and 5-hydroxytryptamine release from rat peritoneal mast cells by chlorpromazine was described by FRISK-HOLMBERG[6] and JANSSON[7], we suggest that antihistamines act upon rat mast cells both by a lytic and by an energy-requiring mechanism. It is possible that the preparation procedures for isolating mast cells used by these authors may have enhanced the lytic effect, for the use of a Ficoll gradient has been described as causing cellular damage, and even a single washing of the cells with Hanks solution removes a surface protective material[8]. Moreover toluidine blue itself causes damage to rat peritoneal mast cells and releases histamine by a mechanism which is also dependent on cell metabolism[9]. Glucose was ineffective when toluidine blue was used at a concentration higher than that necessary to prevent the damaging action of promethazine. This same observation has been made with compound 48/80, chlorcyclizine and diphenhydramine, but the concentrations of toluidine blue at which glucose was ineffective varied with the different drugs[1]. These results suggest that toluidine blue inhibits rat mast cell damage by another mechanism, besides blocking Krebs cycle enzymes. The fact that chlorpromazine and chlorpromethazine required higher concentrations of the stains than promethazine for their action to be inhibited may be due to their higher lipophilic nature[10].

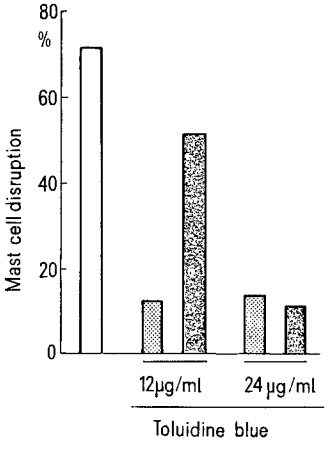

Fig. 4. Effect of toluidine blue on rat mast cell damage caused by promethazine (□, 0.1 m*M*) in the absence (▨) or presence (■) of glucose (4.4 m*M*). Mean of 9 experiments performed at pH 8.4.

Resumen. Se demuestra que la ación de los antihistamínicos prometazina, clorprometazina, y clorpromazina sobre los mastocitos de la rata es inhibida por el azul de toluidina y el azul de metileno. La glucosa revierte la acción inhibitoria de estos colorantes sobre la clorpromazina y la prometazina, sugeriendo que ellos actúan bloqueando el metabolismo celular. Se concluye que además de su acción lítica sobre los mastocitos de la rata, los antihistamínicos también actúan por un mecanismo que necesita de energia.

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In vitro Effect of a 5-Fluorodeoxyuridine Albumin Conjugate on Tumour Cells and on Peritoneal Macrophages

Conjugation with albumin gives to amanitin and to phalloidin a selective toxicity for cells having a high protein uptake[1-4]. The ultrastructural lesions produced in these cells by amanitin-albumin and by phalloidin-albumin conjugates are identical with those caused in hepatocytes by free amanitin and by free phalloidin respectively[1,4]. Since proteins after penetration into the cells are rapidly broken down by lysosomal enzymes[5], it is likely that the conjugates exert their toxic action within the cells after digestion of the protein moiety and release of the free toxins from conjugates. It was suggested[3] to try conjugating albumin with compounds which selectively damage cells in proliferation. If a compound with such a property is released in active form after penetration of the carrier protein into the cells (as in the case of amanitin and phalloidin), the resulting conjugate should specifically damage the dividing cells with a high protein uptake and could be useful in the therapy of histiocytic tumours. It is worth noting that 1 normal cells with high protein uptake (cells of macrophage system, granulocytes and kidney proximal tubule cells) do not proliferate[6,7]; 2. neoplastic proliferating histiocytes were found to be highly endocytic[8-10].

Besides neoplastic histiocytes, other types of tumour cells seem to display a high uptake of macromolecules[11,12]. Following this evidence, TROUET et al.[13] attempted to increase Daunorubicin penetration into tumour cells by administering the drug in a complex with DNA. En-

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Table I. Effect of FUDR-RSA, AMA-RSA and FUDR on the neoplastic cells and on peritoneal macrophages

	Number of viable cell $\times 10^6$ 3T3-MSV-H		S 180-A		RTC		Peritoneal macrophages
	0 time[a]	After 48 h	0 time	After 48 h	0 time	After 48 h	(after 48 h)
Controls	1.43	4.51	0.46	1.31	0.29	0.78	1.88
FUDR-RSA (5 μg/ml)	1.43	1.27	0.46	0.03	0.29	0.23	1.79
FUDR-RSA (20 μg/ml)	1.43	0.46	0.46	0	0.29	0.19	—
AMA-RSA (1 μg/ml)[b]	1.43	4.48	—	—	—	—	0.94
AMA-RSA (4 μg/ml)	1.43	4.53	0.46	0.83	0.29	0.54	0
AMA-RSA (20 μg/ml)	1.43	2.16	0.46	0.40	0.29	0.04	0
FUDR (0.5 μg/ml)	1.43	0.99	0.46	0.03	0.29	0.20	1.87
FUDR (2 μg/ml)	—	—	0.46	0	0.29	0.18	1.90

[a] At 0 time the substances to be tested were added to the cultures. [b] AMA-RSA was prepared according to DERENZINI et al.[1]. The molar ratio of amanitin to albumin in the conjugate, calculated as previously described[1], was 2.3.

couraging results were obtained in the treatment of murine leukemia L1210 and of several cases of human leukemia[13,14].

In the present experiments we conjugated 5-fluorodeoxyuridine (FUDR) to rabbit serum albumin (RSA). FUDR, which inhibits deoxythymidylate synthetase[15], selectively blocks DNA synthesis, thus specifically damaging cells in proliferation. The conjugation was performed by using 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (ECDI) as coupling agent[16]. 180 mg FUDR (Fluka) and 30 mg RSA were dissolved in 1 ml H_2O and 210 mg ECDI (Fluka) in 0.36 ml H_2O were added. After 24 h at 30 °C, the conjugate (FUDR-RSA) was separated from free FUDR and from unreacted ECDI by gel filtration on a Sephadex G75 column. The molar ratio of FUDR to RSA in the conjugate was determined in a separate experiment by using ^{3}H-FUDR (Radiochemical Centre, Amersham); it was found to be 28.

Since we were unable to find a line of neoplastic histiocytes growing in culture, the action of FUDR-RSA was tested on 3 lines of neoplastic non-histiocytic cells displaying a good protein uptake as assessed by sensitivity to an amanitin-albumin conjugate (AMA-RSA) (Table I). The cell lines were: mouse fibroblasts (Balb/c) transformed by murine sarcoma virus, Harvey strain[17] (3T3-MSV-H); cells from a methylcholanthrene-induced sarcoma in Fisher rats (RTC); cells from Sarcoma 180, ascitic form (S 180-A). The 3T3-MSV-H and RTC cells were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS). For S 180-A cells, the same medium plus 10% calf serum was used.

Mouse peritoneal macrophages were obtained as already described[2], and cultured in MEM plus 10% inactivated FBS. Cell viability count was performed by Trypan blue staining[18].

The results given in Table I show that FUDR-RSA at a concentration of 5 μg/ml completely hinders the proliferation of 3T3-MSV-H cells and of RTC cells, while it lowers the number of S 180-A cells to less than $^1/_{10}$ of control cells. FUDR-RSA at a concentration of 20 μg/ml greatly

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Table II. Lack of action of RSA incubated with FUDR in the absence of ECDI and inhibition of FUDR-RSA toxicity by thymidine

		3T3-MSV-H Number of viable cell $\times 10^6$	
		0 time[a]	After 48 h
Exp. 1	Controls	1.54	3.42
	RSA (160 μg/ml) + FUDR[b]	1.54	3.61
Exp. 2	Controls	1.84	3.91
	FUDR-RSA (20 μg/ml)	1.84	0.84
	FUDR-RSA (20 μg/ml) + thymidine (16 μg/ml)	1.84	3.40
	FUDR (1.6 μg/ml)	1.84	0.63
	FUDR (1.6 μg/ml) + thymidine (16 μg/ml)	1.84	3.79
	thymidine (16 μg/ml)	1.84	4.69

[a] At 0 time the substances to be tested were added to the cultures. [b] RSA was maintained in presence of FUDR, as for the preparation of FUDR-RSA, but ECDI was omitted. After 24 h at 30 °C, FUDR was removed by gel filtration. No FUDR was found adsorbed onto RSA as assessed by spectrophotometric measurement.

reduces the number of viable 3T3-MVS-H and RTC cells; it kills all the S 180-A cells. As shows in Table II (exp. 1) the toxicity of FUDR-RSA cannot be due to FUDR merely adsorbed onto RSA. The action of FUDR-RSA is abolished by the addition of thymidine (Table II, exp. 2); this indicates that the conjugate acts by releasing FUDR.

At the concentration which completely inhibits the proliferation of the neoplastic cells, FUDR-RSA does not affect the non-dividing macrophages, although it seems very likely that these cells have a much higher protein uptake. In fact macrophages are many times more sensitive than the neoplastic cells to the AMA-RSA conjugate.

The present results indicate that FUDR coupled with albumin is released in active form after penetration of the conjugate into the cells. A different result was obtained by coupling two other antineoplastic drugs, methotrexate and adriamycin, to albumin. The methotrexate conjugate was completely ineffective on the neoplastic cells and on macrophages, whereas the adriamycin conjugate displayed only a very slight action as compared to the strong effect produced by equivalent amounts of the free drug.

In future experiments we intend to test the action of FUDR-RSA on the in vivo-growth of those tumours whose cells take up proteins to a high degree. It is worth noting that FUDR-RSA has a low toxicity; injected i.p. at a dose of 1 mg/10 g body weight (the maximum tested) it does not kill mice, while the LD_{50} (i.p.) of AMA-RSA for the mouse is only 1.5 µg/10 g body weight.

Since only about 1% of input FUDR is conjugated to RSA by our ECDI procedure, some difficulties arise to obtain the amount of conjugate necessary for the in vivo experiments, which should be postponed until a search is made for a more effective method of conjugation[19].

Riassunto. La 5-fluorodeossiuridina, unita covalentemente alla albumina di coniglio, è liberata in forma attiva dopo penetrazione del coniugato dentro le cellule.

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Enhancement of Cerebral Noradrenaline Turnover by Thyrotropin-Releasing Hormone: Evidence by Fluorescence Histochemistry

Thyrotropin-releasing hormone (TRH) has been claimed to improve mental depression[1,2]. TRH potentiates the behavioral effects of L-DOPA plus pargyline in mice[3]; this action seems independent of the release of thyroid-stimulating hormone (TSH), because the potentiation occurs in hypophysectomized mice, as well as in normal mice, and may be connected with a direct potentiation of catecholamine systems. Biochemical findings have been reported[4] indicating that TRH enhances the turnover of noradrenaline in the brain of rats, enhancement observed in thyroidectomized rats as well as in normal rats.

We investigated morphologically the action of TRH on the monoamine neurons of the rat brain. 30 male albino rats of Wistar origin, 200–250 g, were used. The animals were injected i.p. with 300 mg/kg α-methyl-p-tyrosine (α M_PT), TRH + α M_PT, TRH alone and saline (controls). α M_PT was administered 4 h before sacrifice and TRH (20,30 or 40 mg/kg) $^1/_2$, 1, 2 or 3 h before α M_PT. The brains cut perpendicularly in 5 frontal sections were frozen in propane cooled in liquid nitrogen, freeze-dried and treated with formaldehyde, according to the method of FALCK and HILLARP[5]. Sections 10 µm of all these brains were mounted semi-serially (1/10, 1/20); the observation of the sections by the fluorescence microscope under standard conditions permitted us to obtain a subjective morphological appreciation of the changes in the fluorescence intensity of monoaminergic neurons at various cerebral areas.

After administration of α M_PT a marked decrease of the green fluorescence of the dopamine and noradrenaline cell bodies (locus niger and locus coeruleus + lateral pontobulbar nuclei, respectively) occurred. The green fluorescence of the dopamine terminals (neostriatum) and that of noradrenaline terminals of hypothalamus and cerebral cortex was also strongly diminished (Figure b). No changes in the yellow fluorescence of 5-hydroxytryptamine neurons was observed.

TRH in doses of 20–40 mg/kg administered $^1/_2$, 2, 3 h before αM_PT accentuated the decrease of green fluorescence in the noradrenaline terminals of cerebral cortex (Figure). In the hypothalamic terminals, however, the αM_PT-induced decrease of noradrenaline fluorescence was slightly accentuated after 40 mg/kg TRH administered $^1/_2$ or 1 h before αM_PT, but did not change after lower doses of TRH, and if TRH was administered 2 or 3 h before αM_PT.

The αM_PT-induced decrease of the noradrenaline green fluorescence at locus coeruleus cell bodies, and of the dopamine green fluorescence at the locus niger cell bodies and neostriatal terminals, was not influenced by TRH.

The fact that TRH accentuated the αM_PT-induced decrease of the green fluorescence of cortical and hypothalamic noradrenaline terminals suggests that this tripeptide enhanced noradrenaline release. Since no

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