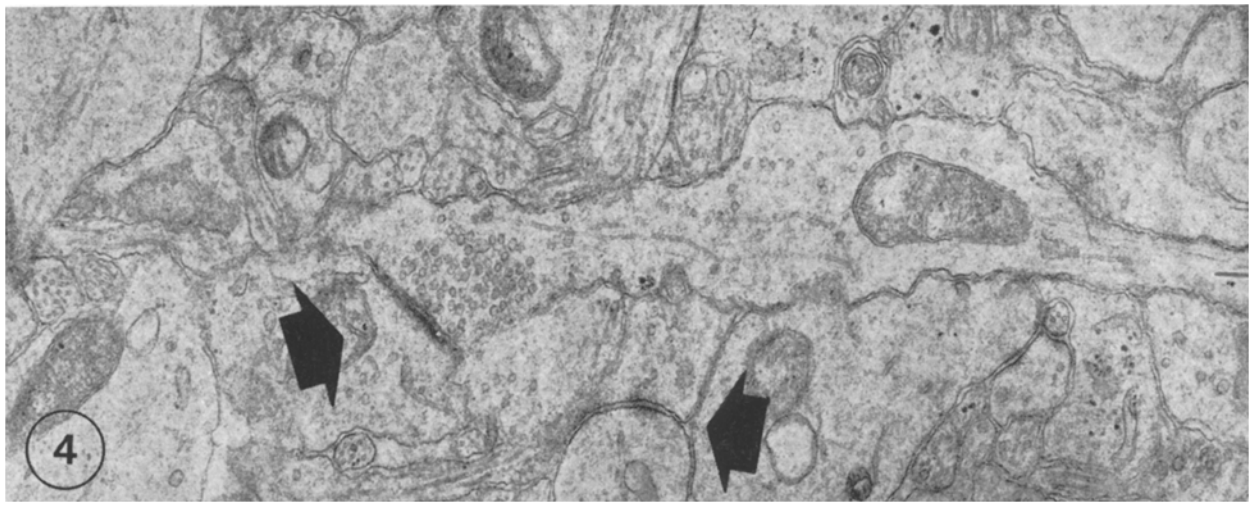

Fig. 4. Two synaptic junctions (arrows) in the layer XI of the optic tectum from an 18th incubation day chick embryo. ×31,000.

typical dilated portions. A small number of terminals in this group could be recognized as segments of fibres running in the stratum opticum and may be considered as retino-tectal synapses: the presence of synaptic terminals from other sources, however, cannot be excluded. The origin of the pre-synaptic terminals in group b) and c) junctions cannot yet be identidied. Whereas fibres of retinal origin are certainly not involved in the synapses of group a)[1,10], they could represent at least part of those of group b). A quantitative evaluation of changes in synaptic density has not yet been done: however, synaptic junctions were few until the 14th incubation day; during the 14th–18th day period similar axodendritic junctions were observed throughout the other layers. A critical increase in number was then observed between the day 18 and the first hours after hatching (Figure 4).

Our observations may be summarized thus: the synaptic junctions in the developing chick optic tectum displayed the essential features of developing synapses previously observed in other parts of the CNS[11–13]. They appear at only a relatively late stage of tectal neuron maturation, particularly in the case of the large multipolar cells of the stratum griseum centrale. They could be recognized when cell proliferation has ceased in the neural epithelium[7] and the essential steps in the distribution of the migrating neurons into the 6 major strata of the tectum[14] have been accomplished. In addition, a marked delay between the time of arrival of retinal fibres and the appearance of retino-tectal synapses was observed.

Although the identification of most pre- and post-synaptic components is still difficult in many junctions, one may safely claim that fibres of very dissimilar origin (such as those of groups a) and c)) form synaptic connections in the same period of time.

Lastly, it should be noted that the first junctions between retinal fibres and the tectal neurons appear at an earlier stage than that in which the formation of intra-retinal synapses was demonstrated[15].

Riassunto. Giunzioni sinaptiche compaiono in differenti strati plessiformi del tetto ottico dell'embrione di pollo, a partire dal 12° giorno di incubazione: aumentano di numero fra il 12° ed il 18° giorno e, in maniera critica, fra questo e le prime ore dopo la schiusa.

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Human Spleen Inhibitor of Leukaemic Cell Growth

In recent years much information has been obtained to indicate that certain tissues contain substances termed chalones, which depress or inhibit cell division acting in a negative feedback mechanism[1–4].

The possibility that the spleen may produce a diffusiable substance with inhibitory or cytotoxic effects on haematopoietic cells has been suggested on the basis of clinical observations. We have been investigating whether or not such a factor was present in the spleens of 2 patients suffering from chronic myelocytic leukaemia (CML), 2 with primary hypersplenism and 4 normal persons undergoing surgery as a result of trauma. For comparison, 2 bovine spleens were also studied. Another important objective of our project was to develop long-term cultures of human CML cells and multiple myeloma cells to test the effect of the splenic inhibitor (SI). Each of the spleens were surgically removed, processed and tested separately. The spleens were homogenized in distilled

water and the cells disrupted by sonication. The homogenate was centrifuged at 16,000 *g* for 20 min, and the supernatant partially deproteinized by adding ethanol to a concentration of 65%. The precipitate was discarded and the supernatant evaporated under vacuum. The powder was dissolved in Ringer's solution and the undissolved material removed by centrifugation at 2,000 *g* for 15 min. The supernatant was purified by column (2.5 × 100 cm) chromatography on Sephadex G-50 medium (Pharmacia Fine Chemicals, Piscataway, N.J.) and eluted with 0.01 *M* NH_4HCO_3. Alternatively the supernatant was subjected to molecular sieving via membrane filtration (Diaflo PM-10, Amicon Corporation), a process that yielded substances with a molecular weight (MW) of less than 10,000 daltons. The void volume (Vo) of Sephadex column was determined with blue dextran 2,000. The cytotoxic fraction eluted by gel-filtration between 330 and 430 ml (Vo = 165 ml) or by ultrafiltration was lyophilized and further purified by use of Sephadex G-25 fine. The cytotoxic fraction was eluted with NH_4HCO_3 between 250 and 430 ml (Vo = 212). The active protein-free SI was lypophilized and stored at –20°C until used. Its MW was estimated by the Andrew's technique on previously calibrated columns of G-25. All procedures were carried out at 4°C.

The active fraction isolated by gel-filtration on Sephadex G-25 represents 2% of the wet spleen weight. This fraction is a white powder highly soluble in water and insoluble in organic solvents, which contains approximately 40% of ninhydrin positive substances and 6% to 8% of carbohydrates (anthrone reagent). The SI does not contain proteins, hexosamines and pentoses. It has a MW of less than 1,000 daltons. These results indicate that the SI is a small peptide or glycopeptide.

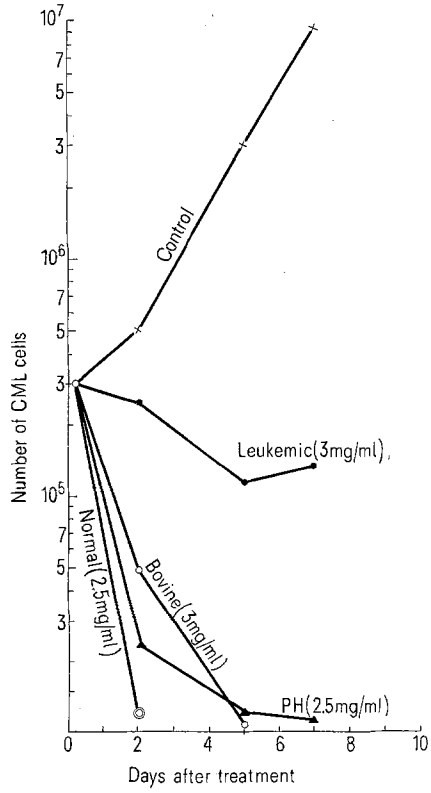
The CML cell-line developed in our laboratory is on the 79th serial passage and has the following characteristics: 1. it proliferates very rapidly in the medium as suspension cultures, 2. the number of cells increases 10 times in 8 days on each passage, 3. the majority of the cells are highly undifferentiated, and the minority are promyelocytes or myelocytes with basophilic granules, 4. the karyotype of all the cells examined showed the Philadelphia chromosome and a long marker D(Dq+), which represents a clonal evolution of the leukemia.

Cytotoxicity was tested by exposing 3×10^5 CML cells to various amounts of the SI in 20 ml of the medium. The total number of viable cells was measured by the trypan blue dye exclusion technique. The results are illustrated in the Figure. Cultures of CML cells used as controls were grown with and without the addition of fractions eluted by gel-filtration before and after elution of the active fraction. At the dose of 0.25 mg/ml of the culture medium the SI from a leukemic spleen produces no effect on the growth of CML cells. A decrease in the total number of viable cells was observed 2 days after exposure of the cells to 0.5 mg/ml of the medium. The lethal effect was significantly greater, however, after exposure of the same number of cells to 1.0 and 2.5 mg/ml of the SI. At the highest dose used (5 mg/ml) the number of viable leukemic cells was greatly reduced at 24 h and all the leukemic cells were dead at 48 h. Normal spleens have twice as much cytotoxic activity when compared to the abnormal spleens on dry weight basis.

The effect of the SI on nucleic acid synthesis was investigated by pulse labeling 3×10^5 CML cells with 15 μCi of ^{3}H-thymidine (^{3}HT) or ^{3}H-uridine (^{3}HUr). The incorporation of labeled precursors was analyzed by autoradiographic techniques at various intervals after the addition of 2.5 mg/ml of the SI to the cultures. The incorporation of ^{3}HUr was impaired as early as 3 h and suppressed after 15 h, a time at which 50% of the cells were unlabeled and another 48% had less than 20 grains. The mitotic index fell to 2% (control 7%–8%). At the same time, 65% of nontreated control cells had more than 200 grains and only 4% were unlabeled. No change in DNA synthesis was seen before 24 h. At this time, marked decrease in the ^{3}HT incorporation was observed. The majority of the labeled cells had less than 100 grains, while all control labeled cells had 300 or more grains. The number of unlabeled cells was similar (57%) in treated and control cultures. By 72 h, the majority of the cells showed degenerative changes and many cytoplasmic masses without nuclei were observed.

Recently, the inhibitory action of protein-free spleen extracts on granulocytic cells in vitro have been ascribed to the presence of cytotoxic polypeptides in those preparations[5,6]. The chemical composition of those extracts, however, has not yet been explored.

Specific inhibitors of cell proliferation have been isolated from different normal mammalian tissues. These



Cytotoxicity of the SI, obtained from normal spleens and those from patients with chronic myelocytic leukemia (CML), primary hypersplenism (P.H.) and bovine spleens on CML cells containing the Philadelphia chromosome as a marker. The lines represent the number of viable cells. The dose of the SI used is given in mg/ml of the culture medium. 20 ml of the medium was used in each experiment.

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substances, called chalones[7–10], inhibit DNA synthesis without affecting RNA or protein synthesis. They are tissue-specific but not species-specific.

A non-specific inhibitor of cell growth identified as a methyl-glyoxal analog has also been reported[3,4].

The inhibitor of the RNA synthesis isolated at our laboratory has a MW lower than chalones and higher than ketoaldehydes. There are also marked differences in their mode of action. Methyl-glyoxal and other α-ketoaldehydes seem to interact with SH groups, inhibiting cell proliferation by interference with protein synthesis and, only moderately affect DNA and RNA synthesis[3,4]. All known chalones have been found to inhibit DNA synthesis, while the inhibitor described herein affects primarily RNA synthesis, thus initiating a series of events leading to the inhibition of DNA synthesis and cell death.

The presence of a negative feedback mechanism which is capable of regulating cell growth through inhibition of RNA synthesis has not been previously reported. The inhibition of RNA synthesis may, however, be of significance in the control of malignant growth since differences in RNA metabolism have been found between normal and cancer cells[11].

The results indicate that, indeed, a cytotoxic factor for CML cells is present in human spleens. In addition, a line of CML cells is made available for the first time[12].

Resumen. En este trabajo se describe la purificación de un inhibidor de la síntesis de ARN que afecta secundariamente la síntesis de ADN y actividad mitótica. El factor ha sido aislado de ocho bazos humanos, comprendiendo dos de enfermos con leucemia mielocítica crónica (LMC) dos con hiperesplenismo primario, y cuatro de personas sanas, en los cuales se efectuó la esplenectomía por ruptura traumática del bazo. El inhibidor esplénico posee fuerte citotoxicidad sobre células en cultivo provenientes de enfermos con LMC. Estas células tienen el cromosoma de Philadelphia. El inhibidor esplénico tiene un peso molecular de alrededor de 1,000 y es probablaemente un péptido o glicopéptido.

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Inhibition by Terephthalic Acid of Spontaneous Mammary Tumorigenesis in Mice

It is generally believed that spontaneous mammary tumors in mice are more resistant to chemotherapy than transplanted experimental tumors. None of several antitumor agents involving 5-FU and mitomycin-C, which are widely used for clinical purposes, showed any inhibitory effect on the autografts of spontaneous mammary tumors in Swiss mice[1]. Terephthalic acid (TPA) has been found in rats to prevent *p*-dimethylaminoazobenzene (DAB) metabolizing enzymes in the liver from the decrease of activities by DAB, and to result in suppression of protein-bound dye formation, although TPA did not increase the activities of these enzymes under the normal conditions[2,3]. TPA encouraged the growth of the fowl when it was fed on undernutritional food[4]. No toxicity of TPA was ascertained in mice[5]. These results strongly suggest an important role of TPA in maintenance of homeostasis of the body. The present experiment was carried out in order to investigate whether or not TPA

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Mammary tumor incidence in each group

Group	Total No. of mice examined	Mammary tumor incidence at each months of age (%)[a]												
		4	5	6	7	8	9	10	11	12	13	14	15	16
Control	116	1.7 (2) [113]	6.9 (8) [105]	20.7 (24) [86]	37.1 (43) [61]	51.7 (60) [44]	64.7 (75) [17]	72.4 (84) [17]	75.0 (87) [12]	77.6 (90) [9]	81.0 (94) [5]	81.0 (94) [5]	83.6 (97) [2]	85.3 (99) [0]
TPA	31	0 (0) [28]	0 (0) [28]	3.2 (1) [25]	9.7 (3) [23]	16.1 (5) [19]	32.3 (10) [14]	41.9 (13) [10]	45.2 (14) [9]	48.4 (15) [8]	51.6 (16) [6]	58.1 (18) [4]	61.3 (19) [3]	64.3 (20) [0]
Significance of difference between groups by χ^2-test		NS	NS	$P < 0.01$									$P < 0.05$	

[a] Mammary tumor incidence = (Cumulative number of mice with tumors / Total number of mice examined) × 100. All the mice had mammary tumors or died by the end of 16 months. ( ), Cumulative number of mice with tumors; [ ], Number of surviving mice without tumors.