scribed in these papers, the resulting 2 or 3 branches are about equal in diameter while the 'buds' observed in our material receive less than 5% of the mass of the mother fiber, resulting in a very unequal distribution of fiber material.
Preliminary results of investigations on the generation of new fibers in the red fiber complex indicate the occurrence of a similar budding process.

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Effect of the calmodulin inhibitor R24571 (calmidazolium) on rat embryos cultured in vitro

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Summary. The possible effects of inhibition of the calcium-binding protein, calmodulin, on mammalian morphogenesis have been investigated by culturing rat embryos in vitro from 9½ to 11½ days of development in the presence of R24571 (calmidazolium), a specific inhibitor of calmoldulin. Embryos cultured in 10^{-2} mM R24571 for 48 h show inhibited development and exhibit a range of morphogenetic abnormalities including assymetry and neural tube defects. Embryos exposed to R24571 for the first 24 h of a 48 h culture are more severely affected than embryos exposed to R24571 for the last 24 h.
Key words. Morphogenesis; mammalian embryo; calcium; calmodulin.

Morphogenesis, tissue shaping during embryogenesis, is accompanied by changes in the shapes of the individual cells of the tissue undergoing a change in form. In some examples of morphogenesis such as neurulation and gastrulation microfilaments appear at the time when cells are changing shape[2]. Since some morphogenetic cell movements are inhibited by cytochalasin-B[3], which prevents microfilament contraction, it is reasonable to suggest that morphogenesis is effected, at least in part, by co-ordinated changes in cell shape brought about by microfilament contraction. Indeed computer simulations have shown that co-ordinated changes in cell shape offer a sufficient explanation for the tissue movements seen in neurulation and other morphogenetic movements[4].
In non-embryonic systems the contraction of microfilaments is initiated by changes in the level of intracellular free calcium, and so calcium is implicated as being important to the control of morphogenesis. A number of observations support this idea. For example papaverine, which is thought to inhibit calcium fluxes, causes abnormal morphogenesis in amphibian and chick embryos, and its effect can be ameliorated or reversed by administration of the divalent cation ionophore A23187[5,6], which increases the permeability of biological membranes to calcium[7]. In a number on non-embryonic cell types the control of cellular functions by calcium is mediated via the calcium-binding protein calmodulin[8]. Thus the possibility is raised that calmodulin might play a part in morphogenesis. One of the ways in which the possible role of calmodulin has been investigated in cellular systems has been by the use of calmodulin inhibitors. Early experiments employed trifluoroperazine (TFP) but recently a more specific inhibitor of calmodulin, R24571 (calmidazolium), has become available[9]. Here we report the results of experiments to test the effects of R24571 on the morphogenesis of rodent embryos grown in vitro.
Materials and methods. Embryo culture. Rat embryos were obtained from random-bred Wistar rats at 9.5 days of gestation, timed from midnight preceding the morning on which vaginal plugs were observed. Embryos at the headfold stage were explanted in Hank's balanced saline containing 4 mM sodium bicarbonate (Flow Laboratories, Irvine, U.K.). Before culture and after 24 h of culture a small rent was made in the yolk sac and amnion to facilitate drug penetration. R24571 (Boehringer) was prepared as a stock solution at 14.5 mM in spectroscopic grade ethanol and used at a final concentration 10^{-2} mM. The membranes of control embryos were also opened and control cultures contained an equivalent concentration of ethanol. The culture medium was rat serum obtained from blood centrifuged immediately after withdrawal. The serum was inactivated immediately prior to use by heating at 56 °C for 30 min. The embryos were cultured for 48 h in rotating glass bottles[10]. Initially the bottles were equilibrated with 5% O_2; 5% CO_2; 90% N_2 gas mixture. After 24 h the cultures were re-equilibrated with a 20% O_2; 5% CO_2; 75% CO_2 gas mixture and after 43 h they were equilibrated with 40% O_2; 5% CO_2; 55% N_2. In the first experimental series embryos were exposed to R24571 for the whole of the 48 h culture period. In subsequent experiments embryos were exposed to

Table 1. Effect on the development of rat embryos cultured in vitro of the calmodulin inhibitor R24571

		Number of embryos	Apparent embryonic age after 48 h in vitro ± SEM	Yolk sac diameter (mm) ± SEM	Crown-rump length (mm) ± SEM	Protein content (μg) ± SEM
	Controls (+ ethanol)	49	11.47 ± 0.03	3.3 ± 0.08	3.0 ± 0.06	164 ± 9
10^{-2} mM R24571	24–48 h	25	11.18 ± 0.06	3.1 ± 0.08	2.8 ± 0.11	–
	0–24 h	26	10.90 ± 0.03	3.1 ± 0.08	2.0 ± 0.06	–
	0–48 h	54	10.90 ± 0.03	2.8 ± 0.08	2.1 ± 0.08	57 ± 6

R24571 for only part, the first 24 h or the last 24 h, of the 48 h culture period.

At the end of the culture period the yolk-sac diameters and crown-rump lengths of the embryos were measured and any abnormalities were noted. The embryos were scored according to the system of Brown and Fabro[11]. This system uses 17 morphological criteria to assess embryonic development and to ascribe an 'apparent embryonic age' to the embryo. After scoring some of the embryos were prepared for scanning electron microscopy, others were used for protein estimations.

Scanning electron microscopy. Embryos were fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3[12]. They were washed in changes of buffer and dehydrated in a graded ethanol series. The absolute ethanol was replaced with liquid CO_2 and the embryos were dried using the critical point method. The dried specimens were mounted on stubs and coated with a gold-palladium mixture. Finally the embryos were observed and photographed using a Phillips 501B scanning electron microscope (Pye Unicam, Cambridge, U.K.).

Protein determination. Unfixed embryos were dissected free from their membranes and the protein content of embryos was determined using the method of Lowry et al.[13].

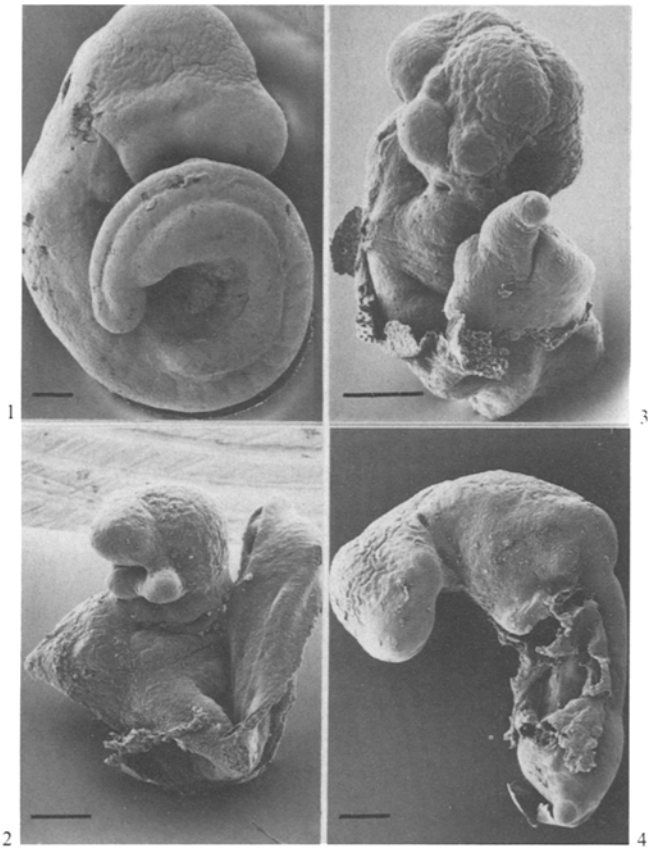

Figure 1. Scanning electron micrograph of rat embryo explanted at 9.5 days and cultured for 48 h in vitro in serum plus 0.07% ethanol. Embryo is typical of 11.5 days of development being fully rotated and showing well developed brain vesicles. Bar = 200 μm.

Figures 2–4. Scanning electron micrographs of rat embryos explanted at 9.5 days and cultured for 48 h in vitro in serum plus 10^{-2} mM R24571 (calmidazolium). Fig. 2. Embryo cultured for 48 h in presence of R24571 shows abnormal rotation and open posterior neuropore. The pericardium region is expanded, brain expansion is reduced and the branchial bars are asymmetrical. Fig. 3. Embryo cultured for the first 24 h in presence of R24571 has failed to rotate and the rhombencephalon is open. Fig. 4. Embryo cultured for first 24 h in presence of R24571 has failed to rotate and shows reduced brain expansion. Bars = 200 μm.

Results and discussion. Controls. Preliminary experiments were undertaken to determine whether ethanol at the concentration used, 0.07%, had an effect on embryonic development. Embryos cultured in serum alone showed normal development and when scored by morphological criteria showed an apparent embryonic age of 11.60 ± 0.04 (n = 14). Embryos from the same batches cultured in serum plus 0.07% ethanol showed no fetal abnormalities and had an apparent embryonic age of 11.49 ± 0.05 (n = 18). These values are not statistically significantly different. Similarly measurements of yolk sac diameters and crown-rump lengths of embryos grown in serum were not significantly different from those of embryos grown in serum plus 0.07% ethanol. In the subsequent experiments embryos cultured in R24571 were compared with controls cultured with ethanol.

Effects of R24571 from 0 to 48 h. The development of embryos exposed to 10^{-2} mM R24571 for the entire culture period was inhibited (table 1); embryos grown in R24571 had a significantly lower apparent embryonic age than controls ($p < 0.001$) and yolk sac expansion was reduced in the treated embryos ($p < 0.001$). Crown-rump lengths were also less in treated than control embryos ($p < 0.001$) although the abnormal rotation of some treated embryos (see below) meant that not all of this group could be scored. However, estimations of the protein content of embryos, normally taken as an index of embryonic growth, showed that the growth of embryos exposed to R24571 was significantly less than that of control embryos ($p < 0.001$).

In addition R24571 caused a range of developmental abnormalities. All of the embryos grown in R24571 showed some malformation; many showed multiple abnormalities. A main class of abnormality observed was embryonic assymetry, that is unequal development of paired structures. Various organs were affected in this way; somites, optic vesicles, fore-limbs, neural tube and brain (table 2). A second main class of abnormality was neural tube defects. Open neuropore, failure of fusion of the neural folds, microcephaly and abnormal brain morphology were observed (table 2). Many embryos showed abnormal rotation. In part this may have been due to the tearing of the embryonic membranes, since some control embryos also showed abnormal rotation, but the incidence of abnormal rotation was higher in treated than in control embryos (table 2). In addition the vascularization of the yold sac was inhibited in many of the treated embryos ('Retarded yolk sac', table 2).

Effects of R24571 from 0 to 24 h and from 24 to 48 h. Scoring

Table 2. Summary of types of abnormalities observed in embryos cultured in vitro with the calmodulin inhibitor R24571

	% of embryos showing abnormality			
	Control	10^{-2} mM R24571		
		24–48 h	0–24 h	0–48 h
Abnormality	(n = 49)	(n = 25)	(n = 26)	(n = 54)
Asymmetry abnormalities (total)	2	20	38	65
Neural tube	0	12	19	30
Telencephalon	0	4	11	20
Optic vesicles	0	4	0	7
Somites	2	12	11	13
Fore limbs	2	4	4	28
Neural tube abnormalities (total excluding asymmetry)	8	36	50	52
Unfused brain	2	24	4	19
Abnormal brain expansion	2	12	38	35
Microcephaly	0	0	0	4
Open neural folds	4	12	15	9
Failure to rotate	10	40	70	58
Retarded yolk sac	4	44	100	93

of embryos to determine apparent embryonic age showed that exposure to R24571 for the first 24 h inhibited development as severely as exposure for the whole of the 48 h culture. Development was also inhibited in embryos exposed to R24571 for the last 24 h of culture, but to a lesser extent than in embryos exposed for the entire 48 h culture (table 1). Measurement of crown-rump lengths gave similar results; growth of embryos exposed to R24571 for the first 24 h of culture was more inhibited than that of embryos exposed for the last 24 h. Comparison of the frequency and types of abnormalities suggested that embryos exposed to R24571 for the first 24 h of culture showed similar types of abnormalities to those exposed for the entire culture period. Embryos exposed to R24571 for the last 24 h of culture showed similar abnormalities but at a lower frequency (table 2).

Thus the present results show that R24571, which inhibits the activity of the calcium-binding protein calmodulin, inhibits the growth of rat embryos in vitro and causes a number of morphological defects, including defects of the nervous system. Recently it has been shown that interference with calcium ion fluxes in mammalian embryos can perturb neural tube formation[14], suggesting that, as in amphibian embryos, neurulation in mammalian embryos is brought about by cell shape changes affected by calcium-activated microfilaments. the demonstration that an inhibitor of calmodulin activity causes abnormal neurulation and perturbs other morphogenetic movements suggests that calmodulin may play a part in the control of calcium levels which are important to microfilament-mediated changes in cell shape during morphogenesis.

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FSH receptor binding inhibiting activity associated with low molecular weight inhibin from sheep, human, rat and chicken

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Summary. Low molecular weight inhibin (1500 daltons) was obtained from sheep, human, rat and chicken testes by sequential chromatography on Sephadex G-100 and G-25. In addition to its ability to suppress circulating FSH levels in adult castrated male rats, it also exhibits binding inhibition of I^{125}hFSH to rat testicular receptors.
Key words. Testes; inhibin; FSH receptor; FSH binding inhibiting activity.

Inhibin appears to regulate circulating levels of FSH either by its direct modulatory action on pituitary responsiveness to hypothalamic GnRH[1] or via the hypothalamus where it may have a regulatory role in GnRH output[2]. Available evidence suggests that in addition to its regulatory action on circulating FSH, inhibin has inhibitory effect on the action of FSH at the gonadal level, in that it inhibits binding of FSH to gonadal receptors in vitro[1,3-5]. In our earlier studies we demonstrated that low molecular weight peptides with inhibin activity obtained from sheep ovaries and testes exhibit FSH-binding inhibiting (FSH-BI) activity. The present study was designed to examine whether the FSH-BI like property is common to inhibin preparations obtained from other species.

Materials and methods. Inhibin preparations were isolated by fractionating the high-speed supernatant derived from 40% homogenates of human, ovine, rat and chicken testicular tissues, sequentially on Sephadex G-100 and G-25 columns as described earlier[6]. The human, ovine, rat and chicken inhibin preparations used in the present study were equivalent to Hm-3-II, Rm-3-II, Rt-3-II and Ch-3-II respectively[6] and are peptides of about 1500 daltons molecular weight. Inhibin activity of these preparations was assessed by their ability to suppress circulating levels of FSH in castrated adult male rats. Adult male rats of the Holtzman strain, bilaterally castrated 2 weeks before the assay, were injected i.m. once daily with 1.0 ml of saline or the inhibin preparations, for 3 days. 4 h after the last injection, the animals were bled under light ether anesthesia and their sera collected. The levels of FSH and LH in the sera were estimated by RIA using NIAMDD systems and expressed in terms of NIAMDD-Rat-FSH-RP-1 and NIAMDD-Rat-LH-RP-1 respectively. Serum FSH and LH levels in the groups of animals treated with the test material were compared with those in the saline-treated group. Significant suppression of FSH levels were taken as an index of inhibin activity.

The FSH-BI-like activity of these preparations was tested using a system containing ^{125}I-hFSH and rat testicular receptors as described by Reichert and Abou-Issa[7]. The receptor preparation was prepared from testes of mature (90-day-old) rats of the Holtzman strain and was equivalent to fraction R-1 of Reichert and Abou-Issa[7]. Human FSH (LER-1575C) was iodinated to a specific activity of 10–12 μCi/μg using chloramine T by the method described by Reichert and Bhalla[8]. The assay was carried out in 10 × 100 mm glass centrifuge tubes to which was added 500 μl of receptor preparation followed by an appropriate amount of inhibin dissolved in 400 μl of 0.05 M Tris-HCl buffer, pH 7.5 (containing 0.1% BSA, 0.1 M sucrose and 5 mM MgC12) and 5 ng ^{125}I-hFSH in 100 μl. The tubes were incubated in a metabolic shaker at 37°C for 3 h. The incubated tubes were centrifuged at 1500 × g for 15 min and the supernatant discarded. The tissue pellets were resuspended in 1 ml of chilled Tris-HCl buffer and subjected to a 2nd centrifugation. The supernatants were decanted, the tubes were drained and the radioactivity bound to the pellets counted in a gamma ray spectrometer. All statistical evaluations were performed using Student's t-test. The relative potencies of different inhibin preparations were calculated by the method of Borth[9].