Table 4 Effect of various inhibitors on choline transport

Inhibitor	Choline uptake (% of control)
None	100
Hemicholinium (0.1 mM)	131
Na^+ replaced by Li^+	81
Na^+ replaced by sucrose (0.25 M)	66
Ca^{2+} replaced by Mg^{2+}	93
2,4-dinitrophenol (1 mM)	40
Ouabain (1 mM)	38
Incubation at 0° C	10

Neuroblastoma cells (NS20 clone) were grown to confluence in 75 cm^2 plastic flasks and depleted of choline metabolites as described for Table 3. The cultures were preincubated with the anticholinesterase agent and the indicated metabolic inhibitor in Hank's BSS for 15 min. Then 2×10^{-3}M ^{3}H-choline was added and after a further 15 min incubation the cells were collected as described in Table 2.

As would be expected for any energy-dependent cellular process, uptake is inhibited by low temperature and 2,4-dinitrophenol. But this should not be interpreted as evidence that choline is taken into neuroblastoma cells by active transport. We have never observed an intracellular choline concentration greater than that in the medium and inhibition of transport by such nonspecific agents could have other explanations. In erythrocytes there is a facilitated diffusion system for choline[10] and we have no evidence against the operation of such a mechanism in neuroblastoma. These inhibitor effects do not eliminate the possibility that the characteristic properties of cholinergic choline transport emerge when a universal uptake system is coupled to choline acetyltransferase. If coupling were absent in neuroblastoma cells, choline kinase and acetyltransferase could compete for their common substrate.

Although the absence of the brain synaptosomal high affinity choline transport system may simply be correlated with the absence of synapses in neuroblastoma cultures[15], there are other explanations of our data. In particular, synthesis of choline acetyltransferase may not be an expression of more generalised cholinergic potential of the NS20 clone. This view is supported by the existence of acetylcholine receptors in neuroblastoma cells[16], a finding that suggests a postganglionic origin for the cells and seems incompatible with the concomitant presence of cholinergic synaptic structures. Nevertheless, the generation of a high affinity uptake system possessing such properties as preferential synthesis of acetylcholine and sensitivity to appropriate inhibitors could provide a convenient biochemical tool for investigating the differentiation of neuroblastoma cells *in vitro*.

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Binding of interferon to gangliosides

RECENT evidence suggests that the antiviral action of interferon is triggered by interaction with the cellular membrane. Mouse interferon covalently bound to Sepharose beads (IF-Sepharose) retains its antiviral potency and only direct contact with these particles produces the antiviral effect[1,2]. Preincubation of mouse L cells with *Phaseolus vulgaris* phytohaemagglutinin (PHA) blocks interferon action[3]. The inhibitory action of PHA can be almost completely reversed by washing PHA-treated cells with fetuin, a glycoprotein of high affinity for this plant lectin[3]. These data suggest that membrane sites interacting with interferon are carbohydrate-containing molecules that bind to PHA, although other explanations for the inhibitory action of PHA based on nonspecific steric or charge effects might be possible. To substantiate further that glycoside-containing membrane components bind to interferon, we have investigated the effect of gangliosides on interferon binding and action.

As Table 1 shows, preincubation of IF-Sepharose with a ganglioside mixture from bovine brain completely blocked antiviral activity. When tested individually, mono- as well as di- and trisialogangliosides were inhibitory. The concentration in the preincubation mixture resulting in maximal inhibition of antiviral activity was lowest for G_{M2} and G_{T1}, suggesting somewhat stronger binding of these two gangliosides. Thin-layer chromatography on silica plates in chloroform–methanol–water (65:45:9) followed by resorcinol spray[4] revealed one band for G_{M2}, but showed that commercial G_{M1}, G_{D1a} and G_{T1} were somewhat contaminated with other gangliosides although free of G_{M2}. In separate experiments using G_{M1}, G_{D1a} and G_{T1} which were 82–87% pure, essentially similar results were obtained, though G_{M1} and G_{D1a} appeared slightly less inhibitory. G_{M3}, a mixture of the acetyl and the glycolyl neuraminic acid derivatives, was only tested at one concentration because of the small amount available. Its inhibitory action appeared slightly less than that of G_{M2} and G_{T1}, but more than that of the other two gangliosides. In our determinations of viral yield only differences greater than twofold were considered significant. Therefore, although Table 1 compares μg quantities of gangliosides instead of μmol, the accuracy of the assay does not allow conclusions based on molecular weight differences of individual gangliosides. Inhibition of IF-Sepharose by individual gangliosides was the same regardless of whether preincubation was carried out for 1 h or 18 h. When the temperature of the preincubation mixture was varied, however, there was a consistent increase of the inhibitory effect of gangliosides with increasing temperatures. Thus, EMC yield by plaque-forming units (PFU) in response to IF-Sepharose preincubated with mixed gangliosides at 4° C was one-third of that found after preincubation at 37° C. Washing ganglioside-treated IF-Sepharose with PHA solutions partially reversed ganglioside inhibition, suggesting that PHA and interferon have common binding sites (Table 2). It was not possible completely to restore antiviral activity, however, indicating that under the conditions

Table 1 Inhibition of interferon-Sepharose by gangliosides

Ganglioside in preincubation mixture	Viral yield after preincubation of IF-Sepharose with different amounts of ganglioside (μg)					
	400	200	80	40	20	0
Ganglioside mixture	4,096 (4,096)	ND	512	ND	64	64 (4,096)
G_{T1}	ND	ND	2,048	256	64	32/64 (2,048)
G_{D1a}	ND	1,024	256	128	64	32/64 (2,048)
G_{M1}	ND	2,048	256	64	32	32/64 (2,048)
G_{M2}	ND	2,048	2,048	256	64	32/64 (2,048)
G_{M3}	ND	ND	256	ND	ND	4/8 (512)

Interferon covalently bound to Sepharose (IF-Sepharose), prepared as described previously[1,2], was treated with gangliosides by incubating 5×10^4 beads with 0.4 ml ganglioside solution in PBS for 75 min at 25° C. The beads were then washed once with 4 ml PBS and suspended in 1 ml of Eagle's minimal essential medium plus Hanks salts without serum (MEM). The ganglioside mixture was obtained from bovine brain (Sigma, type III). Individual gangliosides were obtained from Supelco Inc. Mouse L cells were cultivated in 35 mm plastic dishes (5×10^5 cells per dish) in MEM, containing 10% calf serum. After removal of medium the cells were incubated for 5 h at 37° C with a 1-ml suspension of IF-Sepharose or control Sepharose 4-B in MEM at a ratio of one bead per 10 cells. After removal of the beads, the cells were challenged with encephalomyocarditis virus (EMC) at a multiplicity of infection of 0.1. Viral yield was determined after 16 h of incubation at 37° C by haemagglutination of human red blood cells of type O in serial twofold dilutions of virus suspensions[10]. The numbers represent the reciprocal of the highest dilution that showed haemagglutination. The individual gangliosides were designated according to Svennerholm[11]. Numbers in brackets represent experiments in which control Sepharose 4-B was used instead of IF-Sepharose. ND, Not done.

Table 2 Effect of PHA on ganglioside inhibition of interferon-Sepharose

Ganglioside in preincubation mixture	Viral yield without ganglioside pretreatment of IF-Sepharose	Viral yield after ganglioside pretreatment of IF-Sepharose	
		Before PHA	After PHA
Ganglioside mixture (400 μg)	64	4,096	512/1,024
G_{M2} (80 μg)	32/64	2,048	512
G_{M1} (200 μg)	32/64	2,048	512
G_{D1a} (200 μg)	32/64	1,024	512
G_{T1} (80 μg)	32/64	2,048	512

After treatment of IF-Sepharose beads with gangliosides as described under Table 1, they were suspended in 0.3 ml of PHA (phyto-haemagglutinin from *Phaseolus vulgaris*, M form, Grand Island Biological Co.) at a protein concentration of 4.1 mg ml^{-1}. After incubation for 60 min at 25° C the lectin solution was removed; the beads were washed once with 4 ml PBS, and suspended in 1 ml MEM without serum. Further experimental details were as described under Table 1.

used the affinity of gangliosides for interferon is greater than for PHA. Preincubation of L cells with gangliosides did not result in stimulation of the antiviral effect of interferon, as might be expected if gangliosides became incorporated into the cell membrane to produce additional receptor sites. Such stimulation of activity was observed with cholera toxin[5], which has been shown to bind strongly to G_{M1} (ref. 6).

Incubation of interferon solutions with gangliosides bound covalently to a Sepharose-polylysine copolymer as described by Cuatrecasas[7] resulted in complete adsorption of antiviral activity to these beads (Table 3). In contrast, under identical conditions control beads that only contained covalently bound polylysine did not bind interferon to any great extent. Binding of interferon to Sepharose-bound gangliosides was prevented by preincubation of these beads with PHA, in accord with previous results suggesting that PHA and interferon bind to common constituents on the cell membrane[3]. Washing PHA-treated ganglioside beads with fetuin, a protein of known affinity for PHA which reversed PHA inhibition of interferon action[3], failed to restore measurable interferon binding to ganglioside beads, even at a concentration of 60 mg ml^{-1}. It appears, therefore, that PHA has a higher affinity for Sepharose-bound gangliosides than for fetuin in solution. Ganglioside-Sepharose beads also completely inhibited the action of soluble interferon, as Table 4 shows.

Gangliosides are common constituents of cellular membranes. The occurrence of disialoganglioside (G_{D1}) and haematoside (G_{M3}) in the cell membrane of L cells has been reported[8]. Inhibition of mouse interferon action by preincubation of

Table 3 Binding of interferon to ganglioside-Sepharose beads

Interferon preincubated with	Interferon titre in supernatant
Nothing	1,024
Control beads	1,024
Control beads Pretreated with PHA	1,024
Ganglioside beads	< 16
Ganglioside beads Pretreated with PHA	1,024

Mouse interferon was prepared by induction of mouse L cells with ultraviolet-irradiated Newcastle disease virus in serum-free MEM plus Earle's salts as described before[12]. Crude interferon solutions were concentrated tenfold by pressure dialysis at *p*H 3 and used as such after changing the *p*H to 7. They contained 0.2 mg ml^{-1} protein[13]. Polylysine (molecular weight 30,000, Sigma) was coupled to CNBr-activated Sepharose (Pharmacia) as described by Sica *et al.*[14]. Mixed gangliosides from bovine brain (type III, Sigma) were attached to polylysine-Sepharose as described by Cuatrecasas[7]. Covalently bound gangliosides correspond to 0.15 mg ml^{-1} packed beads, as judged from the amount of carbohydrate in the soluble portion of the reaction mixtures after completion of the reaction[15]. In the experiments with PHA, 0.2 ml of packed beads was incubated for 30 min with 0.5 ml lectin solution (4.1 mg ml^{-1} protein, see Table 2) for 30 min at 25° C. They were then washed twice with 1 ml of 1 M NaCl and twice with 1 ml of PBS. In each of the experiments listed 0.2 ml packed beads (washed twice with 1 ml of NaCl and twice with 1 ml of PBS immediately before use) were suspended in 0.5 ml of interferon solution and incubated for 45 min at 25° C with occasional stirring. The supernatant of the beads was collected, and the beads were washed twice with 1 ml of 1 M NaCl and twice with 1 ml of PBS. Original supernatant and washings were combined and the interferon titre was measured by determining the cytopathic effect in serial twofold dilutions on mouse L cells after infection with vesicular stomatitis virus (VSV). Control beads represent polylysine-containing beads before ganglioside attachment. In the control experiment without beads interferon solution was incubated alone and diluted with corresponding amounts of 1 M NaCl and PBS. The numbers represent the reciprocal of the dilution of combined supernatants and washings showing a cytopathic effect of 50%.

L cells with PHA suggests involvement of carbohydrate-containing cell membrane constituents in interferon binding and/or action[3]. Our data show binding of interferon to gangliosides and inhibition of interferon action by exogenous gangliosides. They also demonstrate that inhibition by and binding to gangliosides is partially or completely reversed by PHA, in accord with our previous results[3]. Binding to gangliosides has been shown for certain toxins, like cholera toxin and tetanus toxin, for hormones like seretonin[6], and for viruses like Sendai[9]. Our data suggest that binding to gangliosides might also play a role in interferon action *in vivo*.

Table 4 Inhibition of interferon activity by ganglioside-Sepharose beads

Beads used	Bead to cell ratio	Viral yield (PFU $m^{-1} \times 10^{-5}$) No interferon added		Interferon added	
		I	II	I	II
None	—	1,200	740	5	3
Control beads	1:8	ND	340	ND	2
Ganglioside beads	1:8	ND	430	ND	170
Control beads	1:10	800	ND	5	2
Ganglioside beads	1:10	800	ND	200	170
Control beads	1:20	ND	ND	ND	2
Ganglioside beads	1:20	ND	ND	ND	32

Polylysine-Sepharose (control) or ganglioside-Sepharose beads prepared as described under Table 3 were suspended in 1 ml of MEM without serum and layered on to mouse L cells in 35 mm plastic Petri dishes (5×10^5 cells per dish). Mouse interferon prepared as described under Table 3 was added and the cells were incubated at 37° C for 24 h. After removal of medium (derivatised Sepharose beads remained tightly attached to the cells) the cells were infected with VSV at a multiplicity of infection of 0.1 and further incubated for 16 h at 37° C. Viral yield was measured directly by determination of plaque-forming units (PFU). I and II represent two different sets of experiments. ND, Not done.

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Evidence for ATP action on the cell surface

IT seems that Mg^{2+}–ATPase (EC 3.6.1.3) and 5′-nucleotidase (EC 3.1.3.5) are prevalent plasma membrane marker enzymes in eukaryotic cells[1]. Our studies on the 'sidedness' of plasma membrane macromolecules suggested that the two enzymes function as ecto-enzymes[2], although the substrates presumably were not present on the cell surface. We assumed that the substrates were present, but obscured within the intercellular spaces and by rapid metabolism. The specificity of the ecto-ATPase is low but we suspected that translocated cytoplasmic ATP might be a primary substrate.

We have recently obtained evidence in tissue cultures for apparent ATP translocation from the cytoplasm into the medium (unpublished data). Reports by McIlwain *et al.* that adenosine, and to a lesser extent adenylates, were released from electrically stimulated brain slices support this possibility[3–5]. Pull and McIlwain had speculated[4] that ATP was the source of the released adenosine. We postulated that the translocation of cytoplasmic ATP might be part of a common physiological phenomenon. During a study of the effects of ATP application to the membrane surface I have now found that the addition of ATP to monolayer cultures of mammalian cells produces a biphasic change in membrane permeability. *In situ* this event may be initiated by the translocation of cytoplasmic ATP and terminated by ecto-nucleotidephosphoesterhydrolases.

Changes in membrane permeability were measured by determining the extrusion of isotopic ions from pulse labelled monolayer tissue cultures. I shall discuss the permeation of isotopic species because measurements of net fluxes have not been made yet. The basic methodology was briefly as follows. Established tissue culture cell lines were grown in 250 ml Falcon flasks in the appropriate medium. When the cultures reached confluency they were exposed for 2 h to 5 ml of medium containing $^{32}P_i$, $^{42}K^+$ or other isotopic compounds. After removal of the isotopic medium by several brief rinses, the cultures were superfused with 4 ml of serum-free medium at 37° C in an air/CO_2 atmosphere (95/5, v/v). During incubation the flasks were rotated horizontally once every 150 s for 6 s at 50 r.p.m. to provide stirring action. Extrusion of isotopic species was measured by sampling the medium at intervals and by scintillation counting. Protein content of the cultures was determined in NaOH digests according to Lowry *et al.*[6].

Using ^{32}P pulse labelled cultures of mouse fibroblasts (L929), HeLa cells (human choriocarcinoma) or KB cells (human carcinoma of the nasopharynx) I found that ^{32}P efflux into the medium increased significantly when ATP reached about 5×10^{-4} M. In monolayer cultures of neonatal Syrian hamster astrocytes, the effect of ATP is particularly evident and typical dose responses are illustrated in Fig. 1. When the superfusate

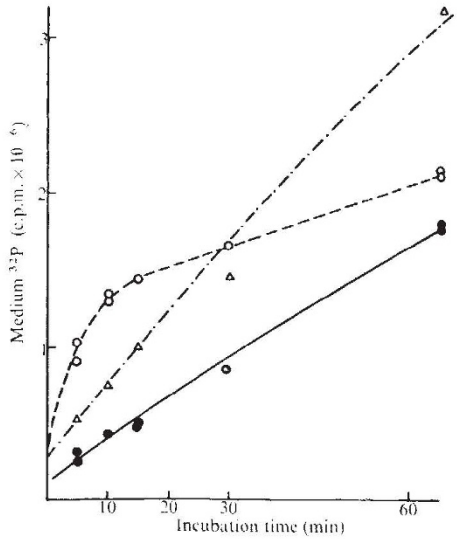

Fig. 1 Effect of medium ATP concentration on ^{32}P extrusion of pulse labelled NN astrocyte cultures. Cultures labelled with $^{32}P_i$ for 2 h, rinsed three times and superfused with Hanks' minimum essential medium containing ATP as indicated. Curves show cumulative ^{32}P in medium. △, 5×10^{-3} M; ○, 1.2×10^{-4} M; ●, control.