Adult specimens of *Procambarus* were anesthetized by cooling. Abdomens were perfused with 4% paraformaldehyde-4% glutaraldehyde in phosphate buffer (pH = 7.3, 4°C). Abdominal ganglia were removed, immersed in fixative (overnight), post-fixed in OsO_4 (2%, 2 h), dehydrated through an ethanol series, stained 'en bloc' with uranyl acetate and finally embedded in araldite. Thin sections of ventral areas of ganglia were stained on grids with lead citrate.

Transmission electron microscopy of neuron perikarya reveals the presence of modified double-walled endocytotic vesicles (300–700 nm). They are formed by apposed neuronal and glial membranes which enclose small extrusions of the glial cytoplasm (fig. 1). The external membrane is of neuronal origin, whilst the internal one is glial. These two membranes are separated by a narrow cleft of 10 nm as in a normal neuron-glia interface[11,12]. These membranes do not show in general gap-like junctions, but can bear neuronal subsurface cisternae. In some cases, a thin lamina of dense neuronal cytoplasm underlies endocytotic figures. Free double-walled vesicles are frequently observed in neuron cell bodies (figs 2 and 3). Their content shows the same density as glial cytoplasm and is lighter than neuron mitochondrial matrix or neuronal cytoplasm. These vesicles are interpreted as modified endocytotic vesicles moving to internal cytoplasm. Both endocytotic vesicles and internalized double-walled vesicles are found in most of the neuron cell bodies, irrespective of their size or situation in the ganglia. They are not found in glia/axon pairs or in neuropile.

The presence of gap-like junctions[11], and the observed modified endocytosis suggest that the neuronal microenvironment concept is no longer applicable to the intercellular space between perikarya of abdominal neurons of crayfish and associated peri-neuronal glia cells. Gap-like junctions could allow the glia-to-neuron transfer of small proteins up to 1000–1500 dalton[13]. Larger free cytoplasmic macromolecules could be transferred through endocytosis of glial cytoplasmic extrusions. In this case, some coupled mechanism to free the content of the double walled vesicles and to transport it to destination is probably needed[14].

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SV40 transformed fibroblasts recognize the same 140 kD fibronectin chemotactic fragment as non-transformed cells

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Summary. SV40 virus-transformed human embryonal fibroblasts show an enhanced chemotactic response to the glycoprotein fibronectin. However, they recognize the same chemotactic active region as non-transformed fibroblasts. The result suggests that an enhancement of chemotaxis by fibroblasts which have been transformed with Simian Virus 40 is due not to the utilization of further chemotactic domains in the molecule, but to an increased sensitivity of the cells to the chemoattractant.
Key words. Fibronectin; domains; chemotaxis; SV40; fibroblasts.

The high molecular weight glycoprotein fibronectin (FN) is composed of apparently independent domains, which exert different biological activities, among them binding to different biological substances, such as bacterial walls, heparin, gelatin, fibrin, actin and proteoglycans[2]. FN promotes cell attachment to collagen as well as cell spreading on tissue culture substratum. Large FN fragments with cell binding activity have been demonstrated to be chemotactic for fibroblasts[3,4]. Further work has shown that FN peptides which do not contain the cell binding site are also unable to attract fibroblasts[5].

Virus-transformed and tumor-derived cells have been shown to have an enhanced chemotaxis to FN if compared with normal fibroblasts[6,7]. These cells have also an increased random migration in the presence of FN, if compared with control fibroblasts. However the chemokinetic component accounts for only 10% of the migration. An increased chemotaxis by tumor cells is thought to be involved in tumor invasion and metastasis[8,9]. Enhancement of chemotaxis by tumoral cells has been reported in response to other chemoattractants, like collagen[10] or complement derived factors[11]. The major source of chemotactic activity for fibroblasts, leukocytic cells and tumor cells, elicited by the complement, derives from the C5 component[12,13]. However, different fragments might be responsible for the attraction of the different cell types.

In the present study we demonstrate that two different lines of SV40 virus-transformed human embryonal fibroblasts recognize the same FN fragment as non-transformed fibroblasts, although their chemotactic response to the protein is enhanced.

Material and methods. Human embryonal fibroblasts (HEF), derived from a 16-week-old fetus after legal abortion, SV40 virus-transformed human embryonic fibroblasts SV40/WI 38 (American Type Culture Collection, strain ATCC CCL 75.1) and SV40/WI26 (strain ATCC CCL 95.1) were a present from Dr P.K. Müller, Max-Planck-Institut für Biochemie (FRG).

Cells were grown in Dulbecco's MEM supplemented with glutamine, antibiotics and ascorbic acid as previously described[14]. Purified plasma FN[15] was used for the chemotactic assays and for the production of specific peptides. Briefly, the large cathepsin D fragments Ca70, Ca140 and Ca75-S-S-65 were prepared by mild digestion of human FN[16]. Fragments Ca95 and Ca23 were

prepared from a heparin non-binding fraction from a more advanced digestion with cathepsin D and separated by chromatography on Ultrogel AcA 44, as previously outlined[17]. For location of the peptides see figure.

For the chemotaxis assays polycarbonate filters (8 µm pore size, Nuclepore, Concorezzo, Milan, Italy) were coated with gelatin as described[18]. The cells were trypsinized (2 min, 0.025% trypsin, 0.1% EDTA in PBS), suspended in serum-containing culture medium to inactivate the enzyme, centrifuged, washed with medium without serum and resuspended in this medium. 2.5–3 × 10^5 cells/ml[19] were placed in the upper compartment of Boyden chambers (0.8 ml), while the lower compartment (0.2 ml) contained the molecules to be tested for their chemotactic activity, in serum-free culture medium (c = 200 nM). Medium conditioned by human embryonal fibroblasts, which contains cellular fibronectin and other chemotactic factors[14], was used as a positive control; serum-free culture medium was the negative control.

The migration was run for 5 h[19] at 37 °C in 5% CO_2 and 95% air. Non-migrated cells attached to the upper side of the filter were removed mechanically. Migrated cells on the lower side of the filter were fixed by ethanol, stained with toluidine blue and counted at 16 × 10-fold magnification. Each sample was assayed in triplicate and cells were counted in 3 × 5 unit fields. A unit field represents 1/160 of the total surface of the migrated cells. The standard deviations refer to the average values for the three filters.

Results and discussion. The chemotactic response to FN and FN peptides, of control fibroblasts and of two SV40 transformed cell lines is shown in the table. In the same table the response to fibroblast conditioned medium (FCM) and to serum-free culture medium are also illustrated. The chemotaxis of the two transformed cell lines was 50–60% higher compared to that of control fibroblasts, in response to both conditioned medium and to whole FN. The response to FCM was, for all the investigated cell lines, to some extent higher than the one to FN, as already reported for embryonal fibroblasts[14]. The activity exerted by FN was duplicated by the cathepsin D Ca140 fragment, which contains the cell binding site of FN[20]. The gelatin binding Ca70 and the heparin binding Ca75-SS-65 fragments, which together with the Ca140 represent the whole FN molecule (see fig.), did not elicit a significant stimulation. The Ca95 fragment, obtained by a more advanced cathepsin D digestion, which is unable to promote cell adhesion[20], is also inactive in the chemotactic assay, whereas a small but significant activity (10–20%) was exerted for all the investigated cell lines by the Ca23 peptide, which represents the C-terminal end of the Ca140 fragment. Ca23 reacts (Albini et al., unpublished) with antibodies directed to the cell attachment site of FN[21]. A deterioration of the biological activity of small peptides has been described also for the cell attachment function[21].

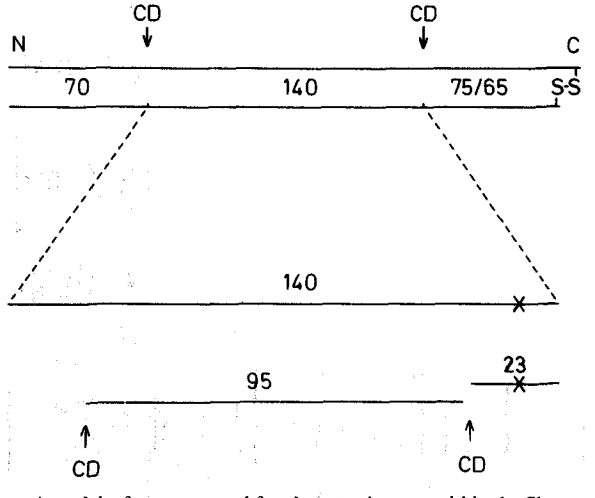
Chemotaxis requires the presence of a concentration gradient. To act as a chemoattractant 'in vivo' cellular fibronectin must be solubilized from the extracellular matrix and this can be achieved, under physiological or pathological conditions, by proteolytic attack which could give rise to the large chemotactic fragment[9]. The decline of the chemotactic potency as a consequence of advanced proteolytic digestion may represent a mechanism involved in the termination of the chemotactic event.

Our results show that, although the chemotactic response to FN of fibroblasts, which have been transformed with SV40, is 1.5–1.6-fold more than that of non-transformed fibroblasts, the chemotactic peptide Ca140 is the only one responsible for the activity. No additional chemotactic sites could be found in the other functional peptides Ca70 and Ca75-SS-65. This could mean that viral transformation renders the fibroblasts more responsive to FN, without inducing the recognition of further FN sites. It appears thus that, in contrast to the C5 complement component, the same FN region is chemotactic for normal, tumoral[5] and viral transformed fibroblasts.

The fact that the Ca23 peptide – which reacts with monoclonal antibodies against the cell attachment FN domain – retains some chemotactic activitiy would confirm the hypothesis that cell adhesion and chemotaxis are closely related phenomena[22].

Number of migrated cells/unit field (magnification × 160) using fibronectin (FN) and FN cathepsin D peptides as chemoattractants (c = 200 nM) in the Boyden chamber

Lower compartment (attractant)	Upper compartment (cells) HEF	SV40/WI38	SV40/WI26
Conditioned medium	69 ± 5	102 ± 7	109 ± 4
MEM'D	1 ± 1	3 ± 1	2 ± 1
FN	56 ± 4	92 ± 6	95 ± 14
Ca70	2 ± 1	4 ± 2	3 ± 1
Ca140	62 ± 1	90 ± 5	84 ± 3
Ca75-S-S-65	3 ± 1	9 ± 4	4 ± 1
Ca95	2 ± 1	4 ± 1	2 ± 2
Ca23	7 ± 2	17 ± 4	11 ± 1



Location of the fragments used for chemotaxis assay within the fibronectin chains. Relevant cleavage sites for cathepsin D (CD) as well as the possible chemotactic domain (x) are indicated. Numbers refer to the molecular mass in kdaltons (kD).

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Utilization of phosphate compounds for growth of *Tetrahymena*

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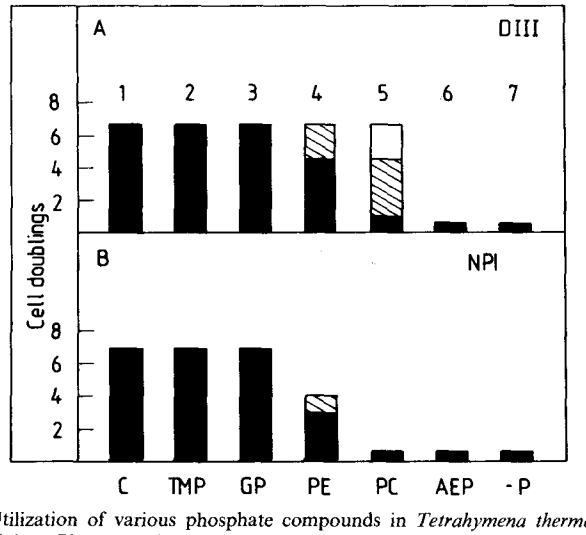
Summary. Taking advantage of a synthetic nutrient medium, we have studied which compounds phosphate-starved *Tetrahymena thermophila* can use as phosphate sources for growth and cell multiplication. Ortho-, trimeta- and α-glycero-phosphate are good sources for both the wild type and a food-vacuoleless mutant; phosphorylcholine is used only by the wild type, and 2-aminoethyl phosphonic acid fails to serve as a phosphate source. Since at least two phosphatases are found in the extracellular fluid these results indicate that *Tetrahymena* can make use of extracellular digestion of nutrients.
Key words. Tetrahymena; growth studies; utilization of organic phosphate; role of phagocytosis.

The ciliate *Tetrahymena thermophila* can be grown in a synthetic medium in which we can control the concentrations and the chemical forms of added compounds[1]. This gives us a possibility to see which compounds – in this case, which phosphate compounds – can support cell growth under certain circumscribed conditions of cultivation.

We have used a synthetic medium[1] supplemented with 0.2 mg tyrosine per ml, prepared without the usual orthophosphates and then supplemented with one of the six phosphate compounds to be tested. When cells from the complete medium are transferred to a phosphate-free medium (at least a 100-fold dilution) they complete 5–6 generations on internal reserves and on phosphates carried over upon subcultivation. Such cells go through at most one cell doubling upon transfer to new phosphate-free medium. The growth and multiplication tests have been carried out on two cell types: a nonphagocytic mutant of *T. thermophila,* NP1, which does not form food vacuoles when grown at restrictive temperature, and the wild type from which it is derived, DIII[2]. The cells were inoculated into test medium at a population density of 5000 cells per ml in small test tubes (10 mm wide, 100 mm high) holding 2 ml sterile-filtered synthetic medium. The cultures were incubated at 38 °C, the restrictive temperature for NP1 and near optimum for the wildtype. Population densities were monitored in an electronic counter on samples removed 18, 42, and 66 h after inoculation. The number of cell doublings was calculated and plotted (fig.).

Tetrahymena can utilize a number of phosphate compounds for growth and multiplication. Ortho-phosphate, trimetaphosphate, and α-glycerophosphate (Nos. 1–3, respectively, fig.) support growth at the rate of more than six cell generations in 18 h in both wild type (frame A) and nonphagocytic cells (frame B). Phosphorylethanolamine (No. 4) supports cell growth better in the wild type than in the mutant, and phosphorylcholine (No. 5) supports growth only in wild-type cells, and the growth rates are low. 2-Aminoethyl phosphonic acid (No. 6) is not utilized by *Tetrahymena,* not even in 10 mM concentrations (Not shown). The cells do not grow in phosphate-free medium (No. 7).

Taking in account that *Tetrahymena* secrets large amounts of phosphatases into the extracellular medium[3], and assuming that only ortho-phosphate can pass the plasma membranes, we may conclude the following from these results: 1) trimetaphosphate, α-glycerophosphate and phosphorylethanolamine are broken



Utilization of various phosphate compounds in *Tetrahymena thermophila. A* Phagocytozing wild-type cells; *B* a non phagocytozing temperature-sensitive mutant derived from the wild type of frame A and grown under restrictive conditions[9]. Phosphate-starved cells were transferred to media containing 1) orthophosphate, 2) trimetaphosphate, 3) α-glycerophosphate, 4) phosphorylethanolamine, 5) phosphorylcholine, 6) 2-aminoethyl phosphonic acid (a structural analogue of phosphorylethanolamine, but containing a P–C bond instead of a P–O–C bond), and 7) no phosphate. The concentrations of the phosphate sources were in all cases 3 mM. Cell multiplication during the first 18 h: ■; during the next 24 h: ▨; and during the next 24 h: □.