contrast, there was no evidence for the secretion of 20-deoxy-makisterone A by brain-ring glands from larvae grown on a diet with cholesterol (fig. 2) or ergosterol[8]. This is clear evidence that the secretion of 20-deoxy-makisterone A results from the presence of campesterol in the diet.

These results are compared in the table in which RIA activity in the HPLC fractions corresponding to 20-deoxy-makisterone A is expressed as a percentage of the fractions comprising the ecdysone peak. Data from previous analyses[8], using brain-ring glands from larvae reared on cornmeal-yeast medium (HPLC fractionation at 1- or 0.5-min intervals) and a defined diet with a mixture of C28 and C29 sterols, including approximately 0.006% w/v campesterol (0.5-min HPLC fractions), are included for comparison. From this it is apparent that a 5-fold increase in campesterol concentration (0.006% w/v to 0.03% w/v) resulted in a comparable increase in the proportion, relative to ecdysone, of 20-deoxy-makisterone A secreted. However, the relative levels of 20-deoxy-makisterone A secreted were lower than in experiments using brain-ring glands from larvae reared on cornmeal-yeast medium.

The original identification of 20-deoxy-makisterone A in *Drosophila* was based on four criteria[8]: a) indications for side-chain modification to an ecdysteroid nucleus from experiments with different ecdysteroid antisera; b) an HPLC retention time corresponding to the major ecdysteroid secreted by *Dysdercus fasciatus* prothoracic glands; c) conversion to a compound with the characteristics of makisterone A by *Drosophila* fat body in vitro; d) the presence of makisterone A in third-instar *Drosophila* larvae. Furthermore, this ecdysteroid had similar properties on gas-chromatography/mass spectrometry to a minor ecdysteroid isolated from *Dysdercus fasciatus* and assumed to be 20-deoxy-makisterone A[5]. That the secretion of this ecdysteroid results from the presence of campesterol in the diet is additional corroboration. On this evidence, campesterol is a substrate for ecdysteroid biosynthesis and, clearly, the C24-methyl group may be retained, resulting in the synthesis and secretion of the 24-methyl analogue of ecdysone. The data suggest that ergosterol, a $\Delta 7$, $\Delta 22$, C24-methyl sterol, cannot act as a substrate for ecdysteroid biosynthesis without prior dealkylation.

Two components of the defined diet used for these experiments were present in relatively large amounts: casein (5.5% w/v) and agar (1.5% w/v). Although the casein used was extracted with petroleum ether by the manufacturer and supplied as 'fat and vitamin free', sterol estimation after saponification showed that this component of the diet contained up to 184 µg sterol per g casein. The agar used contained 48 µg per g. These two components thus contributed 0.0011% w/v sterol to the diet and on this basis the added sterol (0.03% w/v) represented 96–97% of the total. Interpretation of these experiments is therefore limited by the possible contribution of contaminating sterol, particularly from the casein component of the diet (presumably cholesterol), which may be preferentially utilized for ecdysteroid production. Thus, the ecdysone secreted by brain-ring glands from larvae reared on campesterol diet may result either from dealkylation of campesterol or (and these are not mutually exclusive) from some preferential utilization of contaminating sterol.

Two additional facts require comment. Firstly, the chemical characterization of 20-deoxy-makisterone A has not, to my knowledge, been reported and since an authentic standard of this compound was not available, it was not possible to quantify accurately the amount (in terms of moles or pg steroid) secreted. Secondly, on the basis of RIA measurements, the high proportion of 20-deoxymakisterone A secreted by brain-ring glands of larvae reared on cornmeal-yeast diet requires explanation. Assuming campesterol forms approximately 20% of the phytosterol in corn[14] and that cornmeal contains 0.27% w/w free phytosterol and 0.67% w/w saponifiable phytosterol[15], the campesterol and campesteryl ester concentration in cornmeal-yeast diet (11.8 g cornmeal per 100 ml) would be of the order of 0.02% w/v. Thus overall, the campesterol concentration in the cornmeal-yeast diet is likely to be lower than used for the campesterol diet. However, it is possible that local variation in diet constitution and/or selective feeding (on cornmeal solids, for example) by larvae could result in a higher campesterol concentration in ingested food than in the substrate as a whole.

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1 Rees, H.H., Biosynthesis of Isoprenoid Compounds, vol. 2, p. 465. Eds J.W. Porter and S.L. Spurgeon. Wiley, New York 1983.
2 Gilbert, L.I., and Goodman, W., Metamorphosis: a Problem in Developmental Biology. Eds L.I. Gilbert and E. Frieden. Plenum Press, New York 1981.
3 Kelly, T.J., Woods, C.W., Redfern, R.E., and Borkovec, A.B., J. exp. Zool. *218* (1981) 127.
4 Aldrich, J.R., Kelly, T.J., and Woods, C.W., J. Insect Physiol. *28* (1982) 857.
5 Gibson, J.M., Majumder, M.S.I., Mendis, A.H.W., and Rees, H.H., Archs Insect Biochem. Physiol. *1* (1983) 105.
6 Bollenbacher, W.E., Goodman, W., Vedekis, W.V., and Gilbert, L.I., Steroids *27* (1976) 309.
7 Borst, D.W., Bollenbacher, W.E., O'Connor, J.D., King, D.S., and Fristrom, J.W., Devl Biol. *39* (1974) 308.
8 Redfern, C.P.F., Proc. natn. Acad. Sci. USA *81* (1984) 5643.
9 Ashburner, M., and Thompson, J.N., The Genetics and Biology of *Drosophila*, vol. 2a, p. 2. Eds M. Ashburner and T.R.F. Wright. Academic Press, London 1978.
10 Sang, J.H., The Genetics and Biology of *Drosophila*, vol. 2a, p. 159. Eds M. Ashburner and T.R.F. Wright. Academic Press, London 1978.
11 Redfern, C.P.F., J. Insect Physiol. *29* (1983) 65.
12 Reum, L., and Koolman, J., Insect Biochem. *9* (1979) 135.
13 Dittmer, J.C., and Wells, M.A., Meth. Enzym. *14* (1969) 482.
14 Kemp, R.J., Goad, L.J., and Mercer, E.I., Phytochemistry *6* (1967) 1609.
15 Anderson, R.J., J. Am. chem. Soc. *46* (1924) 1450.

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Effects of isaxonine phosphate and analogs on fibroblast metabolism in culture

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Summary. Human skin fibroblasts in confluent cultures were incubated for 24 h in the presence of isaxonine phosphate (Nerfactor) and several related factors. The incorporation of ^{14}C-proline into secreted proteins and the release of collagen into the medium were inhibited. When the cells were incubated for an additional period of 24 h after thorough washing, protein and collagen syntheses were

found to be identical to those of controls, demonstrating that the inhibition of protein synthesis was independent of any toxic effect. When cells were incubated in the presence of both isaxonine and colchicine, the secretion of collagen was more inhibited than by colchicine alone, and proteins accumulated in the cells.
Key words. Fibroblasts; cell culture; protein synthesis; isaxonine; collagen; colchicine.

Isaxonine phosphate, a compound obtained by organic synthesis, is the phosphoric acid salt of N-isopropyl-2-amino-pyrimidine. It has been proposed as a drug for accelerating nerve regeneration and protecting nerves from some neurotoxic injuries[1]. Isaxonine was shown to improve the outgrowth of nerve cells in culture[2]. The biochemical mechanism suggested to explain this effect was an improvement of microtubule formation in nerve cells. As shown by an electron spin resonance study, isaxonine also intercalates between the polar heads of phospholipids in the membrane bilayer, thereby leading to its rigidification[3]; an opposite effect has been shown with vinblastine, a neurotoxic antimitotic drug[3].

Considering the fact that collagen biosynthesis by fibroblasts comprises a step of intracellular migration of the precursor procollagen molecules necessitating the integrity of microtubules that serve as a guide in this migration, and also a further step of membrane extrusion, it was of interest to use the model system of fibroblasts for the study of the effect of isaxonine on protein and collagen syntheses. In addition, we studied the action of isaxonine on DNA synthesis and the combined effects of isaxonine and colchicine, since the latter drug was described as preventing the intracellular transport of collagen[4] and since isaxonine seemed to protect nerve cell microtubules from the action of colchicine[1,2].

Materials and methods. Reagents. Isaxonine phosphate (Nerfactor®), its metabolites, BN 1163 (4-hydroxy-2-isopropylamino-pyrimidine), BN 1314 (5-hydroxy-2-isopropylamino-pyrimidine), and some derivatives, BN 1041 (N_1-oxyde-2-isopropyl-amino-pyrimidine) and 2-aminopyrimidine were generously supplied by IHB Research Laboratories (Paris). All the chemicals, when not specially described, were of reagent grade and were purchased from Prolabo, Paris. Methotrexate (amethopterine) and N-ethylmaleimide were obtained from Sigma. Radioactive precursors were provided by CEN, Saclay; ^{3}H-methyl thymidine ref. TMM 79 B (sp. act. 5 Ci/mmole); uniformly labeled ^{14}C-proline ref. CB 18 (sp. act. 175 mCi/mmole). Medium for cell cultures was bought from Gibco and fetal calf serum was rehatuin, purchased from IBF.

Cell cultures. Skin fibroblasts were explanted from foreskin biopsies and propagated as previously described[5]. For experiments with labeled precursors, cultures were used between 2 and 10 passages.

^{3}H-Methyl thymidine incorporation. The cells were obtained by trypsinization and seeded at a constant number of 10^4 per well in Linbro plates. 24 h later, after the cells had become attached, the medium was replaced by 0.25 ml of Eagle's minimal essential medium with Earle's salts (MEM) and 0.5% fetal calf serum containing 0.25 μCi of ^{3}H-methyl-thymidine and an appropriate concentration of isaxonine. In a 2nd series of experiments, isaxonine was added 24 h prior to ^{3}H-methyl thymidine, the final concentrations of reagents in the wells remaining the same. After 24 h of incubation, the amount of radioactivity in DNA was evaluated as described in a previous paper[5]. In some experiments, methotrexate was added at the concentration of 10 μM.

^{14}C-Proline incorporation. The cells were seeded in 25-cm^2 flasks and grown to confluence. The medium was replaced by MEM containing 0.5% fetal calf serum, 50 μg ml^{-1} ascorbic acid, 50 μg ml^{-1} β-aminopropionitrile, 0.2 μCi ml^{-1} of ^{14}C-proline and a convenient amount of isaxonine, to a final volume of 5 ml per flask. In some experiments, isaxonine was added simultaneously with ^{14}C-proline and incubation was carried out for 24 h. In other experiments, confluent cells were incubated with isaxonine for 24 h prior to the addition of ^{14}C-proline and incubation for an additional 24 h period.

The medium was harvested, dialyzed, and aliquots of the non-dialyzable fraction counted. The remaining portions were precipitated by 176 mg ml^{-1} of $(NH_4)_2SO_4$. The precipitate was digested by bacterial collagenase, and the solubilized radioactivity was counted as collagen. These operations have been described in another report[6].

The cell layer, after 3 phosphate-buffered saline rinses, was harvested by trypsinization, centrifuged, and the pellet was submitted to sonication (3 pulses for 10 sec each at 10,000 Hz, generated by a MSE 150 W ultrasonic disintegrator MK2). The homogenate was precipitated by adding TCA to a final concentration 5% (w/v), left to stand at 4 °C for 2 h, and then centrifuged. The precipitate was suspended in 0.05 M tris-HCl buffer pH 7.2 containing 4 mM $CaCl_2$ and 1.25 mM N-ethylmaleimide. An aliquot was counted for determination of total proteins and the remaining solution was digested by collagenase. The supernatant after 5% (w/v) TCA precipitation was counted as the collagen fraction. All samples were counted in a Tricarb 3003 scintillation counter. Statistical comparisons were made using Student's t-test.

Experiments designed in order to exclude toxic effects. In some experiments, at the end of the incubation period of 24 h in the presence of 10^{-5} M Nerfactor, the cells were thoroughly washed with MEM and incubated for an additional period of 24 h in the presence of ^{14}C-pro in the same conditions as for the first period except that Nerfactor was not added. Control cultures were treated the same way. Labeled collagen and non-collagen pro-

Table 1. Incorporation of labeled proline into medium and cell proteins in the presence of 0.5×10^{-4} M colchicine. Nerfactor was added to the confluent cells at time 0, 0.5×10^{-4} M colchicine was added at time 24 h simultaneously with ^{14}C-proline and the incubation was stopped at time 48 h. X = M ± 1 SD (n = 4)

Concentration of isaxonine (M)	Incorporation of labeled proline into proteins of the medium			Incorporation of labeled proline into proteins of the cell layer		
	Total proteins	Noncollagen proteins	Collagen	Total proteins	Noncollagen proteins	Collagen
0 (control without colchicine)	21,208 ± 1179	13,998 ± 1029	7210 ± 739	27,689 ± 6176	23,647 ± 5534	4042 ± 719
0 (control with colchicine)	20,085 ± 1450	14,945 ± 1101	5141 ± 932*	38,055 ± 4207	31,597 ± 6920	4856 ± 300
10^{-9}	20,455 ± 1253	14,508 ± 1218	5856 ± 259*	41,760 ± 2526***	35,711 ± 2436***	6050 ± 113***[a]
10^{-8}	20,400 ± 657	14,780 ± 465	5570 ± 216*	40,276 ± 2576***	34,534 ± 2593***	5743 ± 208***[a]
10^{-7}	20,260 ± 813	14,092 ± 1285	6169 ± 1395	41,630 ± 5193***	36,396 ± 4564	5234 ± 683
10^{-6}	18,023 ± 1859	14,126 ± 1448	3828 ± 582**	31,045 ± 7609	26,815 ± 6464	4231 ± 1156
10^{-5}	16,578 ± 2672	13,183 ± 2606	3397 ± 344**[a]	33,276 ± 3363	28,900 ± 2983	4376 ± 504

*Decrease significant at $p < 0.05$; **decrease significant at $p < 0.001$; ***increase significant at $p < 0.05$; [a]difference from the control with 0.5×10^{-4} M colchicine significant at $p < 0.05$.

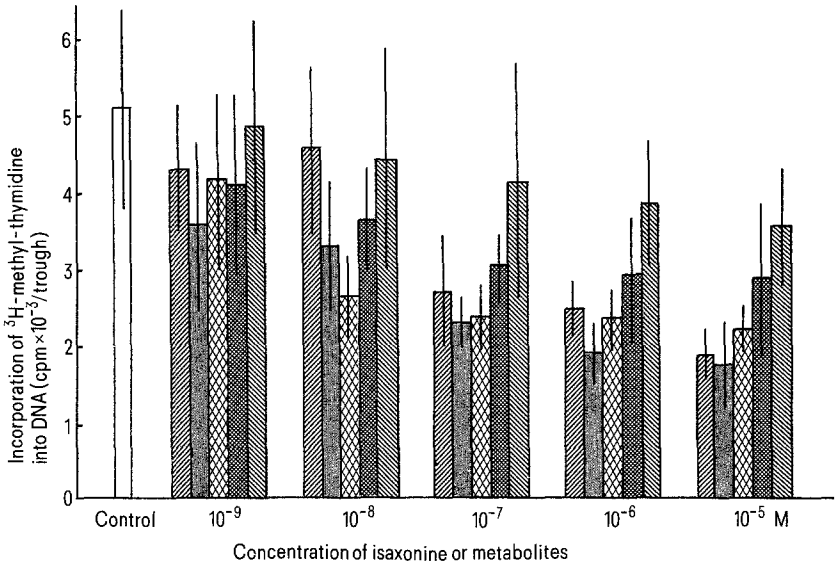

Figure 1. Dose-dependent inhibition by isaxonine and metabolites of ^{3}H-methyl thymidine incorporation into DNA. ▨, Isaxonine phosphate; ▩, 2-amino-pyrimidine; ▩, BN 1314; ▩, BN 1163; ▩, BN 1041. Incorporation of ^{3}H-methyl-thymidine into DNA by non-confluent fibroblasts. Isaxonine or metabolites and methotrexate (10 μM) added at time 0, pulse labeling with 0.25 μCi of ^{3}H-methyl-thymidine started at time 24 h and harvesting the medium at time 48 h. Results expressed as means of 12 determinations. (X = M ± 1 SD)

teins were counted in the supernatant and the number of cells evaluated.

Results. 1) Effects on DNA synthesis. The effects of isaxonine on DNA synthesis were tested in non-confluent cells. When the cells had been in contact with isaxonine for 24 h only, no difference from controls was found. When isaxonine or its structural derivatives had been added to the cultures 24 h prior to the ^{3}H-methyl-thymidine pulse after 48 h of contact with cells, a significant inhibition of the isotope incorporation was obtained (fig. 1). The effect was particularly impressive when the cells were treated with isaxonine at concentrations higher than 10^{-7} M in the presence of methotrexate. Regarding the metabolites of isaxonine phosphate, BN 1041 did not significantly decrease the incorporation of ^{3}H-methyl-thymidine.

2-aminopyrimidine and BN 1314 were more effective than isaxonine and BN 1163 was slightly less effective than isaxonine. Cell numbers did not differ significantly from those of the controls.

Table 2. Results of an experiment showing that, after withdrawal of Nerfactor, cells fully recover their ability to synthesize proteins. The number of cells found in the plates treated with Nerfactor was not significantly different from that of controls (1,151,700 ± 112,168 versus 1,128,600 ± 111,029)

Total proteins		Collagen	
Control	Nerfactor-treated	Control	Nerfactor-treated
1st incubation period			
41,233	31,955	17,575	12,038
42,227	22,182	16,908	7,770
36,995	31,545	15,660	10,532
38,195	31,259	16,824	10,890
Mean			
39,663	29,135	16,742	10,308
Control	Nerfactor withdrawn	Control	Nerfactor withdrawn
2nd incubation period			
26,898	25,040	6,460	6,326
24,924	30,386	5,694	8,634
25,515	24,866	5,812	5,954
25,343	25,773	5,882	5,966
25,670	26,516	5,962	6,720

2) Incorporation of labeled proline into proteins of the extracellular medium. There was no effect when the contact of isaxonine with cells lasted for 24 h only (during the period of pulse labeling). On the other hand, if a preincubation lasting 24 h had been performed prior to adding ^{14}C-proline to the medium, a significant decrease in the incorporation of the isotope into collagen was found for the highest concentrations of isaxonine tested (fig. 2).

3) Incorporation of labeled proline into intracellular proteins. The radioactivity of intracellular proteins was found to be iden-

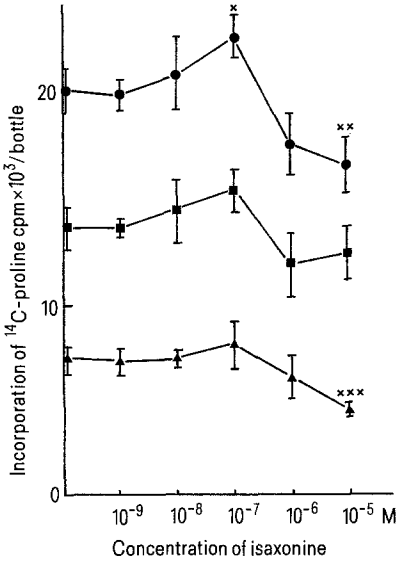

Figure 2. Effects of isaxonine alone on the secretion of proteins into the culture medium. ●—●, Total proteins; ■—■, non collagen proteins; ▲—▲, collagen. Incorporation of ^{14}C-proline into the proteins secreted by confluent fibroblasts into the medium. Isaxonine added at time 0, pulse labeling started at time 24 h and harvesting the medium at time 48 h. X = M ± 1 SD (n = 4). x, significant increase for $p < 0.05$; xx, significant decrease for $p < 0.05$; xxx, significant decrease for $p < 0.01$.

tical to that of the controls after 48 h of incubation with either isaxonine phosphate or its derivatives (data not shown). It was checked in a separate experiment that the trypsin treatment of the cells, under the conditions used, did not alter the amount of radioactivity found in the cell layer.

4) Effects of simultaneous incubation with colchicine (0.5×10^{-4} M) and increasing concentrations of isaxonine. When colchicine was added alone to the cultures, it did not significantly alter the secretion of non-collagen proteins, but it decreased the secretion of collagen by about 30% and slightly increased the intracellular collagen. The simultaneous addition of 0.5×10^{-4} M colchicine and amounts of isaxonine increasing 10^{-9} M to 10^{-5} M demonstrated a synergic effect of the two drugs (table 1).

5) Results of experiment designed for ruling out toxic effects. Table 2 shows the data obtained by incubating the fibroblasts with ^{14}C-pro firstly for 24 h in the presence of to 10^{-5} M Nerfactor and then, for an additional 24-h period, in the absence of Nerfactor. In the 2nd period, the incorporation of labeled pro into collagen and noncollagen proteins matches that of the controls.

Discussion. We used fibroblastic cells cultured from newborn child skin biopsies to study the possible effects of isaxonine phosphate (Nerfactor) and several related compounds, 2-aminopyrimidine, BN 1314, BN 1163 and BN 1041 on DNA and protein synthesis. When these compounds were incubated with the cells in the logarithmic phase of growth for 24 h, no effect was detected. On the other hand, when the total incubation period was increased to 48 h, incorporation of ^{3}H-methyl thymidine into DNA was reduced whereas the cell number was not altered. This effect was dose-dependent, increasing from concentrations of about 10^{-7} M. Addition of methotrexate (which inhibited endogenous thymidine synthesis) markedly sensitized the inhibition, demonstrating that the mechanism did not involve the stage of nucleotide synthesis. Among the compounds structurally related to isaxonine phosphate 2-aminopyrimidine, BN 1163 and BN 1314 also significantly inhibited ^{3}H-methylthymidine incorporation into DNA after 48 h incubation. These results emphasize the properties of some derivatives of isaxonine as inhibitors of DNA synthesis or repair.

Isaxonine, when incubated for 48 h with fibroblasts at concentrations over 10^{-6} M, induced a decrease in the incorporation of ^{14}C-proline into proteins and collagen secreted into the medium. The question arises whether this effect is of specific or toxic origin. We performed experiments involving double incubation of the same cultures with 10^{-5} M Nerfactor and after removal of Nerfactor. During the second period of incubation, the incorporation of ^{14}C-pro was comparable in both treated cultures and controls, ruling out a toxic effect.

The effect of colchicine in conjunction with isaxonine was studied. Colchicine was shown several years ago, by Ehrlich and Bornstein[4], to interfere with the transcellular movement of procollagen by disrupting microtubules. This effect is attested by a decrease in the secretion of collagen. On the other hand, isaxonine was shown to protect cellular microtubules from the disrupting effect of vincristine in nervous cells. Therefore the aim of this study was to examine whether isaxonine has a similar effect on non-nerve cells. In our experiments we observed that colchicine alone, at the concentration used by Ehrlich in the case of newborn rat cranial bones, has a very slight effect on fibroblasts: a significant decrease in collagen secretion was noticed, but did not amount to more than 30% in our experiments. In contrast it was about 50% in the experiment with cranial bones, with a clear increase in the amount of procollagen retained inside the cells[4].

We could not find in the literature any papers showing data for the effect of colchicine on collagen synthesis by skin fibroblasts in culture. Our results suggest that the effect is far less intense than in calvaria.

Surprisingly, very low concentrations of isaxonine enhanced the inhibiting effect of colchicine on collagen secretion. This effect may be explained by the presence of different target sites for colchicine in fibroblasts and in growing nerve fibers. The protective effect of isaxonine on nerve fibers has been shown in numerous cases, in cultures as well as with in vivo animal models and in man[1]. But if colchicine in addition to isaxonine decreases the collagen release, we observed concomitantly increases in the amount of ^{14}C-proline incorporated into the cellular proteins. It remains to be determined whether the synthesis of some structural proteins is specifically triggered.

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1 Cambier, J., ed., Neuropathies Periphériques et Isaxonine, vol. 11, p. 1189. Nouvelle Presse Médicale, Paris 1982.
2 Tarrade, T., Vigier, P., and Hugelin, A., Neuro. Sci. Lett., suppl. *3* (1979) 334.
3 Berleur, F., Roman, V., Jaskierowicz, D., Leterrier, F., Esanu, A., Braquet, P., Ter-Minassian-Saraga, L., and Madelmont, G., Biochem. Pharmac. *33* (1984) 2407.
4 Ehrlich, H. P., and Bornstein, P., Nature, New Biol. *238* (1972) 257.
5 Maquart, F. X., Szymanowicz, A., Cam, Y., Cornillet-Stoupy, J., Randoux, A., and Borel, J. P., Biochem. biophys. Res. Commun. *96* (1980) 595.
6 Maquart, F.X., Szymanowicz, A., Cam, Y., Randoux, A., and Borel, J. P., Biochimie *62* (1980) 93.

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Morphogenesis of human colon cancer cells with fetal rat mesenchymes in organ culture[1]

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Summary. The morphogenesis and cytodifferentiation of human colon cancer cells (LS174T and HT29) were examined by combining cancer cells with fetal rat digestive-tract mesenchyme in organ culture. LS174T cells migrated into the mesenchyme to form glandular structures composed of single columnar cells with their nuclei oriented basally, while HT29 cells formed cell masses with little lumen formation. Immunohistochemical studies with antibodies against carcinoembryonic antigen and secretory components showed that the composition of cell surface glycoproteins was not necessarily reversed to the normal type, even when neoplastic cells exhibited normal glandular structures.

Key words. Human colon cancer cells; morphogenesis; carcinoembryonic antigen; secretory components; organ culture.