The hand of *Anteosaurus magnificus* (Dinocephalia: Therapsida) and its bearing on the origin of the mammalian manual phalangeal formula

T. Rowe* and J.A. van den Heever†

*Department of Geological Sciences, University of Texas at Austin, Texas, USA 78713
and †South African Museum, P.O. Box 61, Cape Town 8000, South Africa.

It is generally believed that reduction followed by loss of manual phalanges is an evolutionary trend that affected many therapsid taxa convergently, and that the ancestral mammalian formula of 2-3-3-3-3 evolved independently a number of times. This is based on previous interpretations of the hand in several dinocephalians, and the presence of tiny disc-like phalanges in some Gorgonopsia and Cynodontia. However, new information on the hand of the holotype of Micranteosaurus parvus *(junior synonym of* Anteosaurus magnificus*) reviewed with recent data on other Dinocephalia shows the formula 2-3-3-3-3 to be more widely distributed than previously believed. When viewed in the context of a therapsid phylogeny based on many characters of the skull and postcranial skeleton, it is most parsimonious to conclude that the formula 2-3-3-3-3 evolved only once, as an apomorphy of the unnamed therapsid taxon comprising Dinocephalia, Gorgonopsia, Dicynodontia, Therocephalia, and Cynodontia (which includes Mammalia). The disc-like phalanges present in some gorgonopsians and cynodonts are most simply viewed as reversals or* de novo *ossifications that evolved convergently in these two taxa. Thus convergence probably did affect therapsid phalangeal evolution, but available data support the conclusion that it was less common and followed a different pathway than previously believed.*

Daar word algemeen aanvaar dat die reduksie en daaropvolgende verlies van falanges in die manus 'n evolusionêre tendens is wat by baie taksa van die Therapsida voorkom en dat dit aan konvergensie toegeskryf moet word. Gevolglik moes die voorvaderlike soogdierformule van 2-3-3-3-3 'n aantal kere onafhanklik ontstaan het. Bostaande is gebaseer op vorige vertolkings van die manus by 'n aantal dinokefaliërs, asook die teenwoordigheid van klein skyfvormige falanges by sommige Gorgonopsia en Cynodontia. Nuwe inligting omtrent die manus van die holotipe van Micranteosaurus parvus *(junior sinoniem van* Anteosaurus magnificus*), tesame met die resente data oor ander Dinocephalia, toon egter dat die formule 2-3-3-3-3 'n wyer verspreiding geniet as wat voorheen besef is. Gesien in die lig van 'n filogenetiese ontleding van die Therapsida, gebaseer op 'n groot aantal kenmerke van die skedel en die postkraniale skelet, is die mees ekonomiese gevolgtrekking dat die formule 2-3-3-3-3 slegs een keer, as 'n apomorfie van die onbenoemde takson Dinocephalia, Gorgonopsia, Dicynodontia, Therocephalia en Cynodontia (wat soogdiere insluit), ontstaan het. Die skyfvormige falanges by sommige gorgonopsiërs en sinodontiërs word dus beskou as omkerings of* de novo*-verbenings wat konvergent by hierdie taksa ontwikkel het. Konvergensie het dus waarskynlik 'n rol in die evolusie van die falanges by die Therapsida gespeel, maar die beskikbare data toon dat dié verskynsel minder algemeen was en op 'n ander manier plaasgevind het as wat voorheen aangeneem is.*

In Amniota ancestrally the manual phalangeal formula was 2-3-4-5-3. This condition persisted in many early members of Synapsida[1] but was transformed in Therapsida. Phalanges were lost from digits III and IV until the formula 2-3-3-3-3 evolved. This formula was present in Mammalia ancestrally, although within various mammalian lineages there has been further loss of manual phalanges. It is generally believed that phalangeal loss, preceded in most cases by a stage where the phalanges became reduced or vestigial, is an evolutionary trend that affected most therapsid lineages to varying degrees.[2–6] The ancestral mammalian formula is thought to have been achieved independently in a number of lineages, including the Late Permian therapsid taxon Dinocephalia. It is currently believed that in Dinocephalia ancestrally there were four phalanges present in at least one digit in the manus, but that one of the four phalanges was lost in descendant dinocephalians, reducing the formula to 2-3-3-3-3. Boonstra[3–5] regarded the ancestral dinocephalian phalangeal formula to be 2-3-4-3-3, whereas Kemp[6] regarded it to be 2-3-3-4-3. Nevertheless, both authors agree that the formula 2-3-3-3-3 was convergently evolved within Dinocephalia, and that phalangeal loss was preceded by a stage in which reduced, vestigial phalanges were present. This argument is based on Boonstra's[3] interpretation of the holotype of *Micranteosaurus parvus*, (junior synonym of *Anteosaurus magnificus*), the only dinocephalian specimen in which four phalanges have been reported in digit III, and the interpretation of unusual phalangeal morphology in *Titanophoneus* by Boonstra[3] and Kemp.[6]

Further preparation of the holotype of *Micranteosaurus parvus* has now revealed that only three phalanges are present in its third digit. In addition, a recent review by Chudinov[7] has reaffirmed Orlov's[8] observation that only three phalanges are present in digit IV of *Titanophoneus*. Consequently, both of these taxa have the same formula as in all other adequately preserved dinocephalians. The formula 2-3-3-3-3 is thus more widely distributed than previously believed. Moreover, when its distribution is viewed in light of recent hypotheses of therapsid phylogeny that are based on a large sample of characters of the skull and postcranial skeleton,[9,10] it is more parsimonious to conclude that this formula evolved only once in therapsid history, as an apomorphy of the unnamed taxon comprising Dinocephalia, Gorgonopsia, Dicynodontia, Therocephalia and Cynodontia. In at least four species of Gorgonopsia and two cynodont species, more than three phalanges are present in digits III and/or IV, and these are usually viewed as vestigial remnants of the ancestral amniote formula. However, when all of the data on therapsid phylogeny are assessed, it is simpler to conclude that there was a parallel secondary increase in the number of digital elements in these species. Hence, convergence did occur in therapsid phalangeal evolution, but probably not in the way usually believed.

Micranteosaurus, and the manus of Dinocephalia

Micranteosaurus parvus (SAM 4323), originally described by Boonstra,[11] is a monotypic taxon consisting of a well-preserved snout with lower jaw, an unprepared partial series of articulated

caudal vertebrae, coracoid, proximal end of humerus, radius, nearly complete articulated manus, femur, fibula, and articulated tarsus.[11,12] Boonstra originally regarded *Micranteosaurus* as an adult specimen of very small body size, hence its designation as a new taxon. Subsequently, Boonstra[13] synonymised *Micranteosaurus parvus* with *Anteosaurus magnificus*, believing it to be juvenile of the latter. Chudinov[7] allowed the taxon *Micranteosaurus parvus* to stand, but King,[14] in her systematic review of the Dinocephalia, recognises the synonomy as do we. However, as a matter of convenience, the name *Micranteosaurus parvus* is used in this paper to refer solely to the type specimen that bears this name. Evidence of the juvenile stature of this specimen lies in its uniquely small size combined with apparently juvenile morphological features in the pectoral girdle, carpus and tarsus. The coracoid is a rounded disc that was apparently completely separate from the scapula and procoracoid, and the proximal carpal and proximal tarsal elements are loosely articulated, subspherical in shape, and lack distinctly formed articular sufaces (though articular facets are well formed on the distal carpals). In larger anteosaurid specimens, the coracoid is subtriangular, becoming firmly sutured or fused to the scapula and procoracoid, and in the few known specimens the proximal carpals and tarsals form complex shapes, with distinct articular facets.

In his initial description of *Micranteosaurus*, Boonstra[11] recorded the phalangeal formula of the manus as 3-3-4-4?-2. He later[15] extrapolated this to be the formula for Anteosauria generally, while in other adequately preserved dinocephalians he believed the manual phalangeal formula to be 2-3-3-3-3. In a subsequent survey of the dinocephalian manus, Boonstra[3] found his earlier description to be in error on several points. He had mistaken the left manus for a right, inverted the position of the radiale and ulnare, and given a reversed phalangeal formula. In his revised description of the specimen[3] and in subsequent papers,[4,5] he regarded its phalangeal formula to be 2-3?-4-3-3. As we argue below, further preparation of this material now suggests additional revision of our understanding of the hand of *Micranteosaurus*, including its phalangeal formula.

Few specimens of the dinocephalian manus are currently known. Although dinocephalian remains are relatively abundant in parts of the South African Karoo, in nearly all instances the skeletons are disarticulated to varying degrees, and the limb extremities are usually missing. Byrne[16] described the first known articulated dinocephalian manus in the moschopine *Moschoides romeri*, stating its phalangeal formula to be 2-3-3-3-3. Boonstra[3] claimed that the formula is 2-3-3-3-3 in an incomplete specimen

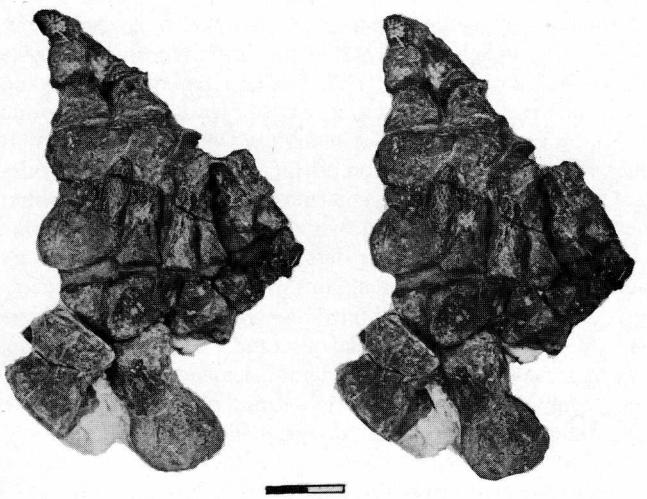

Fig. 1. Holotype of *Micranteosaurus parvus* SAM 4323. Stereophotograph of left manus in dorsal view. Scale 10 mm.

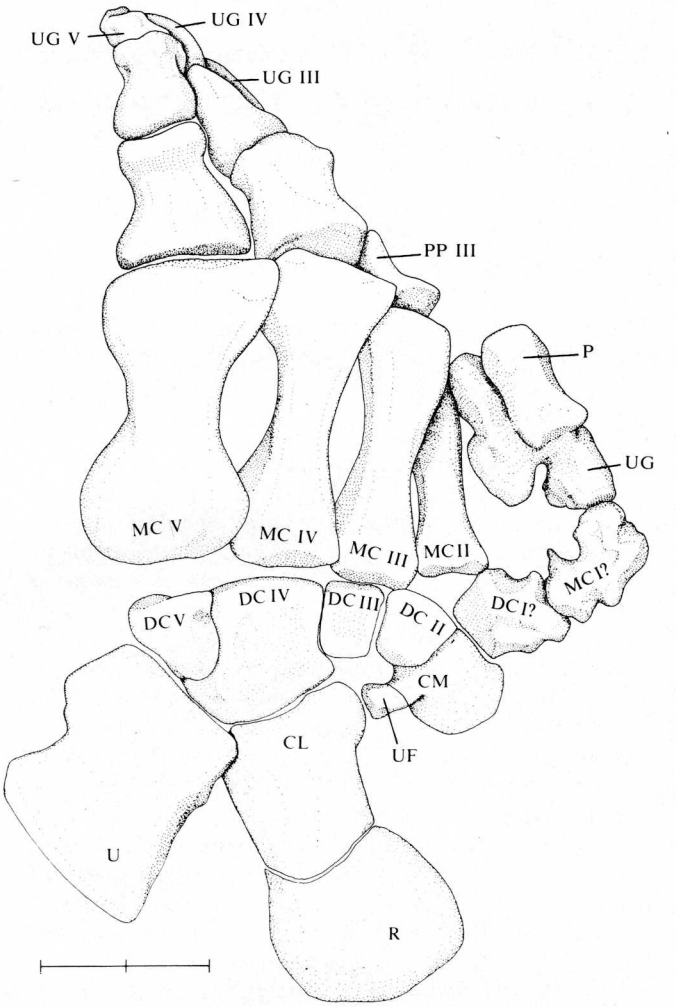

Fig. 2. Holotype of *Micranteosaurus parvus* SAM 4323. Dorsal view of left manus. Scale 20 mm.

referred to *Struthiocephalus* (SAM 12226). However, the third and fourth digits are incomplete in this specimen, and its phalangeal formula is most accurately regarded as 2-3-?-?-3. Boonstra also reassembled a disarticulated hand of *Parascapanodon* (SAM 249) with the formula 2-3-3-3-3, but admitted that 'reassembly as in the figure is an act of faith'.[3] Outside of South Africa, Chudinov[7] reported the phalangeal formula of *Estemmenosuchus uralensis*, a herbivorous dinocephalian from the Late Permian of the Soviet Union, to be ?-?-3-?-?. In addition, in well-preserved specimens of *Titanophoneus*, a carnivorous form also from the Late Permian of the Soviet Union, Orlov[8] and Chudinov[7] report the manual phalangeal formula to be 2-3-3-3-3.

The holotype of *Micranteosaurus* remains the only known articulated anteosaurid manus, as well as the only dinocephalian specimen in which more than three phalanges have been reported in digit III. Consequently, it forms the sole basis of Boonstra's[3-5] argument that the phalangeal formula in Anteosauria is 2-3-4-3-3, and that the formula 2-3-3-3-3 evolved convergently within Dinocephalia.

During the course of a more general review of the therapsid postcranial skeleton by one of us (T.R.), the manus of *Micranteosaurus* was re-examined. Further preparation using a technique not employed by Boonstra has added considerably to our knowledge of this important specimen. Attempts at acid preparation by previous workers had been unsuccessful (but caused little damage to the specimen), and consequently its further development could only be achieved mechanically. The application of a selective stain facilitated distinction between bone and matrix, a notoriously difficult problem in the fossils from the lower strata of the Karoo, from which *Micranteosaurus* was col-

lected. To apply the stain, the specimen was first cleared of all glues and preservatives by brushing it with acetone. It was then etched for 15 to 30 seconds in 10% hydrochloric acid, and thoroughly rinsed in water. Alizarin Red dye, mixed in a 4% solution of potassium hydroxide in distilled water, was then applied to the specimen for approximately 30 seconds. The specimen was then washed a second time in water and allowed to dry. This procedure selectively stains the bone red, while leaving the matrix unchanged. The stain can be quickly removed by reapplying the dilute acid and then washing the specimen. The use of this stain allowed much more detailed preparation, using conventional mechanical techniques, than was previously possible.

The pre-axial portion of the manus of *Micranteosaurus* appears to have been slightly damaged prior to burial because the position of its bones are disturbed and some are shattered (Figs 1 and 2). However, most of the carpus is intact and articulated, and digits III – V are in articulation and complete except for the distal ends of the unguals. Boonstra's second description[3] is accurate in his identifiction of the ulnare as an elongate bone lying along the proximal part of the postaxial edge of the hand. Lying medial to the ulnare, Boonstra[11,3] reported the presence of the intermedium. However, it is now evident that the structure he referred to is composed only of matrix, and that the intermedium is not preserved. Boonstra correctly identified the radiale and lateral centrale, although he described and illustrated them as being fused. It is now apparent that these two bones lie in close contact on the dorsal side of the hand, but then when viewed from below they are widely separated and are neither fused nor sutured. Boonstra also reported that the medial centrale was missing. This element has now been located, displaced slightly to the palmar side of distal carpals IV and V. Distal carpals II through V are present and in articulation, but disarticulation and deformation of the elements in the vicinity of where carpal I should lie has rendered its identity uncertain. Distal carpal V is present as a separate bone approximately half the size of IV, and lies closely appressed to its distolateral face. Metacarpals II through IV are present and in articulation with the carpus. Metacarpal

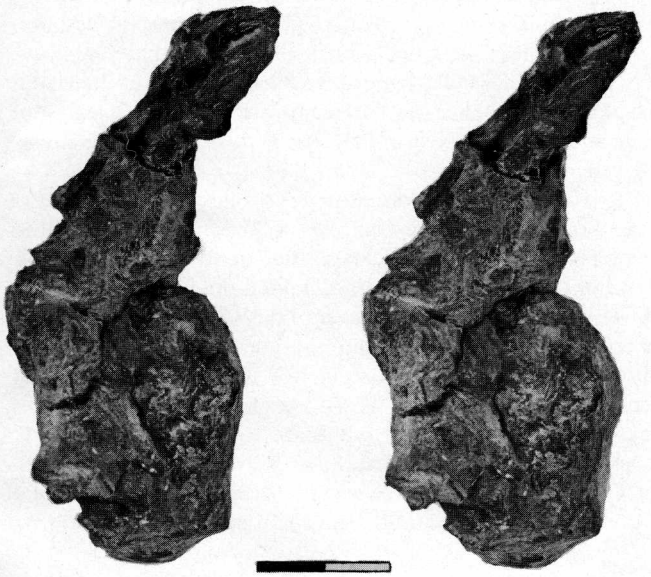

Fig. 3. Holotype of *Micranteosaurus parvus* SAM 4323. Stereophotograph of complete third digit of left manus in medial view. First and second digits have been removed in this photograph. Scale 10 mm. Key: C, crack through second phalanx of third digit; CL, lateral centrale; CM, medial centrale; DC, distal carpal; GR, groove around base of ungual made by original preparator; M, matrix; MC, metacarpal; P, isolated phalanx; PP III, proximal phalanx of third digit; R, radiale; SAM, South African Museum; U, ulnare; UF, unidentified fragment; UG, ungual phalanx.

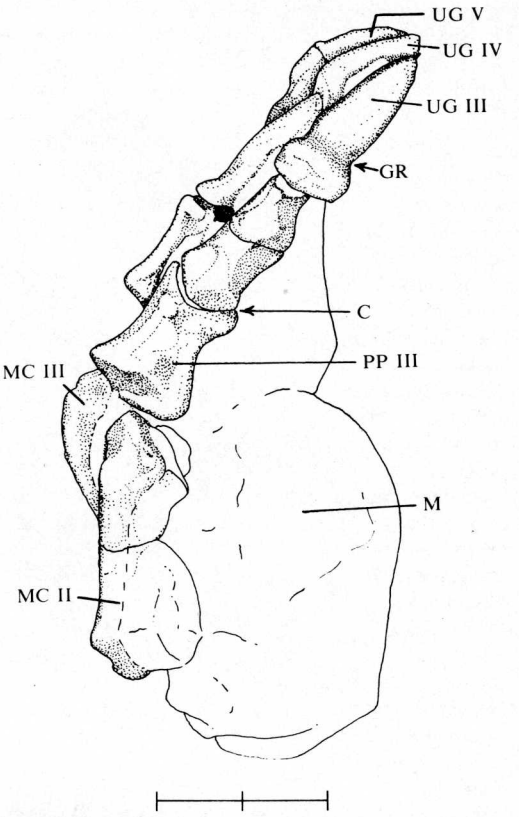

Fig. 4. Holotype of *Micranteosaurus parvus* SAM 4323. Complete third digit of left manus in medial view.

I may be represented by one of the shattered bones of the preaxial edge, but it cannot be positively identified. A claw and an isolated phalanx are undoubtedly parts of either digit I or II, but little more can be said of these digits with certainty.

As a result of the additional preparation and staining, it is clear that in digits III, IV and V there are only three phalanges, as is the case in other adequately preserved dinocephalian specimens. Boonstra's[3] misinterpretation of digit III was due to a groove cut round the proximal portion of the ungual by the original preparator and a crack through the transverse midline of the second phalanx (Figs 3 and 4). Because of the poor distinction between matrix and bone in this specimen, Boonstra[3] was led to interpret the proximal portion of the second phalanx as the complete second phalanx, and the distal portion of the second phalanx together with the proximal portion of the ungual as the third phalanx. The distal portion of the ungual was consequently regarded as the complete fourth phalanx.

The ancestral dinocephalian phalangeal formula has also been confused by an unusual phalangeal morphology present in the second phalanx of digit IV in *Titanophoneus*. Both Orlov[8] and Chudinov[7] reported three phalanges in this digit, but the structure of the second phalanx suggested to them that it formed from the fusion of two separate phalanges. They also implied that phalangeal loss was preceded by a reduced stage. Nevertheless, they explicitly regarded this to be a single adult structure, concluding that only three phalanges are present in both digits III and IV. In contrast, Kemp[6] evidently regarded the unusual structure in *Titanophoneus* as the retention of a separate vestigal phalanx, giving the formula of 2-3-3-4-3 for the primitive dinocephalian taxon Brithopodidae (to which *Titanophoneus* belongs), while stating (p. 81) that in other dinocephalians the formula is 'reduced to the mammalian one of 2-3-3-3-3, there being no longer any vestigial phalanges'. Boonstra[3] followed Orlov[8] in recognizing the formula 2-3-3-3-3 in *Titanophoneus*, a specimen that Boonstra had seen, and argued that 'the reduction in the number of phalanges in the third and fourth digits

Table 1. Phalangeal formula distribution in Synapsida. Phalangeal formula for *Cynognathus* is based on disarticulated specimens.[21]

Taxon	Formula	Reference
Synapsida		
Ophiacodon	2-3-4-5-3	1
Dimetrodon	2-3-4-5-3	1
Sphenacodon	2-3-4-5-3	1
Therapsida		
Biarmosuchus	2-3-4-5-3	7
Dinocephalia:		
all known specimens	2-3-3-3-3	see text
Dicynodontia:		
all known specimens	2-3-3-3-3	6,8
Therocephalia:		
all known specimens	2-3-3-3-3	6,19
Gorgonopsia:		
Lycaenodontoides bathyrhinus	?-?-3-3-?	20
Aelurognathus tigriceps	?-3-4-4-3	21
Lycaenops ornatus	?-3-4-4-3	20
Prorubidgea robusta	?-?-4-?-?	21
Dinogorgon sp.	?-?-4-5-3	21
BMNH 3768	?-?-4-5-?	2
BPI 1210	2-3-4-5-?	21
Cynodontia		
Leavachia duvenhagei	2-3-3-4-3	22, 23
Thrinaxodon	2-3-4-4-3	2, 23
Diademodon	2-3-3-3-3	23, 24
Cynognathus	2-3-3-3-3	23
Exaeretodon	2-3-3-3-3	25
Tritylodontidae	2-3-3-3-3	26
Mammalia		
Monotremata	2-3-3-3-3	9
Theria (ancestrally)	2-3-3-3-3	9

has thus followed a different course in *Titanophoneus* and *Micranteosaurus*, with the condition in the latter more primitive than in the former' (pp. 18 – 19). A consequence of Boonstra's and Kemp's arguments is that within Dinocephalia the phalangeal formula 2-3-3-3-3 must be viewed as having evolved independently of the same formula in other therapsids.

The manus in Gorgonopsia and Cynodontia

The above interpretation is influenced by the well documented occurrence of 1 to 2 separate, reduced phalanges in digits III and/or IV, which raise the phalangeal count of these digits to above three in the manus of some, but not all, Gorgonopsia (Table 1), and the cynodonts *Leavachia duvenhagei* and *Thrinaxodon*. Close inspection of these 'reduced' elements in the gorgonopsian *Aelurognathus tigriceps* (SAM 2342) shows that their structure is considerably modified over that of other phalanges. They form flattened discs, with little or no constriction around their middle, no development of swollen articular surfaces, and lie closely appressed to adjacent, fully developed phalanges. This raises some doubt as to whether they were capable of independent movement, or simply acted as 'epiphyses' attached to adjacent, fully developed phalanges. Moreover, in cross-sections through the articulation of a disc with a fully developed phalanx, it can be seen that the disc lacks the thickened cortical bone characteristic of the articular ends in fully developed phalanges. Hence, these 'discs' are highly modified when compared to the fully developed phalanges that were present ancestrally. Although they do resemble incompletely formed ('vestigial') phalanges, the resemblance itself is not evidence that phalanges actually passed through such a phylogenetic stage before they were lost.

Discussion

When the distribution of the various synapsid phalangeal attributes (Table 1) is analysed in light of recent phylogenetic studies of Therapsida,[9,10] in which a large sample of cranial and postcranial characters were analysed together, a new explanation of phalangial evolution is apparent. The simplest interpretation (i.e. that requiring the fewest evolutionary steps[17]) of those data that are currently available is that the manual phalangeal formula in Dinocephalia ancestrally was 2-3-3-3-3. This is most parsimoniously viewed as having arisen once, as an apomorphy of the most recent common ancestor of the unnamed taxon comprising Dinocephalia, Gorgonopsia, Dicynodontia, Therocephalia and Cynodontia.[9,10] The monophyly of this taxon is supported by 16 additional hypothesized synapomorphies of the skull and postcranial skeleton,[9,10] and is thus independent of any decision on the distribution of phalangeal attributes. The presence of additional disc-like phalanges in four gorgonopsian and two cynodont species represents either a reversal, in which ancestral phalanges reappeared but only partially differentiated, or the appearance of *de novo* ossifications. In either case, they evolved from an immediate ancestor in which the manual phalangeal count was 2-3-3-3-3. This explanation requires only five transformational steps, namely the single transformation of the ancestral formula to 2-3-3-3-3, followed by a minimum of four transformations resulting in additional digital elements in digits III and IV. The conventional hypothesis of convergent evolution of the mammalian formula requires the four transformations leading to the presence of 'reduced' phalanges in gorgonopsians and cynodonts, plus five (or more) additional steps leading to the convergent reduction to the formula 2-3-3-3-3 in Dinocephalia, some Gorgonopsia, Dicynodontia, Therocephalia, and Cynodontia (which includes Mammalia).

Boonstra and Kemp are thus correct in that some convergence in therapsid phalangeal evolution can be postulated. However, the data currently available most strongly support the view that this convergence did not involve reduction, but instead involved the addition of disc-like phalanges. Consequently, the 'mammalian' phalangeal formula probably did not evolve convergently among therapsids, as is generally believed. This is not a trivial distinction, for it affects the way in which we perceive those processes that operated during therapsid phylogeny. The phylogenetic loss of structures may require very different explanation, in terms of developmental mechanics or adaptive role, than the addition of structures that were not present in the immediate ancestor. Convergence does indeed appear to have occurred in the evolution of therapsid phalanges, but it appears to have been less common, and to have taken a different pathway, than previously believed.

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1. Romer A. S., and Price L. J. (1940). Review of the Pelycosauria. *Geol. Soc. Am. Spec. Pap.* **28**, 1 – 538.
2. Parrington F. R. (1939). On the digital formulae of theriodont reptiles. *Ann. Mag. Natural History* ser. **11**, 209 – 214.
3. Boonstra L. D. (1966). The dinocephalian manus and pes. *Ann. S. Afr. Mus.* **50**, 13 – 26.
4. Boonstra L. D. (1967). An early stage in the evolution of the mammalian quadrupedal walking gait. *Ann. S. Afr. Mus.* **50**, 27 – 42.
5. Boonstra L. D. (1971). The early therapsids. *Ann. S. Afr. Mus.* **59**, 17 – 46.

6. Kemp T. S. (1982). *Mammal-like Reptiles and the Origin of Mammals.* Academic Press, London.
7. Chudinov P. K. (1983). The early therapsids. *Trudi Paleon. Inst., Acad. Sci.* U.S.S.R. **202**, 1 – 229.
8. Orlov J. A. (1958). The carnivorous dinocephalians of the Isheevo fauna (titanosuchians). *Trudi. Paleon. Inst., Acad. Sci.* U.S.S.R. **72**, 1 – 114 (in Russian).
9. Rowe T. (1986). *Osteological diagnosis of Mammalia, L. 1758, and its relationship to extinct Synapsida.* PhD thesis, University of California, Berkeley.
10. Hopson J. A. and Barghusen H. R. (in press). An analysis of therapsid relationships, In *Ecology and Biology of Mammal-Like Reptiles*, edit. J. J. Roth *et al.* Smithsonian Institution and National Institute of Mental Health, Washington, D.C.
11. Boonstra L. D. (1954). The smallest titanosuchid yet recovered from the Karoo. *Ann. S. Afr. Mus.* **42**, 149 – 156.
12. Van den Heever J. and Grine F. (1981). The dinocephalian type material in the South African Museum (Reptilia, Therapsida). *Ann. S. Afr. Mus.* **86**, 73 – 114.
13. Boonstra L. D. (1969). The fauna of the *Tapinocephalus* Zone (Beaufort Beds of the Karoo). *Ann. S. Afr. Mus.* **56**,1 – 73.
14. King G. M. (1986). Personal communication.
15. Boonstra L. D. (1955). The girdles and limbs of the South African Dinocephalia. *Ann. S. Afr. Mus.* **42**, 185 – 326.
16. Byrne F. (1938). A preliminary report on a new mammal-like reptile from the Permian of South Africa. *Trans. Kansas Acad. Sci.* **40**, 221 – 231.
17. Maddison W. P., Donoghue M. J. and Maddison D. R. (1984). Outgroup analysis and parsimony. *Syst. Zool.* **33**, 83 – 103.
18. Boonstra L. D. (1966). The girdles and limbs of the Dicynodontia of the *Tapinocephalus* Zone. *Ann. S. Afr. Mus.* **50**, 1 – 11.
19. Boonstra L. D. (1964). The girdles and limbs of the pristerognathid Therocephalia. *Ann. S. Afr. Mus.* **48**, 121 – 165.
20. Boonstra L. D. (1934). A contribution to the morphology of the Gorgonopsia. *Ann. S. Afr. Mus.* **31**, 137 – 174.
21. Sigogneau D. (1970). *Revision systematique des gorgonopsiens Sud-Africains.* Edition du Centre National de la Recherche Scientifique, 1 – 414.
22. Brink A. S. and Kitching J. (1953). On *Leavachia duvenhagei* and some other procynosuchids in the Rubidge Collection. *S. Afr. J. Sci.* **49**, 313 – 317.
23. Jenkins F. A. Jr. (1971). The postcranial skeleton of African cynodonts. *Peabody Mus. Nat. Hist. Bull.* **36**, 1 – 126.
24. Brink A. S. (1980). Notes on additional material of *Titanogomphodon crassus* Keyser (Diademondontidae). *Ann. Geol. Surv. S. Afr.* **14**, 39 – 50.
25. Bonaparte J. F. (1963). Descripcione del esqueleto postcraneano de *Exaeretodon* (Cynodontia — Traversodontidae). *Acta geol. lilloana* **4**, 5 – 51.
26. Sues H. D. (1983). *Advanced mammal-like reptiles from the Early Jurassic of Arizona.* PhD thesis, Harvard University.

Research Letter/Navorsingsberig

Spreading, growth and function of cells on fibrin-coated agarose or polycarbonate

A method is described by which cells may be grown on a thin film of fibrin which is attached either directly to a nutrient agarose underlay or to a transparent Nucleopore filter disc placed on top of it. The procedure allows cell lines like Vero, HeLa, human oesophageal carcinoma and human keratinoma cells, and cell strains like human foreskin fibroblasts, chick fibroblasts and Vervet monkey kidney cells to attach and spread within hours on otherwise 'cell unfriendly' surfaces, and subsequently to grow to confluency.

The nature of surfaces on which cells grow is of importance, not only for attachment and spreading but also for morphogenesis and function.[1,2] *In vivo* proliferation of epithelial tissue takes place only in the basal layers. The growing front of cells is attached to a basal lamina or extracellular matrix, and it seems likely that the intimate contact between cells and non-cellular substrate helps to stimulate growth and differentiation.[3-7] A method is presented here by which the surface of a solid agar gel or a polycarbonate membrane can be modified to permit cell adhesion and growth. The cells are attached to a fibrin net interphase between the substrate and the atmosphere, a situation which allows for differentiation of certain cell types.[8] This system has obvious advantages where entire cell sheets have to be manipulated as intact entities.

Materials and methods

Plasma. Outdated human blood bags or fresh bags of horse blood containing 70 ml of citrate-phosphate-dextrose-adenine buffer CPDA (citric acid 327 mg, sodium citrate 2,63 g, sodium acid phosphate 2,51 g, anhydrous dextrose 2,90 g and adenine 27,5 mg per 100 ml), were used as sources of plasma. After the bags were left standing upright for at least two hours at 4°C, the supernatant plasma was collected and spun at 1000 × *g* for 15 min. Platelets were removed by filtration through a non-sterilizing depth filter (Whatman No. 5), the clear plasma was then sterilized by filtration through a 0,22 μm membrane (Millipore). The plasma was kept at −20°C in aliquot amounts.

Preparation of surfaces. Two per cent molten agarose (Sigma High Gelling Temperature Agarose Type V) was mixed 1:1 with double concentrate Earle's based MEM containing non-essential amino acids (Gibco), penicillin and streptomycin (100 μg/ml each) and 5% heat-inactivated newborn bovine serum (Flow). Occasionally, neutral red (final concentration 0,002%) was added to this mixture. Twenty-four-well culture trays (Costar) and 5-cm-diameter plastic petri dishes were filled with 1 or 2 ml nutrient agarose, respectively, using plastic Pasteur pipettes, and allowed to set at 4°C. Ice-cold human or horse plasma was inoculated with sterile $CaCl_2$ solution (final concentration 0,6%) to neutralize the citrate present in the blood-collecting buffer. The nutrient agarose button in each culture well was covered fairly quickly with calcium-containing plasma, excess fluid was drawn off and the trays incubated at 37°C until the plasma clotted (5 to 10 min). The trays can be stored for up to one week in the humidified atmosphere of a CO_2 incubator, preferably under cover of fluid medium. In addition, 2,5-cm-diameter filter discs, pore size 30 nm or below (Nucleopore Co., Pleasanton), were placed on top of nutrient agarose underlays and treated with recalcifying plasma as described above.

Cell culture. The cells were grown in Earle's based MEM containing non-essential amino acids (Gibco), penicillin and streptomycin (100 μg/ml each) and 10% inactivated newborn bovine serum. Well-growing, near-confluent cultures were trypsinized with Dulbecco's trypsin and EDTA (1:1). The cells were taken up in medium, centrifuged, resuspended, counted in a haemocytometer and, depending on the cell type, the numbers adjusted to 5×10^3 or 10^4 cells/ml. One millilitre of cell suspension was then put on top of a nutrient agarose button or into an empty well. Larger petri dishes were inoculated with 2 ml of suspension. After 3 h the medium was withdrawn and the unattached cells counted. The cells on the agarose button were left 'dry', whereas fresh medium was added to the control cells on the plastic surface. After 48 h, cells growing on the plastic surface were treated with 1 ml trypsin – EDTA mixture and counted with a Coulter Counter Model ZB1. Since trypsin is inactivated by the plasma fibrin film, 1 ml of sterile solution of 5 mg Dispase Grade II (Boehringer) in 12,5 ml Ca^{2+}-free phosphate-buffered saline was used for the 'dry' cultures. All tests were carried out in triplicate wells and the following cells were used: Vero cells, HeLa cells, human oesophageal carcinoma cells SN,[9] human epidermoid carcinoma cells A431,[10] human foreskin fibroblasts after 12th and 13th passage, 12-day chick fibroblasts, and Vervet monkey kidney cells after 2nd passage.