*5*).

One possible mechanism of action for cyclic AMP and dibutyryl cyclic AMP's promotion of neurite maturation might be stimulation of microtubule assembly from a preexisting subunit pool. In order to investigate this possibility, dibutyryl cyclic AMP was applied to established cultures in the presence of concentrations of Colcemid sufficient to prevent neurite elongation but not high enough to initiate a significant retraction (maintenance dose). Under these conditions, dibutyryl cyclic AMP significantly reversed Colcemid's inhibitory effects on axonal development.

Dorsal root ganglia from 8½-day-old chick embryos were explanted onto collagen-coated cover slips and cultured (*6*). In order to insure sufficient neuronal growth all cultures were treated for 48 hours beforehand in standard medium (basal medium 199 supplemented with 10 percent heat-inactivated, fetal calf serum) containing 5 m*M* dibutyryl cyclic AMP. The effective removal of the nucleotide from all cultures was accomplished by three 5-minute washes with standard medium before the experimental treatment. That this was an effective measure can be seen by comparison of those cultures subsequently incubated for 24 hours in standard medium with the other test groups (Fig. 1). The various test agents were incorporated into the standard

medium and applied to the ganglia for 24 hours, after which the average axonal length was measured with an eyepiece reticle to the nearest 25 μm. A representative quadrant was then selected, and the number of axons was determined. Typical cultures were photographed for permanent record on a Reichert inverted microscope adapted with Nomarski optics. The extent of neuronal development necessitated presentation of photomicrographic montages. The *t*-test for a comparison of two sample means was used to compare the various experimental groups. The mean and standard deviations of each group were calculated on an IBM 1130 computer which, with the aid of an IBM 1627 CalComp plotter, was also used to draw the graphs to a Gaussian distribution. Each of the groups reported in Fig. 2 consists of at least 60 cultures.

The effects of 24 hours' exposure to standard medium enriched with 5.0 m*M* dibutyryl cyclic AMP (Schwarz Biological Research) or Colcemid (0.05 μg/ml, Grand Island Biological Company), or both, are shown in Fig. 1. Comparison of the frequency distribution histograms of control and dibutyryl cyclic AMP treated cultures shows significant increases in axonal lengths and numbers produced by treatment with the nucleotide (Fig. 2). The inhibitory effects of Colcemid are clearly shown in Fig. 1c. Analysis of the frequency distribution curves (Fig. 2) shows that Colcemid significantly inhibits the increases in both axonal length and number when compared to both control and dibutyryl cyclic AMP treated cultures.

When both agents are applied simultaneously (Fig. 1d), Colcemid's inhibitory effects are completely reversed—that is, axonal length and number are increased significantly when compared to control and Colcemid treated cultures (Fig. 2). The rounded appearance assumed by most of the glial cells in the presence of Colcemid, as indicated by the arrows (Fig. 1c), is also reversed when both agents are present (Fig. 1d). Comparison of the frequency distribution curves (Fig. 2) for those cultures treated with Colcemid and dibutyryl cyclic AMP with those treated only with dibutyryl cyclic AMP reveals no significant ($P < .05$) differences

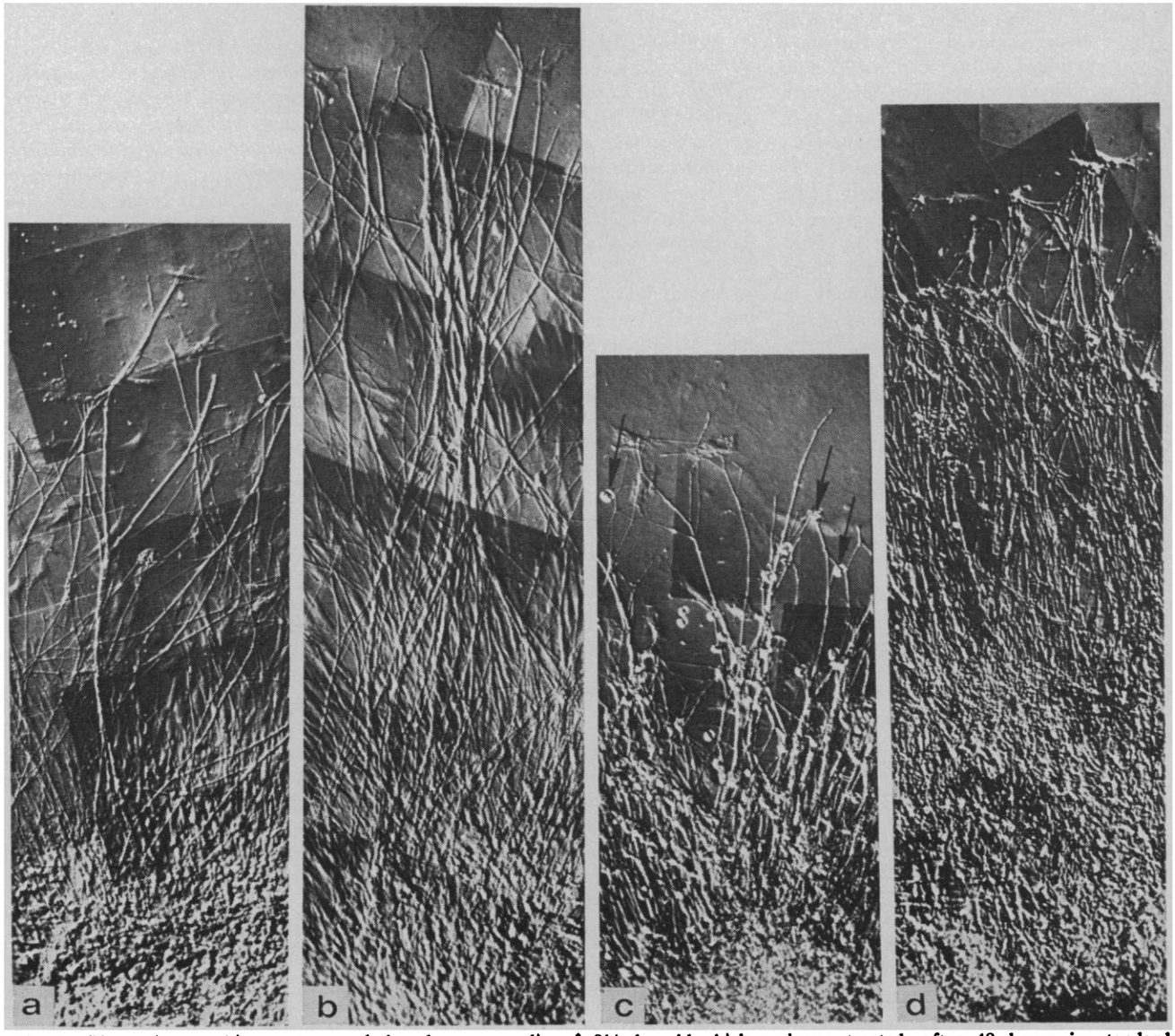

Fig. 1. Photomicrographic montages of dorsal root ganglia of 8½-day-old chick embryos treated, after 48 hours in standard medium (SM) supplemented with 5 m*M* dibutyryl cyclic AMP, for an additional 24 hours in the following ways: (a) SM (control), (b) SM and 5 m*M* dibutyryl cyclic AMP; (c) SM and Colcemid (0.05 μg/ml); (d) SM and 5 m*M* dibutyryl cyclic AMP and 0.05 μg of Colcemid per milliliter. (Nomarski optics, × 165)

between these two treatment groups.

Microfilaments are a second major class of cytoskeletal components present in these neurites. Their essential roles have been shown to be at the level of growth-cone activity, microspike formation, and collateral neurite development (*7*). The possibility that cytochalasin B might act specifically on these microfilaments (*7*) prompted us to attempt a similar reversal of its destructive action with dibutyryl cyclic AMP. In these experiments growth-cone activity was not restored in cultures treated with a maintenance dose of cytochalasin B (4 μg/ml, Imperial Chemical Industries), nor was neurite elongation stimulated significantly.

It is suggested that one action of dibutyryl cyclic AMP on the cytoskeletal elements of developing axons is at the level of the microtubules. Since the initial stages of nerve cell elongation have been reported to be independent of protein synthesis (*8*) it may be that dibutyryl cyclic AMP acts by stimulating microtubule assembly from a subunit pool, rather than by derepressing microtubular protein synthesis; this possibility is currently under investigation.

Our previous speculation that nerve growth factor (NGF), an agent known to stimulate axonal development, might in some way act through a second messenger system (*3*) led to similar experiments in which NGF was applied to Colcemid treated cultures. Nerve growth factor has in every case significantly ($P < .01$) reversed Colcemid's inhibitory effects (*9*). Therefore, in accordance with our hypothesis, one way NGF stimulates axonal maturation is apparently via a cyclic AMP intermediary which stimulates the assembly of microtubules from a *preexisting* subunit pool.

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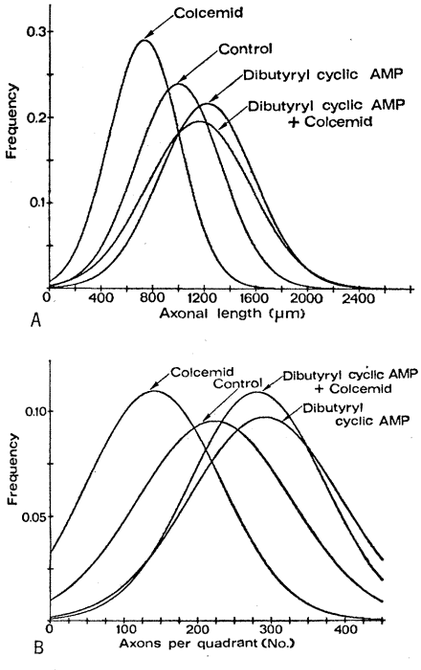

Fig. 2. The effects of Colcemid (0.05 μg/ml) and 5 m*M* dibutyryl cyclic AMP and of both together on axonal maturation are summarized in these normalized frequency distribution histograms based on a minimum of 60 cultures per group: (A) axonal length; (B) axonal number.

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Behavioral Maintenance of High Concentrations of Blood Ethanol and Physical Dependence in the Rat

Abstract. *Rats maintained on an intermittent food schedule with an available ethanol solution drink to excess (13.1 grams of ethanol per kilogram of body weight, daily). Removal of ethanol produces symptoms of physical dependence including death from tonic-clonic seizures. Overindulgence in oral self-administration of an aqueous ethanol solution, resulting in unequivocal physical dependence, approximates a model of human alcoholism.*

The study of alcoholism would be facilitated if an animal model, possessing the major behavioral and physiological features of the human alcoholic, was developed. Recent reviews indicate (*1*) that presently available experimental arrangements fall short of providing such a model. One problem has been that the salient behavioral requirements of the model are quite demanding: (i) animals should orally ingest ethanol solutions excessively and chronically in a pattern that increases the concentration of blood ethanol analogous to that in the alcoholic; (ii) unequivocal physical dependence on ethanol must be demonstrated; (iii) food and ethanol should be available from sources physically separate so that the factors determining ethanol intake are not inextricably bound to those primarily concerned with meeting nutritional requirements; (iv) the experimental arrangement should retain an elective aspect to the ethanol ingestion by not programming extrinsic reinforcing events (for example, shock avoidance, food pellet delivery) contingent upon drinking ethanol. The technique we now describe satisfies all the above criteria.

Previously, one of us (*2*) found that volumes of water three to four times the normal 24-hour amounts were ingested when small food pellets were delivered intermittently to rats on a limited-food regimen. Because there is no increased fluid requirement in this method, polydipsia induced by a food schedule has been the subject of further research and theoretical speculation (see *3*).

Using the schedule-induced polydipsia technique, several investigators (*4*) have presented an ethanol solution as the available fluid instead of water. This research yielded no evidence of physical dependence on ethanol, perhaps because only single daily sessions, usually between 1 and 3 hours in length, were administered. Two studies, however, utilized longer sessions. Lester (*5*) maintained a rat on an excessive level of ethanol ingestion for a single 65-hour period. Ogata *et al.* (*6*) maintained mice on schedule-induced polydipsia for ethanol solutions during a 1- to 2-week period, but found no indications of physical dependence upon withdrawal of ethanol.

Eight male Holtzman rats, with a mean free-feeding body weight of 315.9 g, were reduced to 80 percent of their weights by limiting food rations (Purina Laboratory Chow, pelleted). They were housed in individual chambers under constant illumination. Each