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FORMATION OF CELL-TO-SUBSTRATE CONTACTS DURING FIBROBLAST MOTILITY: AN INTERFERENCE-REFLEXION STUDY

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SUMMARY

The formation of close contacts and focal contacts (*ca.* 30 nm and 10–15 nm separation distance respectively) has been studied during the movement of chick heart fibroblasts on planar substrates using interference-reflexion microscopy, and evaluated in the context of spreading and net movement. During spreading the overall advance of the margin of the close contact is steady, punctuated by periods in which it remains stationary, and only 5% of the time is spent withdrawing in contrast to the extreme leading edge. The close contact advances only where a lamellipodium has first extended free of the substrate ($\geqslant$ 100 nm separation distance) ahead of the existing close contact. The new close contact is formed by the lamellipodium lowering to the substrate either progressively from its base forward or distally in patches which later join with the main close contact. New focal contacts are formed successively ahead of existing ones, either by microspikes or lamellipodia contacting the substrate locally ahead of the close contact, or within the close contact usually immediately, but not more than 1–2 μm, behind its margin. Examining the cell margin alternately with interference-reflexion and differential-interference contrast showed that the formation of the focal contact was preceded in 90% of the cases by the development of a linear structure in the form of a microspike (as expected), a short projection (<2 μm long) of the lamellipodium, or a fibre within the lamellipodium, each of which could be traced to the cytoplasmic fibre typically associated with the focal contact. Stress fibres subsequently developed centripetally from these initial fibres. The different forms of the linear structure which preceded the focal contact were interchangeable, giving rise to one another, and we have evaluated that the structure common to each is probably a short bundle of microfilaments.

The following features indicate that the close contact plays a primary role in marginal spreading: it is lost when spreading ceases; it is reformed when spreading resumes but only under the newly spread area; the advance of the margin of the thicker leading lamella closely follows that of the close contact; the advance of both can occur ahead of and is thus independent of existing focal contacts and associated stress fibres. We propose that the close contact provides the adhesion required to transmit to the substrate the forces involved in the forward movement of the marginal cytoplasm. The continual formation of focal contacts and stress fibres at the margin is consistent with their role, suggested by others, in drawing the bulk of the cell forward. These evaluations are discussed in the context of the form and distribution of contractile proteins in the cell margin. A primary role of the lamellipodia and microspikes in extending the cell margin and forming new adhesions, preparatory to further cytoplasmic movement, is established by this work.

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INTRODUCTION

In a previous paper (Izzard & Lochner, 1976), we described the contacts between living chick heart fibroblasts and planar substrates, and the relationship of these contacts to structures in the cell, using interference-reflexion microscopy and differential-interference contrast microscopy. Two distinct types of contact, in which the cell surface came sufficiently close to the substrate for the contacts to be considered significant in adhesion, were recognized under spread cells: focal contacts, separated by 10–15 nm from the substrate, and located under the peripheral regions of the leading lamellae and other extended non-spreading regions of the cell margin; and close contacts, consisting of broad areas of uniform cell-to-substrate separation (*ca.* 30 nm) and typically present under the peripheral regions of spreading leading lamellae. The focal contacts were coincident either with cytoplasmic fibres of the same dimensions which we equated with the cytoplasmic plaques described from electron-microscope studies of the same cells by Abercrombie, Heaysman & Pegrum (1971), or with the peripheral ends of longer birefringent cytoplasmic fibres which we equated with the cytoplasmic plaque and contiguous bundle of microfilaments or stress fibre (Abercrombie *et al.* 1971; Heath & Dunn, 1978). In this report we describe and analyse the formation of these contacts during the movement of chick heart fibroblasts. Special attention was given to the following points during the study.

The motility of fibroblast-like cells *in vitro* can be resolved into 2 major components: (1) the local advance or spreading of the cell margin across the substrate, which involves *de facto* the entrainment of the cytoplasm in forward movement and results in the production of extended, flattened regions of cytoplasm termed leading lamellae (see Ingram, 1969; Abercrombie, Heaysman & Pegrum, 1970*a*); and (2) the relatively more rapid retraction of trailing or other extended cell processes, which results in a significant net movement of the cell. (See for example, Algard, 1953; Trinkaus, Betchaku & Krulikowski, 1971; Wessells, Spooner & Ludueña, 1973.) From the early experiments of Algard (1953), it is clear that considerable tension can be developed between the advancing margin and the bulk of the cell and, implicit in his observations, is the conclusion that the adhesions capable of transmitting the tensile force to the substrate are located at or near the advancing margin. The conclusion is well supported by the microdissection studies of Harris (1973*a*) and there is good circumstantial evidence that the peripherally located focal contacts in chick heart fibroblasts represent these adhesive sites (see Abercrombie *et al.* 1971; Abercrombie & Dunn, 1975; Izzard & Lochner, 1976). It is generally considered that the bundles of microfilaments or stress fibres play a major role in developing the forces required to move the cell mass forward (Abercrombie *et al.* 1971; Huxley, 1973; Wessells *et al.* 1973), and the accumulating information is consistent with this idea, for example the actin (Lazarides & Weber, 1974; Goldman, Lazarides, Pollack & Weber, 1975) and myosin (Weber & Groeschel-Stewart, 1974) content of the stress fibres and especially their ability to shorten in the presence of ATP (Isenberg *et al.* 1976). However, the forces developed by the microfilament bundles can be effective only up to the most peripheral point of insertion of the microfilaments, namely the focal contacts (Heath & Dunn, 1978), and

therefore the bundles cannot, in a simple sense, be considered to entrain the cytoplasm in forward movement beyond the focal contacts. Since the focal contacts remain stationary relative to the substrate (Lochner & Izzard, 1973), 2 requirements necessarily follow: a means whereby the cell margin advances ahead of the existing focal contacts; and the continual formation of new focal contacts and transfer of tension to these as the margin advances.

The leading edge of cultured fibroblasts is highly motile, undergoing a cyclical protrusion and withdrawal while showing a net advance (Abercrombie *et al.* 1970*a*), or lifting away from the substrate to produce ruffles (Harris, 1969; Ingram, 1969). This motile sheet of cytoplasm, termed the lamellipodium (Abercrombie, Heaysman & Pegrum, 1970*b*), extends ahead of the microfilament bundles (Abercrombie *et al.* 1971) and the focal contacts (Izzard & Lochner, 1976). However, it is not known how, if at all, the lamellipodium establishes new adhesions with the substrate, or if extension of the lamellipodium alone is sufficient to overcome the limitation of the microfilament bundles in forward advance. In the context of these questions, we have examined in chick heart fibroblasts, using interference-reflexion and differential-interference microscopy, the nature of the advancing margin of the cell and the mode and sequence of formation of close and focal contacts, and have evaluated the relative roles of the 2 contacts in spreading and net movement.

METHODS

Cell cultures

Primary explant cultures of chick heart fibroblasts were prepared on coverglasses from fragments of 7-day-old chick embryonic heart as previously described (Izzard & Lochner, 1976). Cultures were used between 18 and 36 h of incubation. The culture medium consisted of Basal Medium (Eagle) in Hanks' salts (pH 7·4) supplemented with 10 % horse serum, 10 % embryo extract and 50 U./ml penicillin and 50 μg/ml streptomycin. For routine observation the coverglass carrying the culture was inverted over a 35 × 50 mm coverglass (no. 1½) bearing the pool of fresh medium and supported on spacers cut from no. 1½ coverglasses. The preparation was sealed with Valap (Vaseline:Lanolin:Paraffin wax 1:1:1). When extra thin preparations were required (see below), the coverglass carrying the culture was inverted over a no. 00 coverglass and spacers cut from no. 0 or 1 coverglasses. Corning brand coverglasses were used throughout. They were cleaned by repeated dipping in boiling water, then in 95 % ethanol, and dry-heat sterilized. The preparations were maintained on the microscope at 37 °C with a Sage air curtain incubator.

Optical techniques

The interference-reflexion microscope (IRM) was set up on a Ziess Photomicroscope II equipped with a type II C vertical illuminator, a reflector insert with aperture stop and a 100/1·25 epiplanachromat POL oil-immersion objective, and used as described previously (Izzard & Lochner, 1976). The illuminating numerical aperture (INA) for particular applications is given in the text.

To correlate changes in the cell-to-substrate contacts during movement with changes in the cell it was necessary to be able to examine the same cell alternately by IRM and differential-interference contrast (DIC) and to change rapidly from one to the other. The conventional Zeiss equipment for transmitted-light DIC could not be used simultaneously with the above IRM set-up. However, we were able to construct an alternative transmitted-light DIC system, compatible with the IRM, from Zeiss components designed for reflected-light DIC. Its construction was based on the location of the beam-splitting Wollaston prism immediately above the objective, and the principle that symmetrical objective and condensing lens systems would

require a pair of identical Wollaston prisms. Thus the 100/1·25 epiplanachromat POL objective and its appropriate interference-contrast attachment for reflected-light were mounted on the vertical illuminator. A second identical objective and interference-contrast attachment were used in place of the conventional DIC condenser for transmitted-light and attached to the sub-stage via a plate manufactured to accept the quick-change mount of the interference-contrast attachment. The short working distance of the condensing objective required the use of extra thin specimen preparations (see above). A rotating analyser in the tube slot and polarizer below the sub-stage completed the basic transmitted-light DIC system. An auxillary lens was not required below the sub-stage. When the field stop in the base of the microscope was brought to focus in the specimen plane by adjusting the height of the condensing objective, Köhler illumination for the mercury arc was obtained without further adaptation of the microscope. The correct orientation of the lower Wollaston prism was found by rotating the whole condensing system with its mounting plate in the sub-stage carrier until the exit pupil of the objective was most fully and uniformly extinguished. At best an area slightly less than two thirds the diameter of the exit pupil of the objective was extinguished. Since a variable aperture stop could not easily be included in the condensing system, an aperture 2 mm in diameter drilled in a blackened brass disk was centred and taped in place at the back of the condensing objective so that it was conjugate with the aperture of the objective. The diameter of the inserted condenser aperture was 0·6 of the objective exit pupil, which reduced the resolution of the system slightly below the optimal level. However, it was selected to coincide with the central extinguished area of the objective exit pupil, thus giving workable extinction in the field of view. Measured extinction factors ranged from 250 to 300. Bias retardation most suitable for the specimen detail was introduced by lateral movement of the upper Wollaston prism.

With this optical system we could switch rapidly from the IRM to DIC images of the same cell merely by moving the lever on the microscope to divert the illuminating light from the reflected to transmitted paths, i.e., in less than 1 s. In the IRM mode both incident and reflected light passed through the upper Wollaston prism, but no differential-interference effect was produced in the IRM image since the incident light was unpolarized. The only problem initially encountered with the combined IRM/DIC system was stray light, which originated as a back-reflexions from optical components located below the specimen and degraded the IRM image. The most serious reflexion originated from the planar surface of the polarizer and was eliminated by tilting the latter a few degrees so that the reflexion returned off-axis and not through the optical train.

Monochromatic light for the IRM and DIC techniques was supplied by a 200-W/2 mercury arc with a d.c. power supply (Opti-Quip, Highland Mills, N.Y.) and a 546-nm Pil interference filter (Zeiss). Heat-reflecting filters (Calflex, Zeiss) were used in the reflected and transmitted light paths.

Film recording and analysis

Monochromatic IRM and DIC images were recorded alternately and in continuous sequence on Kodak Panatomic-X 35 mm film processed in Diafine developer (Acufine Inc., Chicago, Ill.). The individual exposures were initiated at every 10 or 12 s in the different sequences, which lasted for 12–27 min. The frequency with which exposures could be initiated was limited by the exposure times required (7–9 s). Coincidence of structures in the DIC and IRM images was determined by superposition of enlarged transparencies made on sheet film from the 35-mm negatives.

Time-lapse cine records of the monochromatic image from the basic IRM (i.e. without the DIC attachments) were made on 16-mm Plus-X negative stock (Eastman Kodak Co.) using an Arriflex 16S camera driven by a Sage Arri Animation Motor. The negatives were developed in Diafine. The framing rate was 60 frames/min and the exposure time 0·25 s.

Frame-by-frame analysis of the cine films was performed with a Photo-Optical Data Analyzer, Model 224A (L-W Photo Inc., Van Nuys, California), the image being projected via a 45° mirror onto a tracing desk at a final magnification of 5000 times.

Quantitative analysis of movement at the leading edge of the cell was performed on a Vanguard Motion Analyzer, Model C-11 (Vanguard Instrument Corp., Melville, N.Y.) at a final magnification of 3500 times. The method of Abercrombie *et al.* (1970*a*) was followed. Three transect lines were marked on the screen in the Y-axis of the analyser at the equivalent of 6·25

μm apart. The image was rotated to bring the direction of spreading parallel to the lines. The position where the feature of interest intersected each line was recorded by setting the *Y*-axis cross-hair at the intersection and reading to the nearest 0·01 in. (0·0254 cm) on the screen (equivalent to 0·07 μm real distance in the cell).

RESULTS

Formation of cell-to-substrate contacts during continued spreading

The basic features of the formation of cell-to-substrate contacts during the continued spreading of chick heart fibroblasts, and the relationship of the contacts to the structure of the cell, are illustrated in the sequence of paired DIC and IRM images in Fig. 1 (see legend and Methods for details). The sequence is representative of 12 similar sequences using the same methods. The central cell (Fig. 1A) possessed a typical fan-shape leading lamella which was largely devoid of cell organelles and extended ahead of the cell body containing the nucleus, mitochondria and other refractile organelles. In the consecutive IRM image (Fig. 1B) the close contact (*ca.* 30 nm separation distance) extended uniformly under much of the leading lamella and generated a reflexion of lower intensity than that from the surrounding coverglass-medium boundary. Lamellipodia produced a single high-intensity reflexion extending ahead of the close contact in these high INA interference-reflexion images (see along the upper, right, and lower edges of the leading lamella in Fig. 1B). The focal contacts (10–15 nm separation distance) produced elongate, very low intensity reflexions in the IRM image and were present, as is typical for spreading chick heart fibroblasts, under the leading lamella within the area occupied by uniform close contact (Fig. 1B). The focal contacts coincided spatially with cytoplasmic fibres of the same dimensions or with the peripheral ends of longer fibres (compare Fig. 1A and B). For further details and explanation of the correlated DIC and IRM images see Izzard & Lochner (1976).

Movement of the cell across the substrate can be followed in Fig. 1 by reference to the small granules attached to the substrate (horizontal arrow) and the larger ovoid granule lying between the 2 cells.

The leading lamella of the central cell had been spreading toward the lower right prior to Fig. 1A and continued to do so at a rate of 1 μm/min throughout the sequence to produce a progressively narrower extension. However, the central portion of the right edge of this leading lamella stopped spreading and retracted slightly (Fig. 1A–E) while the upper margin of the leading lamella began to spread actively toward (Fig. 1A–G) and then beneath (Fig. 1H–L) the adjacent cell. (The extreme suitability of the interference-reflexion technique for following the underlapping of one cell by another is clearly illustrated in Fig. 1H and J.)

The continued spreading of the leading lamellae of chick heart fibroblasts, such as that illustrated in Fig. 1, was paralleled by the formation of close contact with the substrate under the newly established regions of the leading lamella. On this timescale of analysis the close contact typically developed across the whole advancing margin of the leading lamella rather than at discontinuous segments of the margin (e.g. see the newly formed close contact lying below the level of the horizontal arrow in Fig. 1D, F, H). Once formed the separation distance of the close contact remained stable for a period of time while the leading lamella continued to advance, as judged

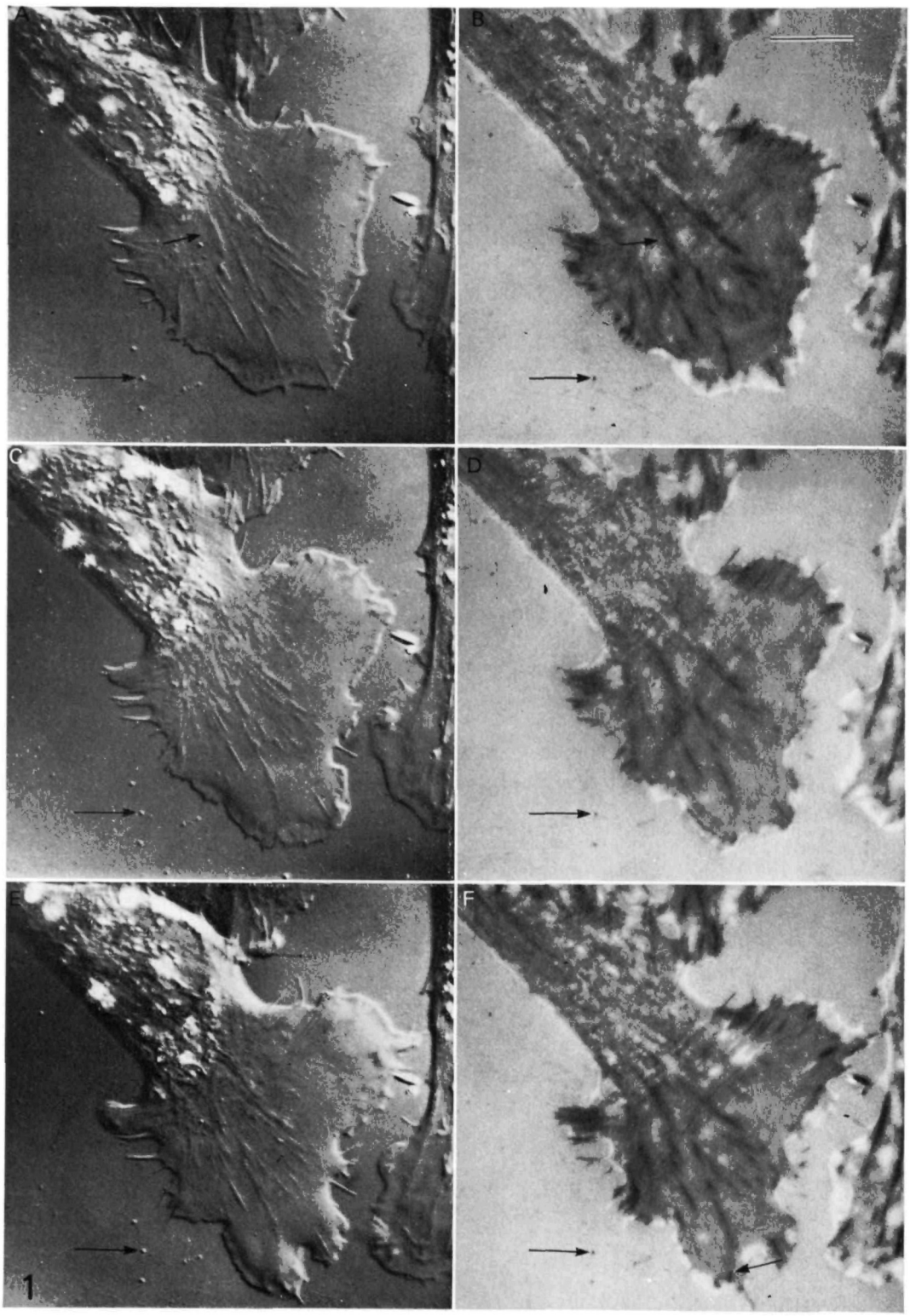


Fig. 1. For legend see page 88.

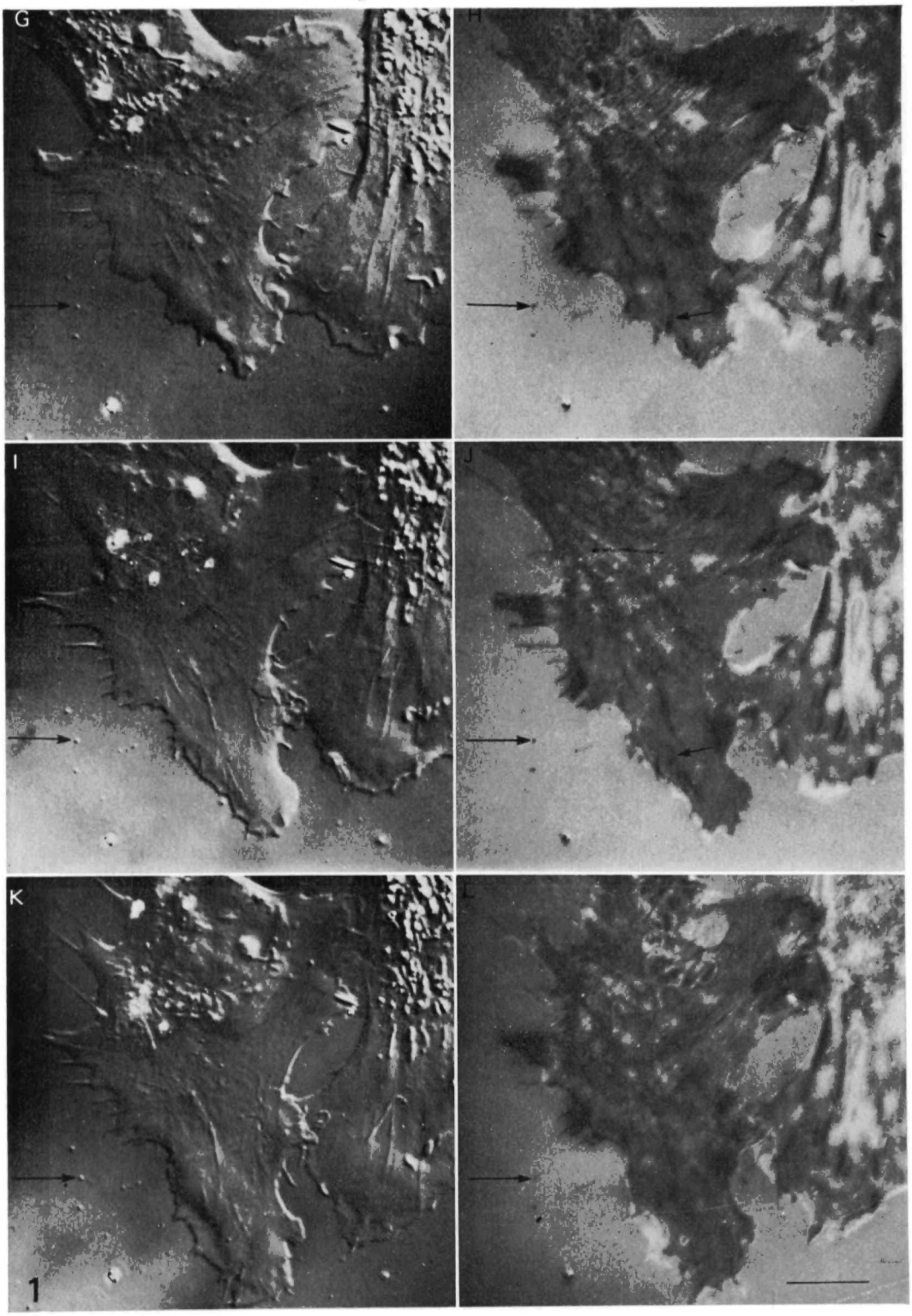


Fig. 1. For legend see next page.

from the intensity of the reflexion in the IRM image (compare the area level with the horizontal arrow in Fig. 1 D with the same area in Fig. 1 F and H). The net effect of these 2 features was the continued development of a broad area of undissected close contact under the advancing leading lamella. Lamellipodia were present across varying portions of the edge of the leading lamellae at each of the stages illustrated in Fig. 1. The detailed relationship between the advancing margin of the close contact and the lamellipodia is examined in a later section.

New focal contacts developed intermittently ahead of existing focal contacts as the leading lamella spread forward. For example, during the 6-min interval between Figs. 1 B and 1 F the lower margin of the leading lamella of the central cell had advanced 3–4 μm ahead of the reference arrow. By the stage in Fig. 1 F, a short focal contact had formed and now lay within the area of close contact immediately behind its leading margin (small arrow, Fig. 1 F). With the continued spreading of this region of the leading lamella, a single new focal contact formed to the left and a group to the right and slightly ahead of the focal contact described above (see Fig. 1 J). Discrete cytoplasmic fibres had formed in association with each of these focal contacts (Fig. 1 I). Similarly, as the upper-right margin of the leading lamella spread toward the adjacent cell, a new row of small, closely spaced, focal contacts developed ahead of an existing row (compare Fig. 1 B and F), a group of prominent focal contacts formed just above the ovoid reference granule (Fig. 1 F, H), and another group formed at the uppermost margin of this portion of the leading lamella as it approached and underlapped the adjacent cell (Fig. 1 H, J). (In Fig. 1 the apparent absence or poor contrast of cytoplasmic fibres associated with the focal contacts in the upper portion of the leading lamella was due to the orientation of the fibres parallel to the direction of shear in the DIC image.) Inspection of Fig. 1 shows that the focal contacts and the portion of the cytoplasmic fibre associated with each focal contact remained stationary relative to the substrate as the leading lamella advanced. This characteristic of the focal contacts was confirmed during the analysis of the cine films (see Fig. 3).

Fig. 1. Chick heart fibroblasts spreading on a glass substrate. Selected pairs of micrographs of consecutive DIC (left columns) and IRM (right columns) images. Direction of shear in DIC images is N.E. to S.W.; INA = 1·0 for IRM images. Exposures for left–right pairs were initiated 12 s apart; exposure time for both 9 s. The pairs were selected from a continuous sequence so that DIC micrographs were separated by 3 min (A, C, E, G, I) or 2·5 min (I, K). Reference points fixed relative to the substrate: small granule (horizontal arrow); and large ovoid granule between the 2 cells. The preparation was moved relative to the edges of the figure between F and G. The major features illustrated are described in the Results. Scale bar, 10 μm.

Arrows in A and B mark respectively a cytoplasmic fibre and associated focal contact, both of which had lost contrast in E and F, and disappeared in I and J.

Small arrow in F marks a new focal contact which is similarly identified in H and J. Note the additional new focal contact to the left, and a group to the right and ahead of the marked focal contact in J. Compare with I to see new cytoplasmic fibres associated with each of these focal contacts. Note the advance in I and J of the leading lamella and close contact 5 μm ahead of these new cytoplasmic fibres and focal contacts.

The cell at the right resumed spreading (E, F) and produced uniform close contact under the newly spread area of the lamella (G–L).

As a leading lamella continued to advance and form new focal contacts, previously formed focal contacts therefore came to lie further behind the advancing margin of the leading lamella. With time these latter focal contacts and their associated cytoplasmic fibres were lost. For example, the large focal contact located centrally in the leading lamella in Fig. 1B (arrow) and the group of 3 small ones immediately ahead had lost contrast 6 min later (Fig 1F) and were no longer visible after a further 6 min (Fig. 1J). At the same time the cytoplasmic fibres associated with each of these focal contacts (see Fig. 1A) were similarly lost (Fig. 1E and I). In addition the row of focal contacts at the upper margin of the leading lamella in Fig. 1B retained good contrast for 9 min in the interference-reflexion series (Fig. 1H) but had lost contrast 3 min later (Fig. 1J) and were no longer clearly visible after a further 2·5 min (Fig. 1L). (Although in this example the focal contacts were lost as the trailing cell body advanced, many other examples recorded on cine film showed a similar loss of focal contacts under the leading lamella without a parallel advance of the cell body.) In this manner the characteristic distribution of the focal contacts within a zone extending 3–20 μm behind the edge of the leading lamella (Izzard & Lochner, 1976) is maintained as the cell continues to advance relative to the substrate (see also Fig. 3, p. 93).

The involvement of the lamellipodium in the formation of the focal contacts and the close contact could not be followed in sufficient detail from a series of alternating DIC and IRM images recorded on 35-mm film because of the rapid movements of the lamellipodium. Instead cine films of the IRM image had to be used for this purpose. Therefore the extent to which the behaviour of the lamellipodium could be followed by the interference-reflexion technique is evaluated in the following section before proceeding to the detailed analysis of the development of new contacts.

Detectability of the lamellipodium in the interference-reflexion image

Lamellipodia generate at their base a high intensity reflexion in monochromatic light (546 nm) corresponding to a zero-order maximum. This reflexion may extend for several micrometres ahead of the close contact when the lamellipodium lies parallel to the substrate, or pass into a series of higher-order minima and maxima when the lamellipodium extends from the leading lamella at an angle to the substrate (Izzard & Lochner, 1976). In either case, the transition in reflected intensity in the interference pattern from that of the close contact to that of the lamellipodium is relatively abrupt (Fig. 1B) and corresponds to an increase in the cell-to-substrate separation distance from 30 to 100 nm. Therefore the boundary between the close contact and the base of the lamellipodium could be followed with ease in the cine films. However, 2 major factors determine whether or not the full distal extent of the lamellipodium can be detected in the interference-reflexion image: the INA; and the angle at which the lamellipodium is inclined to the substrate.

The close and focal contacts are most clearly distinguishable when a high INA is used, but the contrast of the first- and higher-order interference fringes is lost under these conditions (Izzard & Lochner, 1976). Therefore the distal portion of a lamellipodium inclined at an angle to the substrate cannot be detected in the image at high INA. Hence in some of the studies described below a compromise INA of *ca.* 0·8 was

used to increase the detectability of the lamellipodium while retaining a suitable definition of the close and focal contacts.

Even at an INA $\leqslant$ 0·8 one cannot be certain that the full distal extent of a lamellipodium is generating contrast in the image. The interference fringes are of the Fizeau type (i.e. dependent on variations in the thickness of the thin film of medium) and therefore can be detected only if the angular inclination of the reflecting boundaries is small. The extent to which the angular inclination of the lamellipodium limited its detectability was evaluated empirically at an INA of 0·8. The following examples are illustrative of the detectable behaviour of the lamellipodium and define the limitation due to angular inclination.

From the outline and spacing of the interference fringes the shape of the lamellipodium in living chick heart fibroblasts can be reconstructed as gently curved and petaloid, which is in good agreement with that seen in scanning electron micrographs of various fibroblast-like cells (e.g. Revel, Hoch & Ho, 1974). The undulatory behaviour of the extended lamellipodium can be inferred from changes in shape and spacing of the interference fringes and followed directly at the microscope without recourse to time-lapse cinematography. Similarly the characteristic inclination of the lamellipodium away from the substrate (Ingram, 1969; Harris, 1969) can be followed in the IRM image by the development of an increasing number of progressively more closely spaced higher-order interference fringes. A typical example taken from a 16-mm cine film is illustrated in Fig. 2. Bending of lamellipodia toward the substrate was detectable as the converse of the events in Fig. 2.

Movement of the fringes is rapid – the sequence in Fig. 2 took only 7 s – and the lamellipodia *appear* to be undergoing extensive undulations. However, in Fig. 2G the distance in the plane of the substrate between the minima at the base and tip of the lamellipodium was 3·9 μm. Over this distance there is an increase in optical path difference of 4 λ, corresponding to an increase of 817 nm in the thickness of the film of medium at λ = 546 nm. Based on these figures the lamellipodium in Fig. 2G was inclined at an angle of only 11° 50′ to the substrate. The smallest centre-to-centre spacing measured for successive minima under a lamellipodium was 0·6 μm, corresponding to an angle of inclination of 18° 47′. The contrast of the fringes was very weak at this angle (see the loss of contrast between Fig. 2G and H as the lamellipodium approached this angle) and further inclination of a lamellipodium away from the substrate resulted in a sudden loss of the fringes from the IRM image. Therefore, even at the lower INA the contrast of the higher-order interference fringes is retained only within a limited range of angles of inclination between the lamellipodium and the substrate.

In some cases the fringes were lost under the whole lamellipodium, in other cases only under the distal portion of the lamellipodium as this region inclined away from the substrate. In either case, a set of closely spaced fringes frequently reappeared within a few seconds in the same area of the image occupied by the lost fringes, indicating that the loss of fringes was not due to a complete or partial withdrawal of the tip of the lamellipodium but that the latter had persisted and then bent back toward the substrate. Persistence of the lamellipodium immediately after the loss of fringes from the IRM

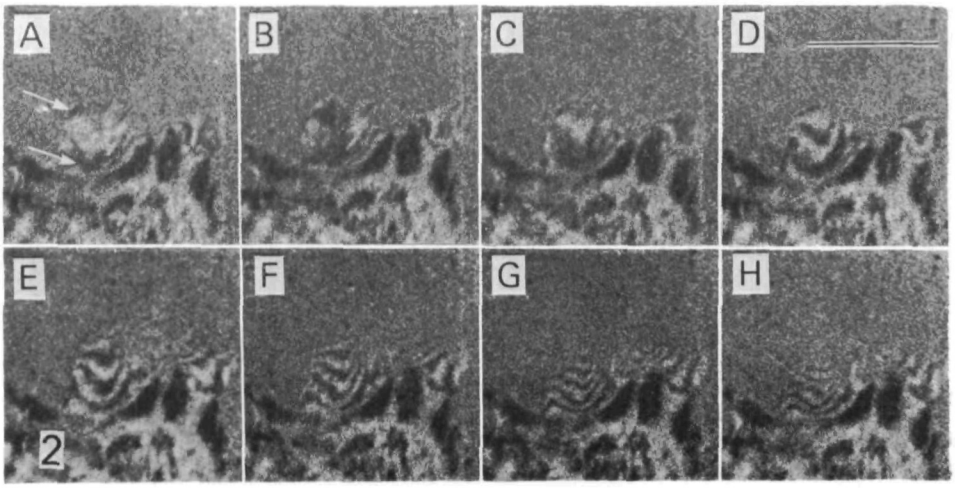


Fig. 2. Inclination of lamellipodium away from the substrate as seen with IRM (INA = 0·8). Prints from 16-mm cine film; 1 s between each print. Scale bar, 10 μm. The central lamellipodium in A showed a minimum near its base and one at its tip (arrows). These 2 minima extended and joined down the centre of the lamellipodium (B) demonstrating that they were of the same order and therefore that in A the lamellipodium was curved either away from or toward the substrate across its centre (the direction of curvature cannot be inferred from the monochromatic image). The central minimum in B moved toward the base of the lamellipodium and became U-shaped, while a new minimum appeared at the tip of the lamellipodium (C). The new minimum similarly moved toward the base of the lamellipodium and was followed in succession by the development and movement of 3 more minima to produce a total of 5 closely spaced minima (D–G). Note the reduction in contrast of the fringes as they become very closely spaced in H.

image was confirmed in many cases through direct observation by switching rapidly (within 1 s) to the DIC image. Nevertheless, in cine films based solely on the IRM image it is not possible to be absolutely certain whether a loss of the lamellipodium from the image represents a real or apparent loss of the structure. However, as it turned out this limitation did not affect the final conclusions to be drawn in this paper. The limitations are documented here for the record and so that the basis of the data presented below is clearly understood.

Formation of the close-contact requirement for the lamellipodium

Movement of the distal margin of the close contact during the advance of the leading lamella across the substrate was analysed from cine films (INA *ca.* 0·8) by recording the position of the margin at 30-s intervals along 3 parallel transects oriented in the direction of spreading. At the same times, the position of the *visible* tip of the lamellipodium was also recorded. The positions of both were plotted against time to provide a graphical summary of the movement of each. A typical example for the 3 transects from 1 cell is given in Fig. 3. Although the method is identical to that used by Abercrombie *et al.* (1970*a*) to describe the movement of the leading edge as seen by phase contrast, we must stress that the fluctuations shown by the tip of the lamellipodium in

the upper trace for each transect in Fig. 3 should not be equated directly with those described by Abercrombie *et al.* (1970*a*) because of the limitations described above on the detectability of the lamellipodium with the IRM. The following information abstracted from these analyses is pertinent here.

The net advance of the margin of the close contact resulted from periods in which it moved forward somewhat steadily, punctuated by periods in which the margin remained essentially stationary, and this pattern was consistent for the 3 transects from any given cell (e.g. Fig. 3). In contrast to the extreme leading edge, which undergoes a repeating cycle of protrusion and pronounced withdrawal (Abercrombie *et al.* 1970*a*), the margin of the close contact did not show similar withdrawals. For the 5 cells detailed below, the margin of the close contact spent only $5{\cdot}5 \pm 1{\cdot}3\%$ (mean $\pm$ S.D.) of the time moving backward. This value is significantly different from that of $19 \pm 9\%$ measured for the extreme leading edge by Abercrombie *et al.* (1970*a*) (for the difference $t = 3{\cdot}3$, d.f. $= 16$, $P \ll 0{\cdot}01$). The duration of the withdrawals was short and the distance withdrawn small. The majority of the withdrawals (94%) continued for only one analysis interval, or 30 s, and the remainder for 1 or 1·5 min, while the distance withdrawn was typically in the 0·75–2·0 μm range but not greater than 4 μm (see Fig. 3). Therefore the behaviour of the margin of the close contact was consistent with the close contact representing a moderately stable adhesion formed between the cell surface and the substrate as the leading lamella advanced. In making these determinations the convention of Abercrombie *et al.* (1970*a*) was followed of disregarding small isolated movements, except that the minimal movement was reduced from their 1·25 to 0·75 μm in 30 s because the speed of movement of the close contact was slower than that of the leading edge. However, all movements are included in Figs. 3 and 4.

Lamellipodia are present typically across varying portions of the advancing margin of the leading lamella in chick heart fibroblasts (Fig. 1). Visual inspection of cine films of the IRM image indicated that new close contact was formed only where a lamellipodium extended ahead of the existing close contact. The graphs from the above analysis largely supported this contention since the upper and lower traces for each transect remained separate over extended periods of time, indicating the repeated presence of the lamellipodium at successive recordings (Fig. 3). However, individual recordings and runs of recordings lacked a *visible* lamellipodium in the IRM image and the 2 tracings coincided at these points (e.g. at 2·5, 22, and 20 min respectively in the upper, middle and lower transects in Fig. 3). Therefore the graphical analyses were used (1) to examine in more detail the overall frequency with which the lamellipodium was present in the image during essentially continuous spreading, and (2) to determine whether or not the margin of the close contact advanced significantly at the specific times when the lamellipodium was absent from the image.

Five cells showing essentially continuous spreading (i.e. not interrupted by stationary periods $\geqslant$ 10 min) were analysed for periods of 30–60 min providing a total spreading time of *ca.* 3·5 h. The net speed of advance of the close contact for the 5 cells ranged from 0·53 to 1·37 μm/min (mean with S.D. $= 0{\cdot}90 \pm 0{\cdot}33$) as measured by the distance moved between the first and last recordings. The frequency with which the lamellipodium was present in the IRM image was high during continued spreading

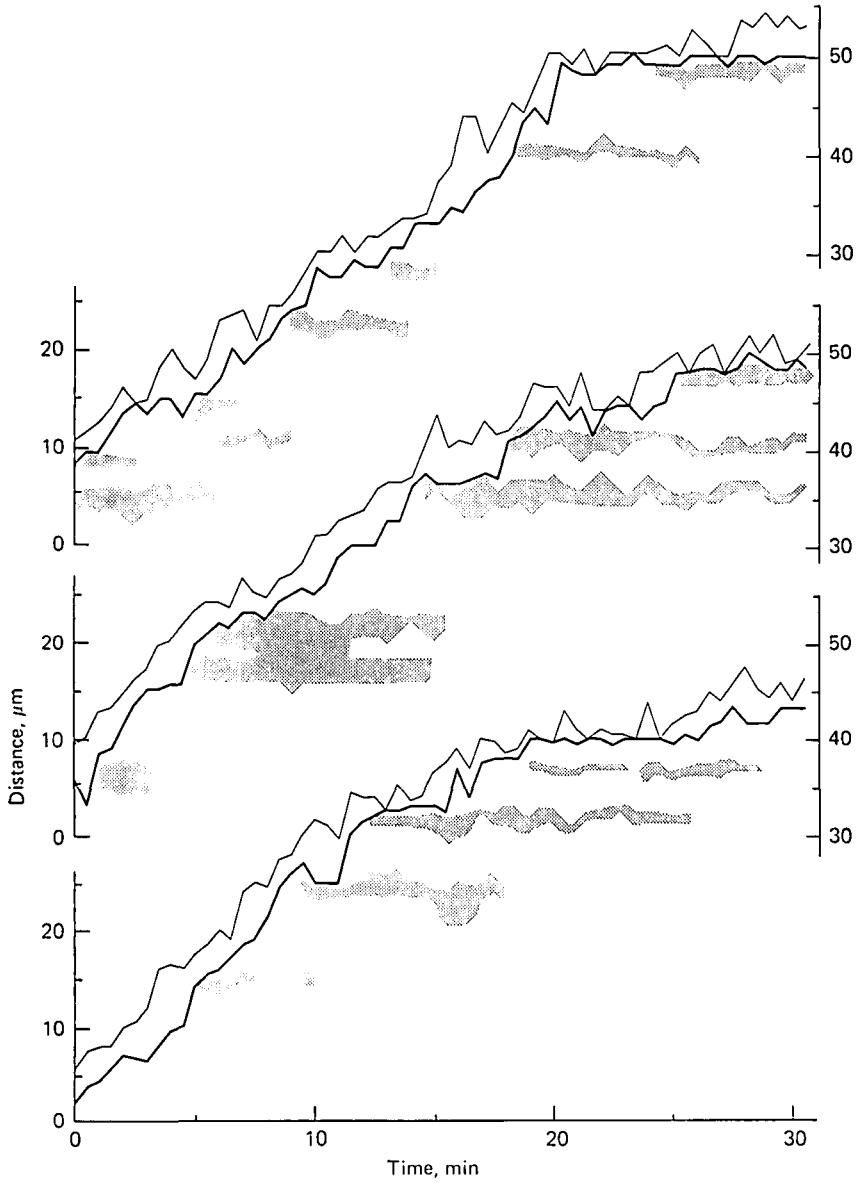


Fig. 3. Plots to show the movement of the visible tip of the lamellipodium (thin line) and distal margin of the close contact (thick line) along 3 parallel transects (upper, middle and lower plots) oriented in the direction of spreading of a leading lamella. Points recorded and plotted at 30-s intervals. See Methods for further details. Coincidence of the 2 tracings in a plot indicates the absence of a visible lamellipodium. The position of the distal and proximal ends of focal contacts along the transects were similarly recorded from their first appearance at the margin to their disappearance under the leading lamella (stippled areas). Note the sequential loss of the focal contacts as the cell margin advances. The overall position of the focal contacts remained stationary. The slight irregularity of the position of the distal and proximal ends of the focal contacts, which outline the stippled areas, was due to the nature of the focal contacts and method of recording. At these ends the transition in density in the film from that of the focal contact to the close contact was gradual and a mid-point was selected for the reading. However, the selection was made independently at each time without reference to the preceding reading and variation arose in this judgement step. The same problem did not arise for the margin of the close contact which is more distinct.

despite the limitation on its visibility in the image. The percentage of the recordings with a lamellipodium, calculated separately for each cell by pooling the 3 transects, had a mean of 84·2% (S.D. 6·9). Thus on average the lamellipodium was not only present, but inclined at an angle $\leqslant 19°$ to the substrate, for 84% of the time. The presence of the lamellipodium varied between 73 and 92% of the total spreading time but there was no obvious correlation between its presence and the net speed of advance of the 5 individual cells.

The recordings without a lamellipodium numbered 187 out of a total of 1235 recordings provided by pooling the 3 transects from each of the 5 cells. Of these 187 recordings 84 (45%) occurred as single instances while the remaining 103 (55%) were grouped in runs of 2–7, or 10, 12 and 15 consecutive recordings forming 34 separate runs. Movement of the close contact associated with the absence of a lamellipodium was determined as the distance between the recordings immediately *preceding* and *following* those without a lamellipodium. This was the only method available for the single recordings but, for these cases and the runs, covered the possibility that the lamellipodium was lost early or reappeared late during the 30-s interval preceding or following respectively the loss of the lamellipodium from the record. Movement was assessed therefore over 1-min or longer intervals and was considered to be significant, on the following basis, if $\geqslant 1{\cdot}5$ μm irrespective of the time interval. A speed of 1·5 μm in 1 min for the single recordings corresponded to the minimal speed of 0·75 μm in 30 s used above as the basis for disregarding small isolated movements. The same distance was used for the longer time intervals of the runs to ensure that short-term steps or slow consistent advances were not overlooked.

Significant advance of the margin of the close contact was associated with only 9 out of 84 single recordings without a lamellipodium (maximum advance 3·0 μm) and with 9 of the 34 runs of similar recordings (maximum advance 3·75 μm). Subsequent frame-by-frame analysis of these 18 cases showed that the close contact advanced only in the presence of a lamellipodium. In 6 single recordings and 7 runs the movement occurred during the 30-s interval preceding the loss of the lamellipodium, the close contact advancing to the visible tip of the lamellipodium and then remaining essentially stationary. In the other 3 singles the movement occurred during the 30-s interval following the recording without a lamellipodium but only after the lamellipodium had reappeared in the image. Advance in the 2 remaining runs occurred between the first and last recordings without a lamellipodium, but frame-by-frame analysis showed that the close contact advanced between recordings and when a lamellipodium was present. From this detailed analysis we conclude that new close contact forms, and therefore the distal margin of the close contact advances, only where a lamellipodium has first extended free of the substrate and ahead of any existing close contact. Stated in another way, we have found no evidence for the extreme distal margin of the cell advancing and simultaneously forming the close contact at the margin.

The presence of a motile lamellipodium at the margin of the leading lamella was not sufficient alone to produce spreading. For example, when leading lamellae became stationary for extended periods (30–50 min), the lamellipodium was not always lost

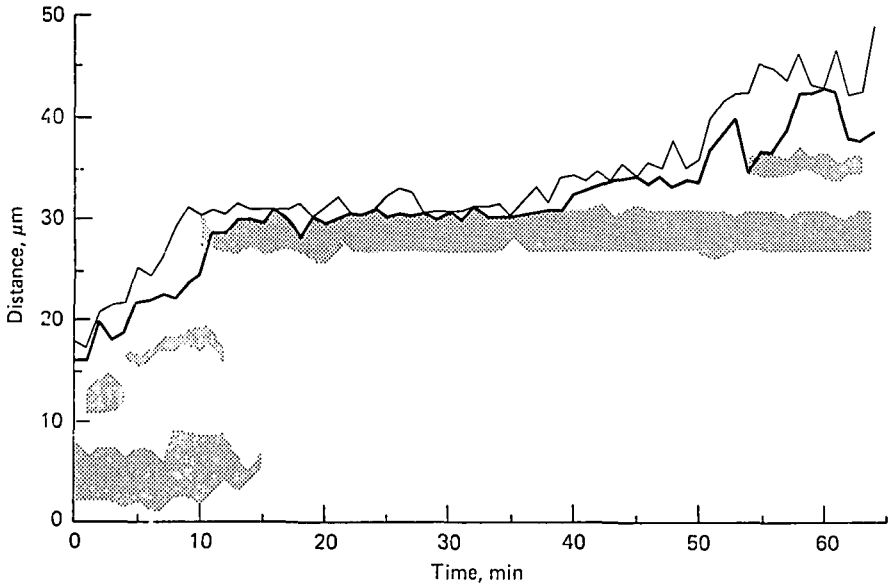


Fig. 4. Plot to show the transition from an actively spreading (0–10 min) to stationary (10–40 min) period, and the resumption of spreading (40 min and on) along a single transect. Details as for Fig. 3 except that the positions were plotted at 60-s intervals instead of 30-s to include the longer time axis in the figure. Omission of the intermediate points did not change the details referred to in the text. Note the increased coincidence of the 2 tracings, and thus decreased presence of a visible lamellipodium, during the stationary period. The most distal focal contact persisted throughout the stationary period, and continued to do so when spreading resumed and new focal contacts were formed.

completely, but the frequency with which it was present in the IRM image did decrease noticeably. Therefore the movement of the visible tip of the lamellipodium and the margin of the close contact was analysed, as above for the spreading cells, along 3 transects in each of 3 essentially stationary cells (mean speed of advance with S.D. = $0{\cdot}1 \pm 0{\cdot}05$ μm/min). The percentage of recordings with a lamellipodium decreased to a mean of 50·8% (S.D. 7·7) for these 3 stationary cells. The transition from a more continuous to intermittent presence of the lamellipodium is clear at the onset of the stationary period at 10 min in the single transect in Fig. 4. The mean for the stationary cells was significantly different from that of 84·2% for the group of spreading cells (for the difference $t = 6{\cdot}36$, d.f. = 6, $P \ll 0{\cdot}01$). The significant difference between the 2 groups, and the lack of correlation within the group of spreading cells between the net speed of advance and the percentage presence of the lamellipodium, suggest that a threshold value exists for the presence of the lamellipodium and onset of spreading. However, the threshold level could represent an increase in the actual presence of the lamellipodium or apparent presence, i.e. greater time spent inclined at less than 19° to the substrate. We are currently investigating this point as a first step to understanding the control of the onset of spreading.

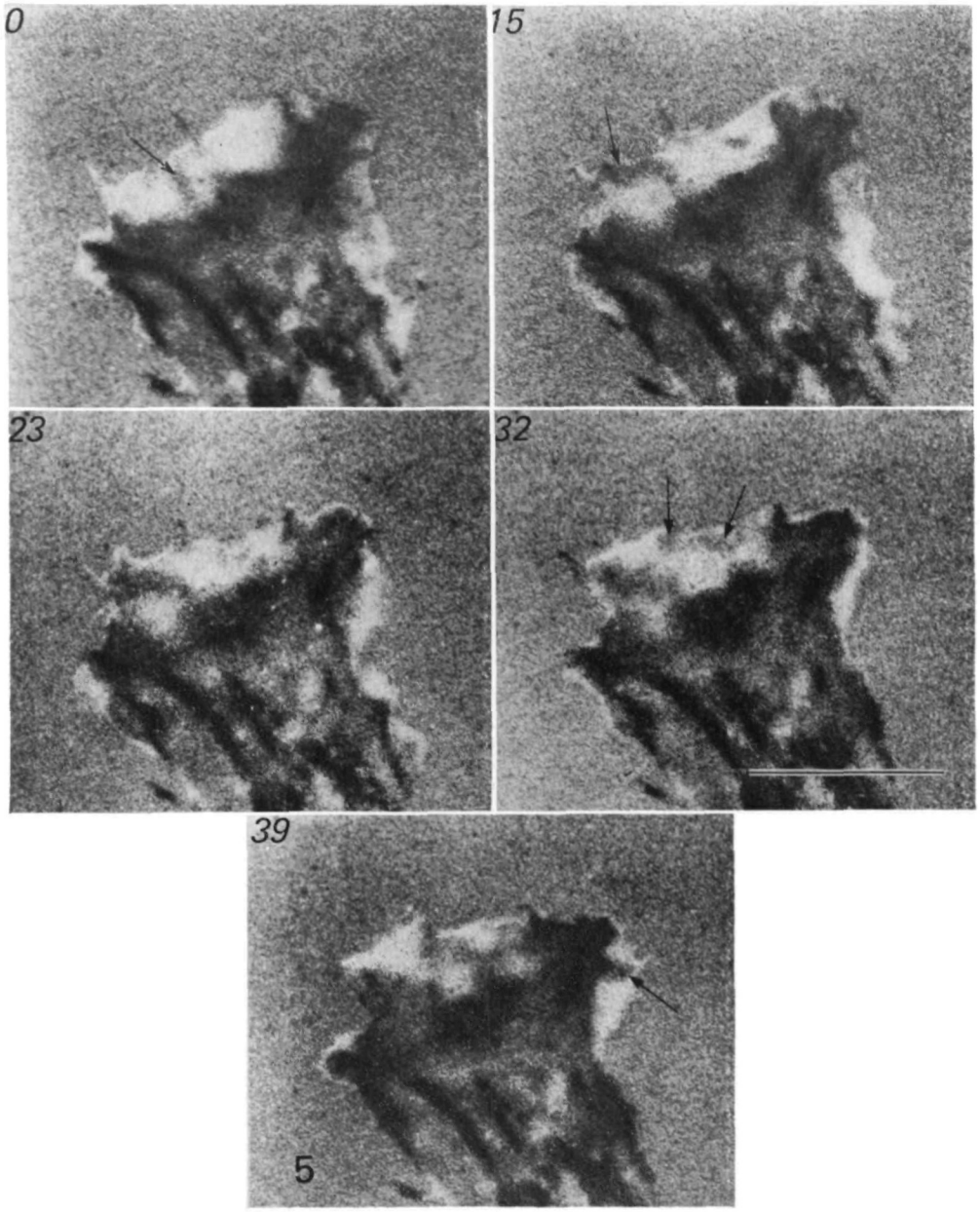


Fig. 5. Formation of close contact at the tip and base of the lamellipodium. Series printed from 16-mm cine film of IRM image (INA slightly less than 1·0). Numbers state seconds elapsed since first print in series. Scale bar, 10 μm.

0 s: A lamellipodium (higher intensity than background) extended up to 2·8 μm ahead of the close contact across the leading edge of the lamella. The elongate patch of lower intensity (arrow) was a transient feature.

15 s: An area, 4 × 1 μm, of intensity between that of the close contact and background had formed at the tip of the lamellipodium (arrow). The area was distinctly separated from the close contact as it formed.

23 s: The same area persisted during the intervening 8 s and spread at its 2 ends to join the pre-existing close contact. This demonstrates that the separation distance of the

Formation of the close-contact role of the lamellipodium

The details of how the new close contact was formed were established from frame-by-frame analysis of segments of another series of films (4 cells, 6 leading lamellae) in which the INA was adjusted to slightly less than 1·0 so that contrast of the first-order minimum at 546 nm was retained under the lamellipodium in addition to the zero-order interference pattern. The adjustment enhanced the visibility of the lamellipodium as compared to an INA $\geqslant$ 1·0 but held to a minimum variations in the intensity of the close contact that result from first and higher orders of interference generated by reflexions from the far exposed side of the cell (see Izzard & Lochner, 1976).

New close contact was formed locally across 1 to 8 μm widths of its existing margin with the result that the outline of the margin changed continually as it advanced. The way in which the new close contact formed varied considerably in detail, but 2 extreme cases could be recognized and serve to demonstrate the role of the lamellipodium in the formation of the close contact. In one extreme, the existing margin of the close contact advanced progressively and therefore the cell surface at the base of the lamellipodium lowered toward the substrate. The expanding area of the contact showed either the same reflected intensity as the pre-existing close contact or less frequently a slightly higher and fluctuating intensity which stabilized to that of the close contact within 15–90 s. These advances occurred over distances of 1–3 μm before hesitating temporarily or stopping for longer periods (1 min or more). Depending on the width of the margin involved, the new areas varied in shape from approximately broad-based segments of a circle to narrower tongue-like areas. Examples of this type of advance are given in Fig. 6 (46, 75, 94 s and 117, 127, 148 s) and in Fig. 5 (39 s). At the other extreme, distal areas of the visible lamellipodium clearly established patches of close contact of varying and often irregular shape ranging initially from 1 to 5 μm in overall diameter. The patches formed distinctly ahead of the existing close contact, the intervening proximal portion of the lamellipodium at first remaining *ca.* 100 nm from the substrate. The patches formed rapidly, the lamellipodium sometimes lowering toward the substrate from a distance of *ca.* 100 to 30 nm in as little as a single frame (1 s). When first formed the reflected intensity within the

Fig. 5. *Continued.*

patch at 15 s was similar to the close contact and thus formed by the lamellipodium bending down toward the substrate. The enclosed area of higher intensity represents the basal region of the lamellipodium which was maintaining its wider separation distance from the substrate.

32 s: The enclosed area at the base of the original lamellipodium had come closer to the substrate and only a small patch remained with an intensity slightly above background. Meanwhile patches of lower intensity had appeared, again at the distal margin of the lamellipodium (arrows) and were separate from the close contact.

39 s: The new patches at 32 s had spread to join each other and the pre-existing close contact. In subsequent frames these areas acquired the close contact intensity. The original area of new close contact (15 s and on) is now completely continuous with the close contact under the lamella. To the right of the leading lamella a small tongue-like area of close contact had appeared (arrow). It formed by the steady advance of the margin of the close contact during the intervening frames.

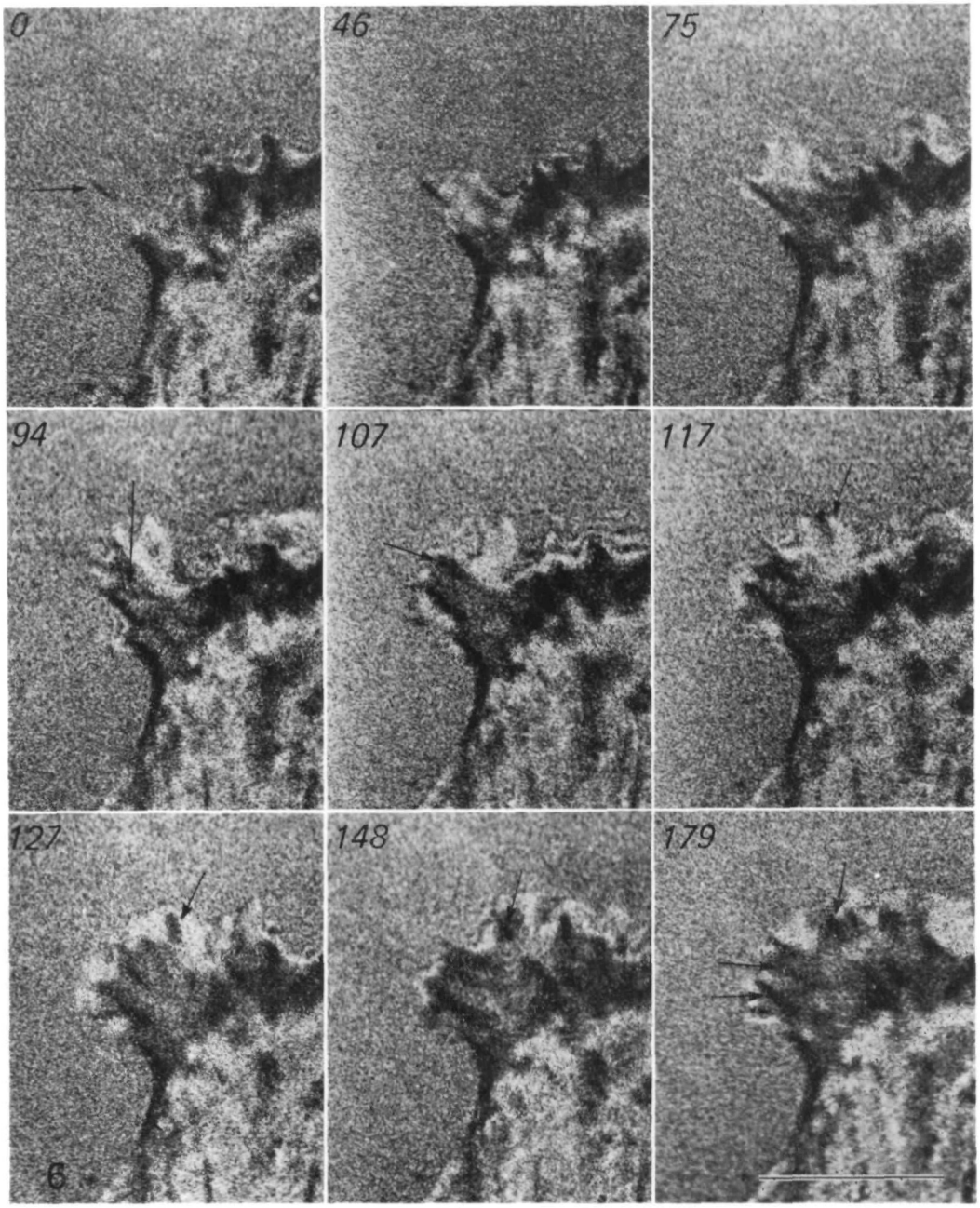


Fig. 6. Formation of focal contacts by a microspike and the lamellipodium, and within the close contact. Series printed from 16-mm cine film of IRM image (INA slightly less than 1·0). Numbers state seconds elapsed since first print in series. Scale bar, 10 μm.

0 s: A microspike made contact with the substrate 8 s before this frame over a length of 1·5 μm close to its tip. By 0 s in the figure the area of contact had darkened considerably (arrow). The contact persisted throughout the series, its length increasing to 2·5 μm and its density to that typical of a focal contact.

46 s: A lamellipodium extended from the cell margin, apparently continuous with

patch often fluctuated locally and ranged between that of the close contact and the surrounding coverglass, i.e. equivalent to a separation distance of 30–50 nm. This labile state lasted for 15–60 s before the contact stabilized and the intensity became uniform and equal to that of the close contact. During or subsequent to the labile period, the proximal portion of the lamellipodium also lowered toward the substrate so that the patch finally became continuous with the previously formed close contact. Examples of this type of advance are given in Fig. 5. Occasionally the lamellipodium would lift away from the substrate during the labile period. Between the 2 extremes were cases in which a labile patch formed rapidly under the more proximal portion of the lamellipodium. These patches were not distinctly separated from the pre-existing close contact, but the margin of the latter contact did not advance progressively as in the first case described above. Other variations mainly involved the shape of the newly formed areas of close contact.

The 2 extreme cases clearly show that much of the undersurface of the lamellipodium is competent to form the close contact. The second extreme case discounts the hypothesis that might otherwise be inferred from the graphs based on analyses at 30-s intervals (Fig. 3), namely that the contact is formed *only* by the cell surface at the base of the lamellipodium progressively rolling down toward the substrate. The relatively rapid time course for the formation of patches of close contact by the lamellipodium would not necessarily be documented in the 30-s interval analyses. However, some of the withdrawals in the transects were clearly noted to occur during the labile period of newly formed close contact.

Fig. 6. *Continued.*

the microspike as it advanced. By this frame it had reached the distal end of the focal contact formed by the microspike.

75 s: The margin of the close contact at 46 s had advanced steadily under the lamellipodium to reach the distal end of the new focal contact and establish a new area of stable uniform close contact. The lamellipodium had continued to extend ahead of the advancing close contact and the new focal contact.

94 s: Steady advance of the margin of the close contact continued during the intervening 19 s to form an irregularly outlined region (long arrow) extending ahead of the new focal contact.

107 s: A second focal contact developed within the new stable close contact formed by 94 s. This frame represents its first appearance (arrow) in the film. The contact can be followed to the end of the series with some changes in its reflected intensity. An undulating lamellipodium, up to 4·5 μm wide, was present ahead and to the right of this new focal contact.

117 s: A very small dark patch (arrow) formed in this frame at the visible tip of the lamellipodium.

127 s: The dark patch formed at 117 s had increased in size (arrow), while the close contact advanced steadily in the intervening frames at the base of the lamellipodium until it had almost reached this developing focal contact.

148 s: The dark patch formed by the lamellipodium at 117 s has now become a distinct focal contact (arrow). Meanwhile the close contact has continued to advance steadily under the lamellipodium to surround this third new focal contact.

179 s: The 3 new focal contacts described above have persisted (arrows) as more than transient structures, while additional focal contacts have formed during the intervening 31 s.

Formation of the focal contacts

New focal contacts were formed only by advancing leading lamellae. Although focal contacts are present under stationary leading lamellae and trailing tapering cell processes, we have never observed the formation of new focal contacts under these processes. The general location at which new focal contacts form, relative to the advancing margin of the leading lamella, was determined during the analysis of the movement of the close contact along parallel transects (see preceding section). At the end of each interval, the position of the distal and proximal ends of the focal contacts lying on each transect were recorded and plotted, together with the positions of the margin of the close contact and visible tip of the lamellipodium (Figs. 3, 4). These initial analyses clearly emphasized that new focal contacts were forming either immediately behind or just ahead of the advancing margin of the close contact (Figs. 3, 4). The same analyses confirmed the stationary position of the formed focal contact relative to the substrate and the sequential loss of older focal contacts as the leading lamella continued to advance (Fig. 3).

The exact position where the focal contacts formed was determined by frame-by-frame analysis of the same set of films used to analyse the details of close contact formation, i.e. 4 cells, 6 leading lamellae and INA slightly less than 1·0 to retain the first-order minimum. The earliest detectable stage of a developing focal contact was taken as an area of slightly greater density in the film than the close contact, located in the same position as, and of size similar to or smaller size than that of the final focal contact. The location of the earliest stage was determined by examining the preceding and subsequent behaviour of the surrounding cell margin. All the focal contacts formed by the individual leading lamellae were analysed, giving a total of 70 focal contacts. It was possible to assign each to one of the categories in Table 1 in terms of position where first formed and consequently mode of formation.

Fifty seven percent of the analysed focal contacts formed within the close contact, i.e. they were first detected within an area of close contact that was stable, distinctly wider than the focal contact and, with one exception (see below), continuous with the main close contact under the leading lamella. A typical example is illustrated in Fig. 6 (107–148 s). The larger proportion of these focal contacts formed in close contact that arose by spreading at the base of the lamellipodium, the remainder in close contact that developed initially as an isolated patch under the lamellipodium (Table 1). However, in all except one of the latter cases the patch became continuous with the main area of close contact before the focal contact was first detected. The majority of the focal contacts began to form immediately behind the margin of the close contact and the others not more than 1–2 μm behind the margin. Seventy percent of the focal contacts which formed within the close contact first appeared between 4 and 58 s after the close contact became stable, while the remainder appeared between 1 and 2·5 min, with one exception which began to form after a 5-min delay.

Forty three percent of the analysed focal contacts formed without the prior establishment of an area of close contact. Approximately equal numbers were formed by a lamellipodium or a microspike bending down to contact the substrate (Table 1). To

Table 1. *Position where focal contacts first detected*

In close contact which formed:		Not in close contact but:	
At base of lamellipodium	29	Under lamellipodium	13
As patch under lamellipodium	9	Under microspike	16
In an unclear manner	2	Other	1
Totals	40		30

achieve the separation distance of the focal contact the cell surface must pass through that of the close contact, but in the case of those formed by the lamellipodium the area involved was essentially the same as that of the developing focal contact. In both groups the proximal ends of the focal contacts were initially 1–9 μm ahead of the margin of the close contact. As the focal contact continued to develop under a lamellipodium the close contact margin advanced, or close contact developed around the focal contact, until the latter was finally located within the main area of close contact (Fig. 6, 117–148 s). Focal contacts were formed by a restricted length of a microspike developing a persistent contact with the substrate, in half of the cases analysed at the visible tip and in the other cases along the length of the microspike. These isolated focal contacts similarly became surrounded by close contact, which developed in the usual manner and in association with a lamellipodium spreading up the length of the microspike (Fig. 6, 0–75 s). When first detected the focal contacts were stable against the clearly observable continued movement of the surrounding lamellipodium or microspike. The first appearance of these stable focal contacts was very rapid. The transition from a freely moving lamellipodium or microspike separated by *ca.* 100 nm from the substrate to one with a stable focal contact occurred in 1 s (or 1 frame interval) in 60% of the cases. In the remaining cases not more than 10 s elapsed before we could be certain that the earliest detectable stage of the focal contact had been reached.

In scanning electron micrographs local substrate adhesions appear to be associated with small processes extending forward from and under the base of the lamellipodium (Revel *et al.* 1974). However, in only one case (Table 1, Other) was a focal contact first detected at the extreme base of the lamellipodium where it developed continuous with, but protruding ahead of the close contact margin. Therefore, if this contact formed in association with a process such as that seen in scanning electron micrographs, the mode of formation is a rare phenomenon. Nevertheless, some 37% of the focal contacts formed within the close contact subsequently and transitorily assumed this relative position within 10–50 s after their initial appearance as the margin of the close contact withdrew slightly. The latter event was frequently associated with the lamellipodium lifting away from the substrate, a condition that would be necessary to view under the lamellipodium in the scanning EM.

The focal contacts took approximately 15–180 s (mean with S.D. = 66 ± 38 s) to attain full density in the film and thus their typical separation distance. Although the initial appearance could be pinpointed to within a few frames and thus seconds, the individual times are considered approximate because the end-point was more difficult to establish visually in terms of full density. However, the wide range of times was

clearly real and not due to this difficulty. The focal contacts were elongate, 1–2 μm long and *ca.* 0·25 μm wide, when first detected, irrespective of where formed, and the long axis was more or less parallel to the local direction of spreading (Fig. 6). The increase in length to the typical 2–10 μm (Izzard & Lochner, 1976) occurred more frequently and to a greater extent at the proximal end of the developing focal contact. Occasionally 2 focal contacts, which formed in succession and in line with each other, would lengthen and become continuous with one another. The developing focal contact usually remained stationary relative to the substrate, but occasionally the orientation of the long axis would change a few degrees as the contact developed. Once fully formed the focal contact remained stationary (Figs. 1, 3).

From this detailed analysis it is clear that the cell surface associated with lamellipodia, microspikes and the newly formed close contact is competent to develop the localized very close and mechanically stable separation distance of the focal contacts, and that each type of cell surface produces significant numbers of focal contacts (Table 1). In addition, from the location and time at which focal contacts were formed in the close contact, there appears to be a limitation on the competency of the marginal cell surface to form focal contacts relative to its initial protrusion as part of a lamellipodium and subsequent incorporation as close contact under the advancing leading lamella.

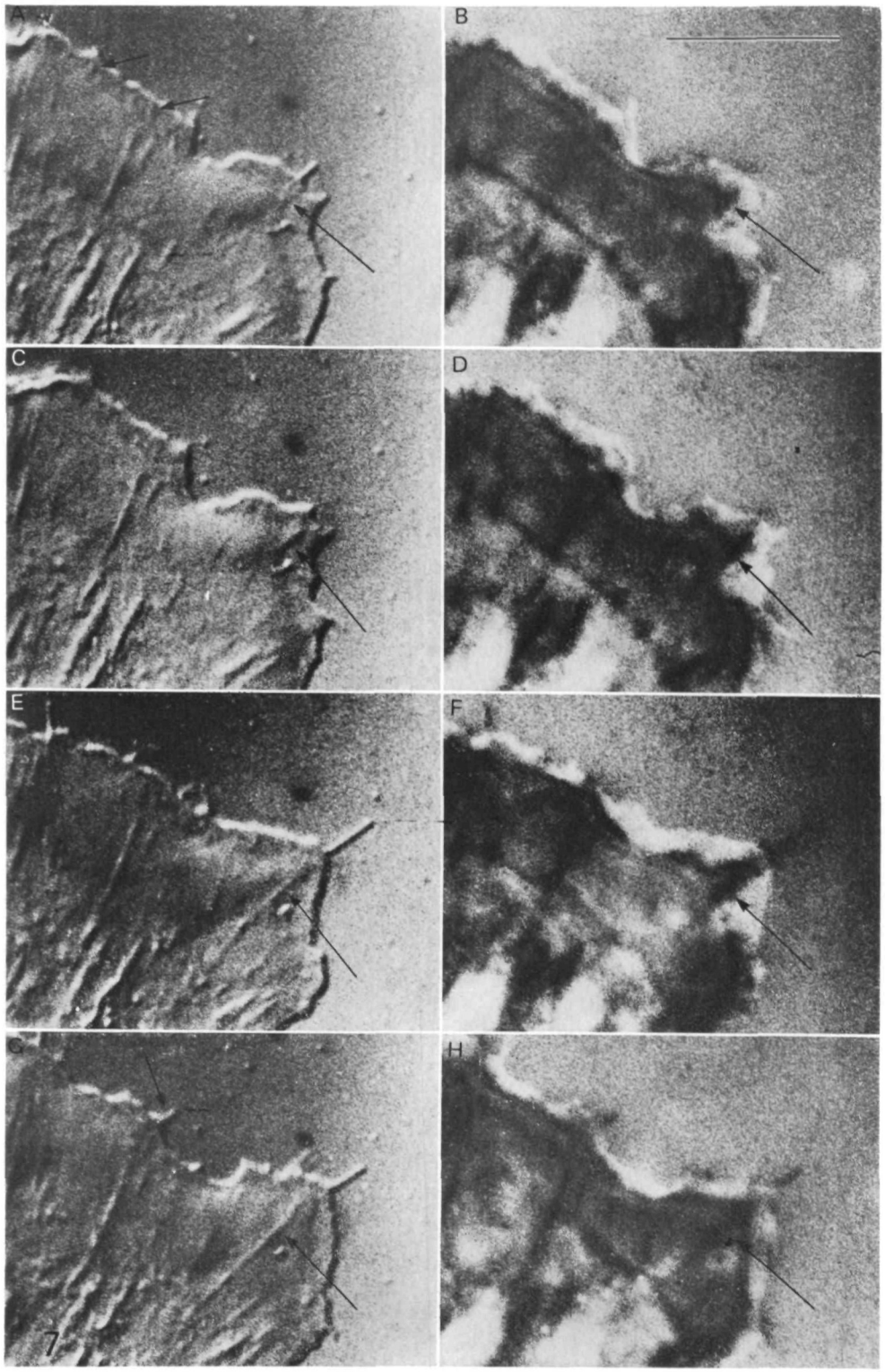
Over a given period of time there was a tendency for focal contacts to be formed preferentially but not exclusively either within the close contact or under lamellipodia and microspikes. This feature was not due to different histotypic origins of individual cells from the explants, since the tendency clearly changed with time for one leading lamella or differed in separate leading lamellae of the same cell. From a qualitative examination of a large number of films, in addition to those analysed in detail, formation of focal contacts within the close contact appears to be prevalent in wide, steadily advancing lamellae with convex margins and often with somewhat short lamellipodia. In contrast, narrow leading lamellae form focal contacts preferentially by their lamellipodia or microspikes. In addition, when the lamellipodia are extensive (8–14 μm in length) focal contacts are formed preferentially beneath them. We have noted that such lamellipodia are common for a short period of time following the rapid advance of the trailing tail of a cell, which is consistent with the observations of Chen (1978), and when spreading is about to be resumed or initiated respectively by a quiescent leading lamella or along the concave side of a cell.

Relationship between formation of focal contacts and associated cytoplasmic structures

The precise coincidence of fully developed focal contacts with cytoplasmic fibres of the same dimensions as the contact or the peripheral ends of longer fibres (Izzard & Lochner, 1976) implies that the formation of both are integrated processes. Therefore we followed the relative time course for the formation of individual focal contacts and their associated cytoplasmic structures using the combined DIC/IRM system described in the Methods. The images in the 2 modes were again recorded alternately at 10 to 12-s intervals on 35-mm film, the exposures occupying 7–9 s of the interval. A total of 49 individual focal contacts derived from 4 different cell preparations were analysed from the 35-mm film records as detailed in the Methods.

A typical example is given in Fig. 7 (A–D are consecutive DIC/IRM images photographed 10 s apart). A small linear structure (1·6 μm long) oriented parallel to the direction of spreading and located within the lamellipodium immediately ahead of the shadow-cast boundary of the leading lamella (Fig. 7A) coincided with a slightly shorter (1·4 μm long) focal contact in the following IRM image (Fig. 7B). The same linear structure was more prominent 10 s later (Fig. 7C) and the focal contact now matched it in length (Fig. 7D). The linear structure remained essentially unchanged for a further 60 s but then elongated centripetally through the leading lamella to form a typical stress fibre 8·2 μm long during the next 80 s (Fig. 7E). The focal contact was now located at the distal end of the fibre (Fig. 7F). The fibre persisted, increasing further in length to 9·8 μm and becoming more sharply defined (Fig. 7G), while the associated focal contact had also increased in length but only to 2·7 μm (Fig. 7H). The sequence shows a close temporal coupling between the formation of the focal contact and initial fibre, both being similar in shape, dimensions and orientation, and the subsequent growth of the stress fibre back into the leading lamella from the initial fibre. Consistent with our previous description (Izzard & Lochner, 1976), not all fibres subsequently elongated; some remained the same dimensions as the focal contact.

If the focal contact in terms of decreasing separation distance developed at the same time as the initial fibre, one would expect with equal frequency that either the focal contact or the fibre would be recorded first in the alternating DIC/IRM photographs. However, in only 5 out of the 49 cases examined was the focal contact recorded first. In the remaining 44 cases (90% of the time) a linear structure of varying form (see below) was present in 1 to 4 DIC images preceding the first IRM image showing a focal contact, i.e. from 10 to 70 s before. Thus there was a clear bias for the formation of the linear structure to occur *before* the formation of a focal contact. In 20 of these cases the linear structure was a distinct microspike (2–6 μm in length, Fig. 7E, G) which formed a focal contact directly as described in the preceding section, and one would expect the observed temporal sequence in these cases. Nevertheless the remaining 24 times that the linear structure preceded the focal contact still represents a strong bias over the 5 times that the reverse order was found. In 6 of these 24 cases the initial structure observed was a microspike which lost its identity as a lamellipodium spread up its length. The focal contacts then formed under or at the base of the lamellipodium in the position of the original microspike and in 3 of these 6 cases under a fibre left within the lamellipodium (presumably the core of the microspike – see below). The linear structure which preceded the focal contact in the remaining 18 cases was either a distinct fibre (1–2 μm long) similar to those in Fig. 7A lying within the lamellipodium approximately parallel to the direction of advance (9 times), a short projection (less than 2 μm long) from the edge of the lamellipodium (Fig. 7G) (6 times), or a projection and fibre combined (3 times). In 8 of these 18 cases the fibre or projection was present in 2 to 4 successive DIC images (30–70 s) before the focal contact appeared. For the most part (15 times) the focal contact was first detected at the boundary between the close contact and the base of the lamellipodium, in the remaining 3 cases it was detected under the lamellipodium.


A
B
C
D
E
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G
H

Analysis of cine films (60 frames/min) of the DIC image showed that the fibres in the lamellipodium, projections and microspikes are interchangeable structures. Fibres could form *de novo* within the lamellipodium and move freely with it. New microspikes often developed by extending from a projection of the edge of the lamellipodium or more rarely in line with a fibre in the lamellipodium. Conversely withdrawal of the lamellipodium on both sides of a projection or fibre left a structure indistinguishable from a microspike which was freely motile and thus not a retraction fibre attached to the substrate. As noted in the 35-mm films, advance of the tip of the lamellipodium relative to that of the microspike invariably in this analysis left a fibre in the lamellipodium in place of the microspike. From these observations we conclude that the fibres in the lamellipodium and the core of the microspikes have a common structural identity.

The conclusion is consistent with EM studies on other cells. Thus the bundle of parallel microfilaments, which forms the core of the microspike, often extends beyond the base of the microspike through the motile cell margins or lamellipodia (Buckley, 1975). Similar bundles of microfilaments lie within the cell margin (Small & Celis, 1978; Small, Isenberg & Celis, 1978). In both cases the bundle of microfilaments becomes less densely packed as it approaches and enters the region of the cell containing microtubules and 10-nm filaments, i.e. presumably the leading lamella. Labelling with myosin subfragment-1 shows these bundles of microfilaments to be actin-like (Small & Celis, 1978) in agreement with the localization by immunofluorescence of actin in microspikes (Heggeness, Wang & Singer, 1977) and in radial fibres in the ruffling margins of various spreading cells (Lazarides, 1976). In addition, HVEM images show short microspikes as little as 0·3 μm in length, the core of which also penetrates into the thin motile cell margin (Buckley, 1975). These latter structures may be equivalent to the projections of the lamellipodia described above in living chick heart fibroblasts.

Fig. 7. Formation of focal contact and associated cytoplasmic fibre. Selected pairs of consecutive DIC (left column) and IRM (right column) images. Exposures for left-right pairs were initiated 10 s apart; exposure time for both, 7 s. The times between the DIC exposures were 20 s (A–C), 140 s (C–E) and 120 s (E–G). Scale bar, 10 μm.

The long arrow in each photograph identifies the cytoplasmic fibre (DIC) or focal contact (IRM) described in the text. In A the fibre lies within the lamellipodium immediately below a second fibre which appears to continue as a short projection (<2 μm long) from the edge of the lamellipodium. The identity of this projection was almost lost 20 s later (C) as the lamellipodium advanced. Short arrows in A identify similar fibres within the lamellipodium. Focal contacts were not associated with these fibres 10 s later (B). The short arrow in G indicates another projection contrasting in length with the microspike in E and G. The dark fringes running parallel to the leading edge in the IRM series resulted from cell thickness and should not be confused with the focal contacts oriented perpendicular to the edge. The distal fringe parallels the shadow-cast edge of the leading lamella in the DIC series.

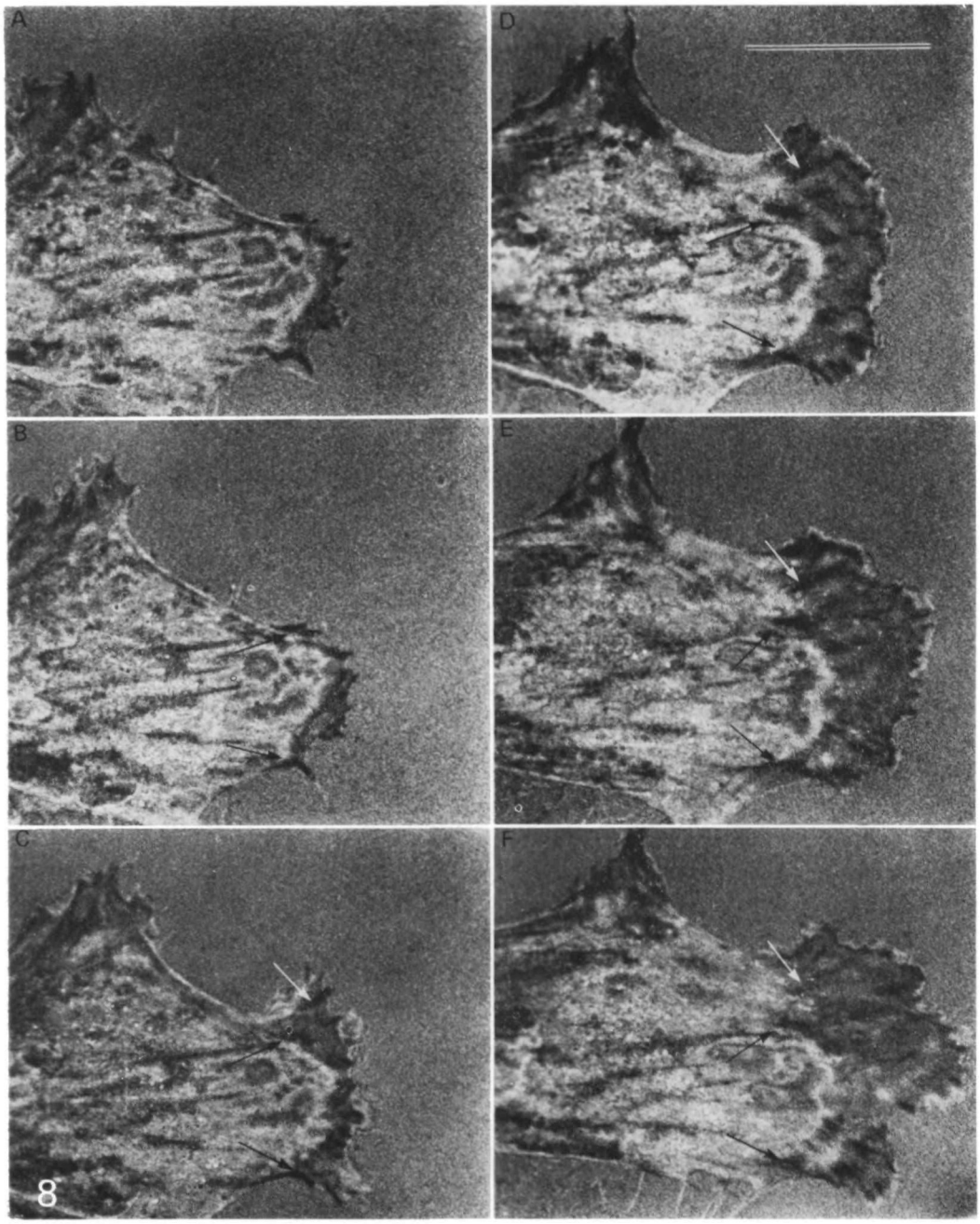

Fig. 8. Changes in cell-to-substrate contacts on cessation and resumption of spreading. Series printed from 16-mm cine film of IRM image; 4 min between each consecutive print. The right leading lamella had been stationary for 20 min prior to A and remained so through B. Arrrows in B mark focal contacts which persisted throughout the stationary period. The same focal contacts are marked in C to F (arrows) and are fading in F. Similarly, the new focal contact in C (uppermost arrow) is fading in F (uppermost arrow). See text for further details. Scale bar, 20 μm.

Specific requirement for the close contact in spreading

The continual formation of close contact across the entire margin of the leading lamella during spreading (Fig. 1) implies a functional relationship between the 2 processes. The relationship is emphasized by changes that occur in the close contact when the leading lamella ceases and then resumes spreading. When spreading ceases there is an increase in the dissection of the close contact by areas with a separation distance of *ca.* 100 nm, first under the nuclear region and then progressively under the leading lamellae, until only a narrow band of close contact, often highly dissected, remains at the tips of the leading lamellae (Izzard & Lochner, 1976). This condition under a stationary lamella is illustrated in Fig. 8A–B. The resumption of spreading along the upper margin of the lamella produced a triangular area underlain by new close contact and a noticeable reappearance of lamellipodia in the image (Fig. 8C). With the initiation of spreading across the entire leading margin of the lamella, a distinct band of uninterrupted close contact was formed (Fig. 8D) and the width of the band continued to increase throughout the illustrated sequence (Fig. 8E, F). By reference to the focal contacts present at the edge of the stationary lamella (Fig. 8B, arrows) and identified in the subsequent stages, it is clear that the increase in the width of the band of close contact occurred solely by active spreading of the cell margin. The close contact did not redevelop centripetally under the leading lamella. Similarly the cell partially included at the right of Fig. 1 had been stationary for the 1·5 min of the record prior to Fig. 1A and probably longer. The close contact under this cell was dissected by areas of greater separation distance (Fig. 1B). The cell began to spread toward the bottom-left of the figure (Fig. 1E–L) and produced an area of undissected close contact but only under the newly spread region of the lamella. The contact under the original stationary lamella was not restored to a uniform close contact. These examples show that both the formation and persistence of the close contact are associated specifically with the spreading process. When the latter ceases, the close contact is lost under the leading lamella.

The role of the close contact in spreading was evaluated further by examining the local formation of the close contact and advance of the leading lamella relative to the distribution of existing focal contacts. Although 41 % of the focal contacts were formed by lamellipodia or microspikes (Table 1) and therefore ahead of the close contact, the advance of the close contact margin was not exclusively dependent upon this spatial relationship. In a given line of advance, as exemplified by the transects in Fig. 3, the close contact could advance up to 10 μm ahead of an existing focal contact before a new focal contact was formed at its margin (see centre transect between 8 and 15 min).This advance of the close contact was not dependent on the prior formation of focal contacts ahead of the close contact and to either side of a given transect. In the illustrated examples of the formation of close contact at the tip (Fig. 5) and the base (Fig. 5, 39 s; Fig. 6, 94 s) of the lamellipodium the new areas of close contact lay ahead of a line drawn between the 2 nearest focal contacts. Sizeable areas of close contact were developed in this manner, e.g. under the whole distal 5 μm of the lower leading lamella in Fig. 1J.

Local advance of the close contact ahead of existing focal contacts did not involve just the apposition of the thin lamellipodium to the substrate; it was also paralleled by the advance of the thicker leading lamella. Throughout Fig. 1 the margin of the close contact, and hence the base of the lamellipodium, corresponds spatially with the edge of a distinct shadow-cast structure running a few micrometres behind the extreme edge of the cell in the DIC image (compare especially A with B and E with F in Fig. 1, and see Izzard & Lochner, 1976). By reducing the INA of the IRM to *ca.* 0·5 so that changes in cell thickness could be evaluated (Izzard & Lochner, 1976), the distal edge of the shadow-cast structure was shown to be the thicker edge of the leading lamella proper, as distinct from the thin lamellipodium extending ahead. The close correspondence referred to above in Fig. 1 and found between each successive DIC and IRM image throughout this and other sequences implies that cytoplasm flows into the base of the lamellipodium as the close contact advances, thus effecting a parallel advance of the leading lamella proper. The close correspondence in the case of Fig. 1 I and J, and in other recorded examples, shows that a local advance of cytoplasm, and thus of the leading lamella, can occur in the presence of only the close contact and does not require the simultaneous formation of focal contacts and associated cytoplasmic fibres.

Since the close contact is formed only at the spreading margins of the leading lamellae, its occurrence under the entire cell in some cases must require the persistence of the contact with the substrate and the eventual movement of the entire cell over the area of the substrate occupied by the close contact. This was observed to occur in several cases. The cell in Fig. 8 continued spreading in the same direction for a further 1·5 h during which time the trailing portion of the cell and the smaller lamella toward the top of the figure both advanced rapidly and the entire cell came to overlie the close contact originally formed at the leading edge in the figure. Persistence of the close-contact separation distance under the centre of some cells, even an hour or more after its initial formation at the leading edge as in the above example, does not result exclusively, and perhaps not at all, from tension developed in the spread cell holding the cell surface close to the substrate. We have repeatedly observed that when the trailing portion of the cell advanced rapidly (e.g. Fig. 1 G, I, K) the close contact separation distance under the centre of the cell and the leading lamellae remained unchanged (e.g. Fig. 1 H, J, L). Persistence of the close contact must result then from a maintenance of adhesion to the substrate, and loss of the close contact when spreading ceases must result from a coordinated loss of adhesion.

Changes in the distribution of focal contacts relative to changes in movement

In the preceding sections we have documented the formation of focal contacts at the spreading margin of the cell, their stationary position relative to the substrate, and their progressive loss under the leading lamella after the margin has further advanced some 5–20 μm. These characteristics are clearly summarized in the transects derived from cine films (Fig. 3). When spreading ceases the progressive loss of focal contacts continues under the leading lamella (Fig. 4). However, the focal contacts last formed, and located at the margin, persist during the stationary period (Figs. 4, 10–40 min; 8 A, B). These focal contacts in the absence of close contact are sufficient to maintain

the spread condition of the cell. When spreading resumes new focal contacts are formed again at the advancing margin (Figs. 4, 8C–F). Those present during the stationary period continue to persist until a field of focal contacts has been formed (Fig. 4, 40–65 min; Fig. 8C–D, arrows). Then they begin to fade (Fig. 8E) and the progressive loss of focal contacts furthest from the leading edge ensues, characteristic of continued spreading. Thus there is a complex control of the persistence of focal contacts relative to spreading such that the persistence differs depending on the position of the focal contact with respect to the margin of the lamella.

If spreading is not resumed by a quiescent lamella, but is initiated elsewhere on the margin of the cell, a spontaneous change in the overall direction of movement occurs (Abercrombie & Ambrose, 1958). The quiescent lamella with time frequently becomes the tail or posterior end of the cell. We have observed the continued persistence of the marginal focal contacts throughout this change in direction of movement, and the origin of the focal contacts under the posterior end of the cell is directly explicable in these terms. [Abercrombie, Dunn & Heath (1977) previously raised the question of the origin of the posterior focal contacts, but concluded that they do not, it seems, represent focal contacts originally formed at the front of the cell, since these fade out as the cell passes over them.] There is usually a further loss of focal contacts at the new trailing end so that the originally wider distal margin of the lamella becomes narrower and the typical tapering shape of the tail is acquired. It should be noted that the final persistence of one or two focal contacts is sufficient to maintain the tail extended and this contrasts markedly with the large numbers present under a spreading lamella on the same cell (up to 100, Izzard & Lochner, 1976). Occasionally lamellipodia or microspikes will reappear transitorily at the trailing end, and only then are new focal contacts formed at this location and by the methods described. In these contexts the overall shape of the chick heart fibroblast spread on a glass substrate at any instant in time can be considered a function of the prior changes in direction of spreading, the formation of focal contacts in these directions of spreading, and the persistence of the focal contacts after spreading ceases.

DISCUSSION

We have analysed the formation of new cell-to-substrate contacts during the marginal spreading of leading lamellae of chick heart fibroblasts. Although considerable attention has been given recently to the organization and function of the structurally overt stress fibres in cultured cells, as we have argued above, these fibres can be effective in entraining the cell in forward movement only up to the most peripheral points of insertion of their component microfilaments, namely the focal contacts (see Izzard & Lochner, 1976; Heath & Dunn, 1978). Accordingly special attention was paid in this study to the nature of the extreme advancing margin of the cell and how new cell-to-substrate contacts are formed relative to the advancing margin and existing contacts.

The characteristic motile behaviour of the leading edge of cultured fibroblasts, long recognized to be associated with net advance of the edge (Abercrombie & Ambrose, 1958), results from the motility of lamellipodia at the leading edge (Abercrombie *et al.*

1970*b*, 1971). By use of the IRM, we determined that the lamellipodium protrudes from the edge of the lamella free from the substrate, i.e. separated by a distance of 100 nm or more (Lochner & Izzard, 1973; Izzard & Lochner, 1976). The free protrusion occurs not only at an angle into the medium as previously described in side view (Ingram, 1969; Harris, 1969), but also when the lamellipodium protrudes parallel to the substrate. The latter could not be determined directly from side-viewing (Ingram, 1969), although Abercrombie *et al.* (1970*a*) inferred that the fluctuating edge of the cell is unlikely to be firmly attached to the substrate at any time, since retraction threads are not pulled out during withdrawal.

The close contact has a relatively uniform separation distance of *ca.* 30 nm from the substrate and extends under much of the leading lamella (Lochner & Izzard, 1973; Izzard & Lochner, 1976), features which could not be readily assessed in EM studies. The data presented in this paper show that the formation of the close contact is singularly associated with the process of spreading, implying that it is an integral component in the mechanism of spreading. Thus the close contact is lost completely or at least becomes highly dissected when spreading of a leading lamella ceases. It is reformed when spreading resumes but then only under the newly spread region of the leading lamella. The close contact can develop ahead of existing focal contacts and therefore its formation is neither temporally nor spatially dependent upon the formation of focal contacts, nor by inference upon stress fibres inserting into the focal contacts.

In our analysis of the advance of the margin of the close contact relative to the presence or absence of a lamellipodium, we have shown that new close contact forms only where the cell margin has first extended free of the substrate as a lamellipodium. The detailed frame-by-frame analyses demonstrated that new close contact is formed by the undersurface of the lamellipodium subsequently lowering to the substrate. As we have stressed in the Results, at no time did the extreme distal edge of the cell advance and simultaneously form new close contact with the substrate. These analyses were based only on the IRM image and thus cell-to-substrate separation distance. However, other criteria support the interpretation that the extending margin is in fact a lamellipodium. Firstly the extending margin undergoes the undulatory behaviour and intermittent lifting away characteristic of a lamellipodium, both of which can be followed with the IRM. Secondly the thickened edge of the leading lamella, seen as a shadow-cast structure in the DIC image (Fig. 1A), typically lies a few micrometres behind the extreme distal edge of actively spreading cells. Thus the extending margin was not the thicker leading lamella advancing free of the substrate. A primary role of the lamellipodium in extending the margin of the cell and forming new close contact with the substrate is established from these studies.

The reason why extension of the cell margin is temporally separate from formation of the close contact in chick heart fibroblasts is not known. Lifting of the cell margin away from the substrate during ruffling has been explained in terms of greater forces applied to the upper surface of the margin (Ingram, 1969; Harris, 1973*b*), but it is not immediately clear how a similar effect could hold the lamellipodium at *ca.* 100 nm from the substrate as it advances several micrometres ahead of the close contact, especially

in view of its ability to undulate along its length. However, the advancing tip of the lamellipodium could initially be non-adhesive and then become competent to form close contact after its extension. The labile periods in the formation of close contact, which were more prevalent under the distal than the proximal portions of the lamellipodium, may be an expression of such a change in adhesiveness.

The protrusion of the lamellipodium involves the forward movement of cytoplasm. However, the volume moved is small compared with the leading lamella since the lamellipodia are thin, typically 110–160 nm (Abercrombie *et al.* 1971). Continued advance of the cell margin must involve the further movement of cytoplasm into the base of the extended lamellipodium in such a way that with time the latter becomes the thicker leading lamella. As we have evaluated in the Results, advance of the thicker leading lamella parallels in time and space the advance of the close contact across the whole spreading margin of the lamella. This parallel includes regions where the close contact has advanced well ahead of existing focal contacts and associated cytoplasmic fibres (Fig. 11 and J). Therefore in the latter specific cases movement of cytoplasm into the base of the lamellipodium was not dependent on the prior formation of stress fibres or focal contacts. Instead, and on the basis of these observations, we propose that the close contact provides the adhesion required to transmit to the substrate the forces involved in the forward movement of cytoplasm and advance of the leading lamella.

The proposal is consistent with and further supported by the following points. First, the limitation of stress fibres in being able to effect marginal spreading beyond their most distal points is overcome. Second, the advance of the margin of the leading lamella is continuous across widths of several micrometres, which is consistent with the pattern of advance of the close contact but not with the discrete and discontinuous nature of the focal contacts and stress fibres. Third, the formation and persistence of the close contact are singularly associated with marginal spreading. Fourth, the establishment of patches of close contact ahead of the existing close contact and margin of the leading lamella shows that the appropriate temporal sequence can occur, i.e. adhesion followed by movement. Finally the proposal depends upon the close contact being adhesive, for which there is evidence. For example, only close contact is formed between glass substrates and rabbit peritoneal neutrophil granulocytes (Armstrong & Lackie, 1975), macrophages, some sarcoma cells and fibroblasts newly emerged from explants (Abercrombie *et al.* 1977). Furthermore, the pattern of close contact advance described here (i.e. without significant withdrawals, Fig. 3) is consistent with its adhesive nature, and retraction fibres can be drawn out from regions where only close contact is present (Izzard, unpublished observations) in addition to where focal contacts are present as described by Abercrombie & Dunn (1975). Interestingly, Radice (1978) reports that focal contacts and microfilament bundles are seen only in retracting or slowly advancing epithelial cells cultured from the epidermis of *Xenopus* tadpoles, and suggests that the close contact in these cells is sufficient to support movement.

While the formation of close contact satisfactorily accounts for the forward movement of the margin of the leading lamella, it is generally considered that tension developed within the stress fibres plays a major role in the advance of the cell body

toward a given leading lamella (Abercrombie *et al.* 1971; Huxley, 1973; Abercrombie *et al.* 1977; Heath & Dunn, 1978) and ultimately of the trailing portion of the cell (Wessells *et al.* 1973). The continual formation of focal contacts at the leading edge and development of stress fibres from the new focal contacts, together with the loss of both behind the leading edge, are consistent with the requirement to transfer tension in the cell progressively to the advancing margin and with the role of the stress fibres in drawing the cell forward. In addition, the formation of focal contacts will strengthen in a punctate manner the adhesion afforded by the close contact, a feature which may be crucial in determining whether or not the ground gained by marginal spreading and close contact formation is retained so that the cell will attain the attenuated fibroblastic form.

The formation of focal contacts was restricted to microspikes, lamellipodia, or to the close contact usually immediately but not more than 1–2 μm behind its margin. Significant numbers were formed at each site (Table 1) and there is probably little intrinsically different in the mechanisms of formation, since the time course for development, initial size and orientation, and final form were not obviously different at the 3 sites. The restricted location at which focal contacts were formed is directly explicable in terms of the development of a linear structure, prior to the first appearance of the focal contact, in the form of either a microspike, short projection or fibre within the lamellipodium, which could be traced to the final fibre associated with the focal contact. Moreover, the initial appearance of focal contacts, as stable adhesions, was very rapid when they were formed by lamellipodia and microspikes. This feature indicates that the external adhesive properties of the focal contact are developed on the lamellipodia and microspikes while they are freely motile. In other words, close approximation of the cell surface to the substrate in these cases does not appear to be the trigger for a series of events leading to the development of the external adhesive properties, as might have been argued had all focal contacts formed within the close contact. If one postulates that the external adhesive properties of the focal contact develop locally together with, or as a consequence of, the formation of the initial linear structures, then the rapid formation, and characteristic shape, size and orientation of the focal contacts when first detected are readily explained in addition to their restricted locations. However, the variable position of the focal contacts along the microspikes, and their relatively shorter length, imply that only a local length of the surface of the microspike develops the adhesive properties of the focal contact.

Whether the linear structures which precede the focal contacts contain the fibrous plaque material and/or microfilaments typically associated with the fully formed focal contact (Abercrombie *et al.* 1971) remains to be determined directly. However, we observed that the microspikes, projections and fibres within the lamellipodium could give rise to each other and, from these changes and by comparison with EM data from other cells, have evaluated in the Results that the structure common to each is probably equivalent to the core of the microspike, i.e. a bundle of actin-like microfilaments. This would be consistent with Heath & Dunn's (1978) observation that every focal contact was associated with a bundle of microfilaments at the HVEM level and explain their failure to find evidence for a delay between the formation of the focal

contact and microfilament bundle in chick heart fibroblasts. These authors do not appear to have examined the reciprocal alternative which we have shown here, i.e. that a cytoplasmic specialization precedes the formation of the focal contact with the substrate. From immunological studies myosin and α-actinin are associated with focal contacts identified by IRM (Badley *et al.* 1978; Wehland, Osborn & Weber, 1979) and α-actinin with the plaque material at the EM level (Schollmeyer *et al.* 1976). However, myosin is absent or severely depleted from the ruffling margin and microspikes of NRK fibroblasts (Heggeness *et al.* 1977) and thus may not be a component of the initial fibres we described (see below). α-actinin is concentrated in the ruffling margin of spreading cells and, although it is not clearly organized into short radial fibres within the margin, as is actin (Lazarides, 1976), could still be involved in the formation of the initial fibre linking the thin filaments to the membrane, as suggested in other situations in non-muscle cells (e.g. Lazarides, 1976; Schollmeyer *et al.* 1976).

Various authors have postulated in general terms that adhesion to the substrate following extension of the cell margin provides an anchorage against which contractile proteins in the cell could pull the cytoplasm forward (Ingram, 1969; Harris, 1973*b*; Huxley, 1973). As we have suggested above, one can now think specifically in terms of the close contact functioning in this role during the marginal spreading of fibroblasts, as distinct from a role of the focal contacts in drawing the bulk of the cell forward. Significant in the context of the role of each contact is the demonstration by Small & Celis (1978) that individual thin filaments, oriented diagonally in a band at the leading edge of human skin fibroblasts and 3T3 cells and grouped into radial bundles of parallel filaments within the band, possess the same polarity, i.e. the arrowheads produced by myosin subfragment-1 point toward the cell body. As these authors state, the polarity of the actin-like thin filaments is such in both cases that an interaction with myosin could move the cytoplasm forward. Although it is not explicitly clear, the band of thin filaments in their preparations is probably derived at least in part from the lamellipodium, since microtubules and 10-nm filaments are excluded from the band (see Abercrombie *et al.* 1971; Buckley, 1975). If this proves correct, then anchorage of unipolar actin filaments in the lamellipodium when the close contact forms could account for the temporal and spatial coupling of the advance of the close contact and leading lamella which we have described. Heath & Dunn (1978) found that close contacts are often associated with a concentration of loosely organized microfilaments in chick heart fibroblasts and suggested that the association may serve a similar function to that of the focal contact-microfilament bundle, i.e. moving the main cell-mass forward. Here we emphasize the role in marginal spreading. The radiating bundles of thin filaments described by Small & Celis (1978) seldom extend beyond the marginal band of thin filaments into the adjacent cytoplasm and, as discussed in the Results, may correspond to the fibres we have described in the living lamellipodium. Thus again, anchorage of a preformed bundle of uniformly polarized thin filaments when a focal contact forms could lead to the development of a force applied locally to the substrate which, by virtue of the greater density of filaments in the bundle, could be greater per unit area of substrate contacted than in the case of the close contact.

We have described the centripetal development of stress fibres from the proximal end of the fibre that precedes and is initially associated with the focal contact. This direction of development is consistent with the polarity of thin filaments in the bundles described by Small & Celis (1978), if the development results from an interaction with myosin and other thin filaments in the cytoplasm. [The same direction of development was described for microfilament bundles from points of cell-to-cell contact in chick heart fibroblasts (Heaysman & Pegrum, 1973)]. Previously Heath & Dunn (1978) speculated that formation of the focal contact provided a fixed point which, together with the meshwork of microfilaments around the nucleus, would allow a near isometric contraction to occur, thus orienting contractile elements in the cytoplasm into a straight bundle. Again the direction of development of the stress fibre is consistent with such an effect, bearing in mind the probable higher density of microfilaments in the initial fibre associated with the focal contact versus the more diffuse microfilaments in the cytoplasm. However, one cannot rule out as a part of the process of stress fibre development polymerization of actin from the plaque region and/or lateral aggregation of filaments through an interaction with, for example, filamin (Wang & Singer, 1977) or other actin-binding proteins (e.g. Bryan & Kane, 1978).

If formation of close and focal contacts anchors actin filaments to the substrate, we consider it likely that the formation of the contacts must also trigger, or at least enhance, an interaction between myosin and the anchored filaments if the marginal cytoplasm and cell are to be entrained in forward movement. The basis for this consideration is that myosin is absent or severely depleted from microspikes and ruffles (presumably non-adherent lamellipodia) in NRK fibroblasts, while actin is concentrated in these structures (Heggeness *et al.* 1977). The exclusion of myosin implies that it is unable to interact with actin in microspikes and lamellipodia, especially in view of the polarity of actin filaments in the cell margin (Small & Celis, 1978). Thus formation of the contacts is the logical step at which the interaction would be triggered or enhanced. This proposal differs from the general hypothesis of Harris (1973*b*) in that development of force is triggered by contact with the substrate, as opposed to an existing force applied to the plasma membrane being converted into a forward traction on the cytoplasm following adhesion of the membrane to the substrate. Chen (1979) reports that the birefringence in lamellipodia and microspikes increases immediately after attachment to the substrate and upon retraction, a change which is paralleled by an increase in alignment of microfilaments at the EM level, and suggests that the changes result from tension applied to the system. These changes are consistent with our discussion above, but at present do not allow a distinction between Harris (1973*b*) alternative and ours.

In summary we propose from our studies and those of other authors discussed above the following basic sequence of events during the spreading and net movement of fibroblasts on planar substrates: (1) extension of the cell margin free of the substrate as a lamellipodium or microspike; (2) formation of close contact by the extended lamellipodium; (3) movement of cytoplasm into the base of the lamellipodium resulting in an advance of the leading lamella, the required forces being transmitted to the substrate via the close contact; (4) formation of focal contacts and stress fibres; and (5)

movement of the cell body through forces developed in the stress fibres. Although (4) and (5) are temporally and spatially superimposed upon, they are not a prerequisite for (2) and (3). The unique properties of the lamellipodia and microspikes in extending the cell margin and forming advanced adhesions preparatory to further cytoplasmic movement are established in this study, a uniqueness emphasized by Bragina, Vasiliev & Gelfand (1976) in the context of the formation of stress fibres.

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