# Characterization of Reptilian Cell Lines Established at Incubation Temperatures of 23 to 36°[1] (34622)

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Preparation of primary reptilian cell cultures has been described by several authors; they employed incubation temperatures of 37° (1–3) or room temperature (18–26°) (4–6). The growth of a cell line derived from the green turtle (*Chelonia mydas*) and incubated at 30 or 35° has also been described (7). Previously, we reported the establishment of cell line TH-1 from the box turtle, *Terrapene carolina* (8), at 23°, certain sublines of which acquired a capability for continuous propagation at 30° (9).

The present report describes the establishment and cultural characteristics of cell lines derived from several species of reptiles, employing incubation temperatures of 23, 30, and 36°. In addition, karyotypes of certain cell lines are presented and compared with those previously reported (10–13) and preliminary data on viral susceptibility are given.

*Materials and Methods. Reptiles.* The reptilian species investigated (Table I) were kindly supplied by Mr. William Leumer, Buffalo Zoological Gardens, or by Dr. Carl Gans, Department of Biology, State University of New York at Buffalo. Other animals were purchased from C. F. McLung, La Place, Louisiana. All animals included in the study were normal.

*Establishment of cell lines.* Animals were sacrificed by decapitation and exsanguination. Visceral organs were washed with amphibian Ringer's solution, minced finely, suspended in Eagle's basal medium (BME), containing 10% fetal calf serum, 100 units of penicillin, and 100 μg of streptomycin/ml, and explanted into plastic (Falcon) flasks. Occasionally, organs were dispersed with trypsin–Versene mixtures according to the method of Shindarov (1) prior to explantation. Flasks were incubated at 23, 30, or 36° and the medium was changed whenever cultures appeared to become acid. At approximately 25 to 50% confluency, the cells were dispersed with 0.25% trypsin and passaged with 1:2 splits as previously described (8), with subsequent 1:2 splits as cultures again became confluent. Both glass and plastic surfaces were successfully employed for reptilian cell culture. Although certain cell types resisted dispersion by trypsin, they were successfully passaged by treatment with 0.02% Versene solution or by scraping with a rubber policeman followed by vigorous pipetting.

*Morphologic studies.* Cells from each line were plated on coverslips in Leighton tubes at 5- to 10-passage intervals. Confluent monolayers were fixed in 5% acetic acid in 70% ethanol and stained with hematoxylin and eosin.

*Karyologic studies.* Karyologic techniques have been previously described (10, 11). In brief, cultures for karyotypic study were pretreated with 0.05 μg/ml of Colcemid, subjected to hypotonic shock (1.0% Na citrate solution), and stained with 2.0% acetoorcein after the method of Moorhead *et al.* (14) with minor modifications.

*Viral studies.* Viruses representative of most of the major recognized groups were

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TABLE I. Reptilian Sources of Cell Lines.

| Species | No. of individuals sacrificed for cell culture | No. of cell lines obtained |
|---|---|---|
| Order Crocodylia (crocodiles) | | |
| *Caiman crocodylus*, spectacled caiman | 4[a] | None |
| Order Amphisbaenia (amphisbaenians) | | |
| *Amphisbaenia camura* | 1 | None |
| *Blanus cinereus* | 1 | None |
| Order Squamata (snakes and lizards) | | |
| *Thamnophis sirtalis*, garter snake | 1 | None |
| *Crotalus cerastes*, sidewinder rattlesnake | 1 | None |
| *Agkistrodon bilineatus*, Mexican moccasin | 2[a] | None |
| *Dipsosaurus dorsalis*, desert iguana | 1 | None |
| *Gekko gecko*, Tokay gecko | 3 | 3 |
| *Mabouya* sp. | 1 | None |
| *Iguana iguana*, green iguana | 3[a] | 4 |
| Order Chelonia (turtles) | | |
| *Chelydra serpentina*, snapping turtle | 1 | None |
| *Kinosternon subrubrum*, musk turtle | 1 | None |
| *Podocnemis unifilis*, side-necked turtle | 2 | 2 |
| *Terrapene carolina*, box turtle | 4 | 7 |
| *Testudo graeca*, Grecian tortoise | 1[a] | 1 |

[a] Indicates juvenile animals; 2 of 3 iguanas were juvenile but 3 of the 4 iguana cell lines arose from the single adult.

employed. The viruses, the host system used for propagation, and the titers of the stocks utilized were as follows (all titers/0.1 ml): vaccinia, primary monkey kidney cells, $10^{6.3}$ tissue culture infectious doses ($TCID_{50}$); herpes simplex HF, chick embryo fibroblasts (CEF), $10^{6.5}$ $TCID_{50}$; pseudorabies, green monkey kidney cell line (GM), $10^{7.5}$ $TCID_{50}$; avian laryngotracheitis, chick embryo chorioallantoic membrane (CE-CAM), $10^{4.0}$ egg infectious doses ($EID_{50}$); adenovirus types 2, 7, and 12, human embryo kidney cells, $10^{5.0}$, $10^{1.5}$, $10^{2.5}$ $TCID_{50}$, respectively; Newcastle disease, CEF, $10^{7.8}$ $TCID_{50}$; Sendai, chick embryo allantoic fluid, 640 hemagglutinin units; canine distemper, human amnion cell line AV3, $10^{4.5}$ $TCID_{50}$; measles, AV3, $10^{5.0}$ $TCID_{50}$; poliovirus type 2, GM, $10^{7.2}$ $TCID_{50}$; vesicular stomatitis, Indiana, CEF, $10^{5.5}$ $TCID_{50}$; Rous sarcoma, Bryan's high titer, CE-CAM $10^{3.0}$ $EID_{50}$.

Two or three tube cultures of each reptilian cell type tested were inoculated with 0.1 ml of the undiluted virus stock listed above and incubated at 36°. The inoculated tubes were observed for at least 14 days. Cultures exhibiting cytopathic effect (CPE) were harvested by freezing and thawing, and a second passage was performed using undiluted whole first-passage material as inoculum. Both CPE-positive and -negative cultures were fixed, stained with hematoxylin and eosin, and examined microscopically.

*Results. Establishment of cell lines.* The reptilian species from which establishment of cell lines was attempted are listed in Table I. (For the purpose of this report, cell lines are arbitrarily defined as cells passaged successfully for at least 12 months and 20 passages.) Cell lines were obtained from 3 of 5 species of turtles studied and 2 of 4 species of lizards, but not from snakes, amphisbaenians, or the spectacled caiman. All but two of the cell lines obtained were derived from adult animals. Male (*Gekko* 1 and 2, *Podocnemis* 1, and *Testudo*) and female (*Terrapene* 4 and 5, *Podocnemis* 2) reptiles both yielded cell lines.

Primary cell outgrowth was obtained from explants of the tissues of each animal at one or more of the incubation temperatures employed—23, 30, and 36°. However, cultures not yielding cell lines degenerated or ceased to grow after 1 to 10 passages. A notable exception was a single caiman heart culture which attained the 25th passage level in 10 months at 30° before growth ceased. Cell cultures derived from one of the green iguanas degenerated after one to five passages, exhibiting syncytial cell changes. A herpesvirus, designated iguana virus, was isolated from these cultures (15).

The designation of each cell line (derived from the genus of reptile and organ of origin), its temperature of cultivation, recent passage level (July 1, 1968), and morphology are listed in Table II. The heart was the most consistent source of cell lines, but lines of spleen, kidney, liver, and lung origin were also obtained.

*Temperature requirements for cell growth.* 30° was the most useful incubation temperature for establishing cell lines from each of the species studied except the box turtle, which yielded cell lines at both 23 and 30°. One cell line, derived from *Iguana* heart was established at the usual homeothermic vertebrate cell incubation temperature of 36°.

The *Terrapene* cell lines 5TSW and 4THW propagated at 30° were the only "lines" observed to stop growing after as long as 12 months in continuous culture. However, a permanent subline grown at 23° was established from the 13th passage of 5TSW. Sublines grown at 23° were also successfully derived from the 30° cell lines 4TSW, TGSW, GL1, and GH1, despite the fact that *Gekko* organs and *Terrapene* spleens did not yield primary growth at 23°.

A subline of the 23° *Terrapene* line TK was successfully established at 30° but other 23° *Terrapene* lines could not be adapted to continuous growth at 30°. Reptile cell lines grown at 30° were capable of limited multiplication at 36°, but ceased growing after 2 to 10 passages.

*Cell morphology.* All primary cell cultures except those derived from trypsinized kidneys began as mixed epithelioid and fibroblastic growth from explants. When the cells were successfully adapted to continuous passage, a dominant cell type usually emerged by the fifth passage. The characteristic morphology of the emergent cell lines varied widely, but epithelioid cells of cuboidal to near-spindle shape predominated. Only two cell lines, TGS and TK, both derived from turtles, demonstrated typical fibroblast-like morphology (Fig. 1a). *Gekko* cell lines (and primary *Gekko* cultures as well) exhibited a unique type of morphology characterized by a reticulated pattern of nests of "contact-inhibited"[4] large epithelioid cells surrounded by multilayered aggregates of smaller spindle-shaped cells (Fig. 1b).

The 30° *Terrapene* cell lines TH4W (Fig. 1c) and TS5W both developed an extremely aberrant cell and nuclear morphology and exuberant growth pattern characterized by formation of necrotic multilayered cell clumps prior to total cessation of growth. Other 30° *Terrapene* cell lines generally exhibited more aberrant cellular and nuclear morphology and less contact inhibition than those maintained at 23° (9, 10).

Unlike *Terrapene* cell lines, *Gekko* cell lines maintained similar morphology at both 30 and 23°. The only cell lines established at 36°, *Iguana* heart-2 exhibited a much more regular morphology (Fig. 1d) than *Iguana* cell lines maintained at a lower temperature (30°). *Podocnemis* cell lines PH1 and PH2, which would grow consistently only at 30°, exhibited a high incidence of nuclear aberrations but maintained epithelial morphology with complete contact-inhibition.

Uniform contact-inhibition persisted in some cell lines but was incomplete in most. Cells with giant nuclei (presumably polyploid) were common in most cell lines, but multinucleated giant cells were rare or nonexistent.

*Karyologic studies.* The karyotypes of *Terrapene, Gekko,* and *Podocnemis* cell lines

[4] For the purposes of the present report, "contact-inhibition" is assumed to be complete when cell growth is restricted to monolayer formation, with no overlapping of cells observed [ see Rubin (16) ].

TABLE II. Cell Lines Derived from Reptiles.

| Cell line | Code | Incubation temp (°) | Months in pass. to 7-68 | Total pass. to 7-68 | Morphology: Cell type[a] | Morphology: Contact inhibition | Karyotype[b] |
|---|---|---|---|---|---|---|---|
| *Gekko* heart-1 | GH1 | 30 | 37 | 91 | ES | — | Bimodal—diploid, hyperdiploid |
| lung-1 | GL1 | 30 | 37 | 66 | ES | — | Bimodal—as in GH1 |
| heart-2 | GH2 | 30 | 20 | 32 | ES | — | Aneuploid (passage 2) |
| *Iguana* heart | IgH | 36 | 23 | 74 | E | + | Aneuploid (1 acrocentric marker) |
| liver-A | IgVA | 30 | 23 | 56 | ES | — | " " |
| liver-B | IgVB | 30[c] | 23 | 59 | FS | ± | " " |
| kidney-17 | IgK17 | 30 | 21 | 26 | ES | + | " " |
| *Podocnemis* heart-1 | PH1 | 30 | 37 | 120 | E | + | Hypodiploid |
| heart-2 | PH2 | 30 | 37 | 117 | E | + | 50% Diploid, 50% pseudodiploid |
| *Terrapene* heart-4 | TH4 | 23 | 36 | 44 | S | ± | Pseudodiploid |
| heart-4W | TH4W | 30 | 32 | 58[d] | S | — | Hypotetraploid |
| lung-4 | TL4 | 23 | 36 | 41 | E | + | nd[e] |
| spleen-4W | TS4W | 30 | 36 | 50 | E | + | nd |
| heart-5W | TH5W | 30 | 36 | 51 | S | — | Pseudodiploid |
| spleen-5W | TS5W | 30 | 24[c] | 24[c] | E | ± | nd |
| kidney | TK | 23 | 36 | 37 | F | + | nd (30° subline pseudodiploid) |
| *Testudo graeca* spleen | TGSW | 30 | 38 | 39 | F | + | nd |

[a] E = epithelial-like; F = fibroblast-like; S = spindle-shaped (short bipolar cells); intermediate forms are designated by combinations of letters.

[b] Diploid chromosome numbers are: *Gekko gecko* —34 (13), *Iguana iguana* —38 (13), *Podocnemis unifilis* —28 (11), *Terrapene carolina* —50 (10), *Testudo graeca* —52 (Huang, C. C., personal communication).

[c] First passage only was cultivated at 36°.

[d] Cell lines degenerated at passage level indicated.

[e] Not determined.

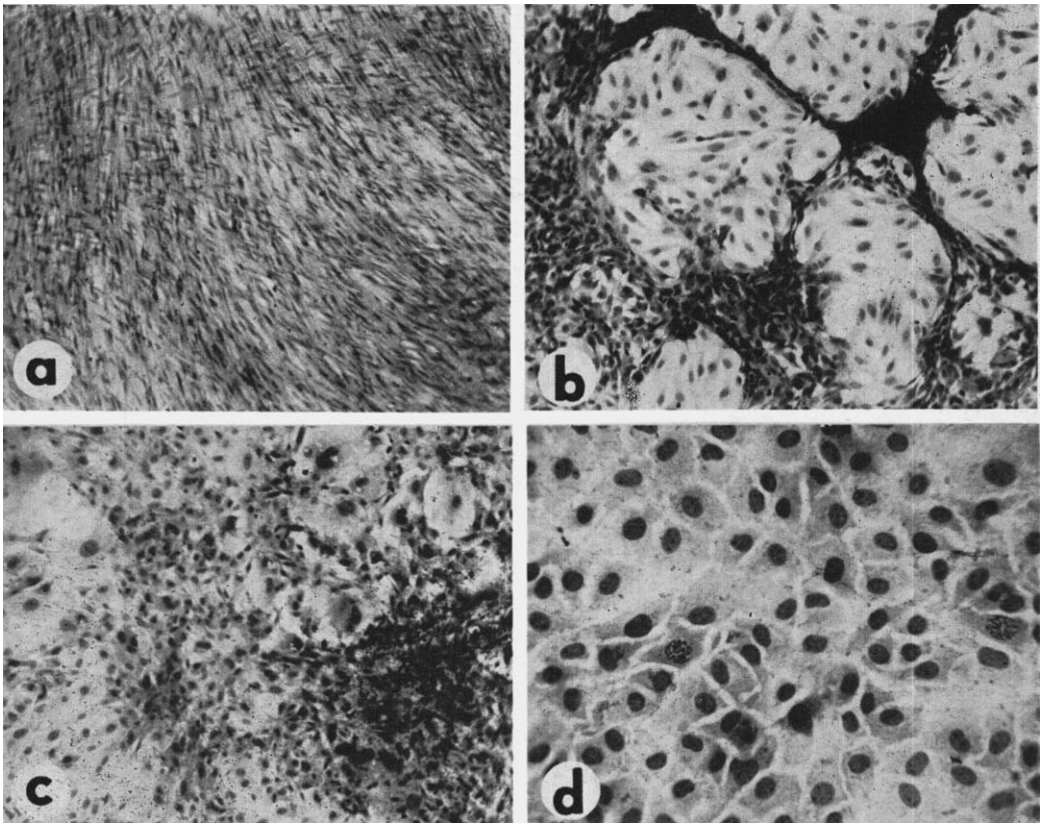


FIG. 1. Morphology of reptilian cell lines photographed at 100× (a, b, c) or 450× (d) magnification. (a) *Testudo graeca* spleen (TGSW), 29th passage. Typical fibroblastic morphology, overlapping planes are apparent. (b) *Gekko* lung 1 (GL1), 65th passage. Foci of epitheloid cells in monolayer surrounded by cords of multilayered spindle-shaped cells. (c) *Terrapene* heart 4W(TH4W), 46th passage. Exuberant disorganized epithelioid cell growth. Cell necrosis is seen in some cell aggregates. (d) *Iguana* heart 2 (IgH2), 40th passage. Uniform epithelioid cell growth, exhibiting complete contact inhibition.

have been described elsewhere (10–12) (see Table II). Cells of each of the four *Iguana* cell lines IgH, IgVA, IgVB, and IgK17 were also subjected to cytogenetic investigation. Originally, cells of each line exhibited a diploid karyotype of 12 macro- and 22 microchromosomes (13). Cell line IgH remained diploid for 40 passages. However, in later passages of all cell lines (73rd passage of IgH, 25th passage of IgVA, IgVB, and IgK17) an extra acrocentric chromosome appeared (Fig. 2). Also, at the 23rd passage level of IgK17, approximately 50% tetraploid cells were observed.

*Virus susceptibility.* The uniform susceptibility of reptilian cell lines to infection with amphibian cytoplasmic viruses has been previously reported (17). A survey of the susceptibility of representative cell lines to typical viruses of homeothermic vertebrates was performed using cytopathic effect (CPE) as an indication of infection. For comparative purposes, the fish cell lines FHM (18) and BF (bluegill fry) (Wolf, K., unpublished), the bullfrog cell line FT (19) and primary spectacled caiman and reticulated python kidney cell cultures were also studied.

In the screening procedure, cells were arbitrarily designated susceptible when CPE was induced in at least two consecutive passages.

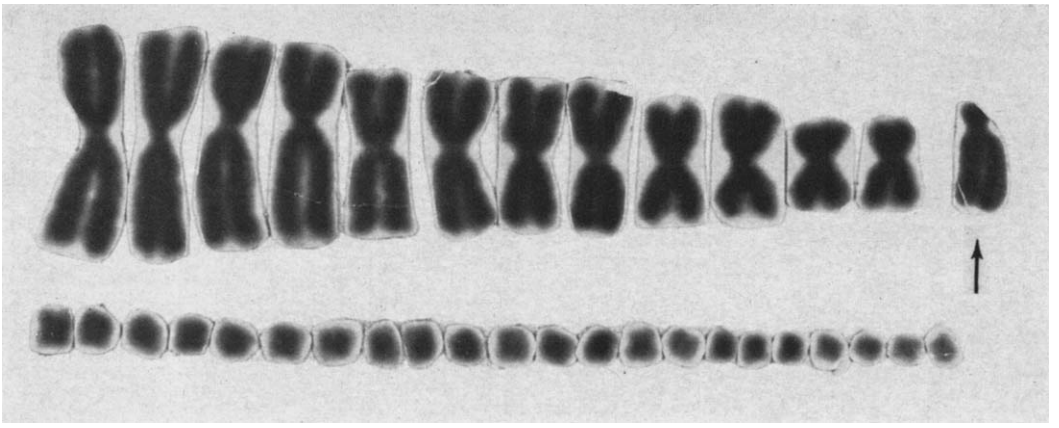

FIG. 2. Karyotype of *Iguana* cell line IgVB, 58th passage. This is identical to the diploid somatic karyotype, except for an additional acrocentric chromosome indicated by the arrow.

When CPE was observed in the first passage only, an abortive infection or a cytotoxic effect was assumed.

Viruses cytopathic for one or more of the test cell systems and their spectrum of infectivity are listed in Table III. Nine viruses of homeothermic vertebrate origin, representing the poxvirus, herpesvirus, myxovirus, rhabdovirus, and arbovirus groups, were successfully propagated in reptile cell lines. The PH1 and PH2 cells, derived from the side-necked turtle, showed maximal susceptibility, each supporting growth of eight viruses. Other reptilian cell lines supported as few as two of the test viruses. There was some variation in the spectrum of viruses supported by different cell lines derived from the same species or even from the same individual animal (TH4, TL4, and TS4W were all established from the same box turtle ). Primary reptilian cell cultures supported the growth of several viruses, but the fish cell lines FHM and BF and the amphibian cell line FT were relatively insusceptible.

Each virus usually produced a type of CPE similar to that characteristic of its growth in susceptible mammalian or avian cells. The herpesviruses: herpes simplex, pseudorabies, and avian laryngotracheitis usually produced CPE consisting of syncytia (Fig. 3a and b) with few to many nuclear inclusions. CPE induced in reptilian cells by the myxoviruses: NDV, CDV, and measles virus was also usually syncytial. Vaccinia virus, Sindbis virus, and VSV caused a cell-destructive type of CPE characterized by

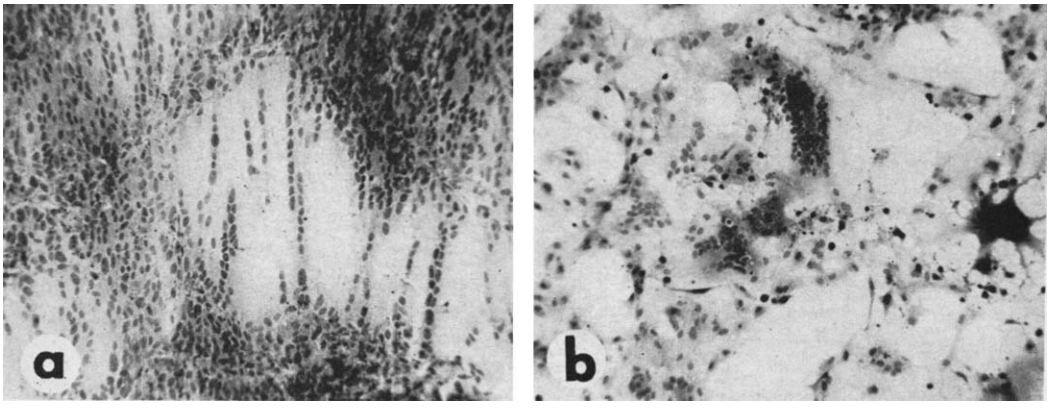


FIG. 3. Syncytial cytopathic effect induced by herpesviruses; photographed at 100×: (a) herpes simplex strain HF in TGSW cells; (b) pseudorabies virus in PH2 cells.

TABLE III. Susceptibility of Poikilothermic Vertebrate Cell Cultures to Viruses of Homeotherms at 36°.[a]

| Virus | Reptilian cell lines | | | | | | | | | | Other cell lines[b] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | GH1 | GL1 | IgH | PH1 | PH2 | TH4 | TL4 | TS4W | TH5W | TKW | TGS | PyK | CyK | FT | FHM | BF |
| Vaccinia | C | C | C | C | T | C | T | T | T | T | C | C | T | — | — | — |
| Herpes simplex | — | — | — | C | C | C | C | C | C | C | C | C | C | — | — | — |
| Pseudorabies | C | C | C | C | C | C | C | C | C | C | C | C | C | — | — | T |
| Avian laryngotracheitis | T | C | T | C | C | T | C | T | T | T | T | — | T | T | — | — |
| Newcastle disease | T | C | T | C | C | T | T | — | T | — | C | C | C | — | C | — |
| Canine distemper | T | T | — | C | C | T | T | — | C | T | T | — | — | — | T | — |
| Measles | — | T | — | — | C | — | — | — | — | — | — | T | — | — | — | — |
| Vesicular stomatitis | C | C | — | C | C | C | — | T | C | T | C | C | C | — | C | T |
| Sindbis | C | C | T | C | C | T | — | T | C | T | C | C | C | — | C | — |

[a] C = cytopathic effect in two consecutive passages; T = cytotoxic effect induced by virus, nonpassageable; — = no change induced by virus.

[b] PyK = primary reticulated python kidney; CyK = primary spectacled caiman kidney; FT = bullfrog tongue cell line; FHM = fathead minnow cell line; and BF = bluegill fry cell line.

typical cell rounding and peeling.

No CPE was induced in any of the cell types listed in Table III by poliovirus type 2, Coxsackievirus B5, or adenoviruses 2, 7, or 12. Rous sarcoma virus was cytotoxic in every cell type tested except BF cells. A preparation of Sendai virus inactivated with ultraviolet light effectively fused primary python kidney cells (Righthand, V.F., and Clark, H F., unpublished observations).

*Discussion.* Cell culture lines were readily established from several species of turtles and lizards, but not from snakes or crocodilians. The failure of crocodilians to yield cell lines is interesting from a phylogenetic viewpoint, as these are the reptiles having the most morphologic affinities to birds (20) which also do not yield cell lines.

Reptilian cells grew well in mammalian cell media and were propagated successfully using ordinary mammalian cell techniques. However, with a single exception, reptilian cells required temperatures below those normally used for propagation of mammalian cells. There was a rough correlation between the optimum temperature for establishment of cell lines and the normal environmental temperature of the species. Thus, the box turtle, occupying in nature the coolest range of any of the species yielding cell lines (21), yielded the only cell lines established at a temperature of 23°. The only cell line established at 36° was derived from the tropical green iguana. The most generally successful temperature for establishing cell lines from reptiles was 30°.

Following a few passages at 30°, cell lines of *Terrapene* spleen and *Gekko* heart and lung were established at 23°. None of these organs yielded cell growth in primary culture at 23°. Possibly, limited exposure to elevated temperatures may provide special stimulus to the adaptation of reptilian cells to continuous culture.

The designation of continuously propagated reptilian cells as "cell lines" is arbitrary. The mammalian dichotomy of "cell strains" with regular fibroblastic morphology, normal karyotype, and finite life span, and derived "cell lines" altered to a neoplastic cell-like morphology, abnormal karyotype

and indefinite growth potential does not seem to apply. Rather, it was usual for reptile cells to adapt gradually to continuous passage *in vitro* through modest alterations. Reptile tissue explants typically yielded primary growth composed of several morphological types. Within a very few passages (usually about five) a single cell type became dominant. At a slightly later period, usually between the 10th and 40th passage, minor chromosomal alterations were detectable, unaccompanied by further morphological change. These changes appeared to stabilize with karyotypes presenting only minor changes from the true diploid karyotype. No cell line retained a true diploid karyotype beyond the 110th passage.

"Alteration" of mammalian cells leading to a capacity for indefinite *in vitro* growth is frequently associated with acquisition of oncogenic potential [reviewed in (22)]. The oncogenic potential of reptilian cell lines has not been studied extensively. In preliminary experiments (Clark H.F., unpublished) cells of a variety of cell lines derived from box turtles, side-necked turtles, Tokay gekkos, and iguanas were inoculated by several different routes into animals of the species from which they were derived. These animals were observed for periods of 1 to 28 months and necropsied at death. All remained tumor-free. However, because of the slow growth of reptile cells *in vitro* and the probable wide genetic disparity between the donor cells and the host, the significance of these observations is tenuous.

The question of "immortality" of these reptilian cell lines is also unanswered. Two cell lines that persisted for more than a year subsequently degenerated, after developing an unusually exuberant growth pattern and abnormal karyotype. It is possible that further experience will reveal that other reptilian cell lines may have a restricted generation potential.

Reptilian cells in culture appear to support the replication of a wider variety of homeothermic vertebrate viruses than do the two fish cell lines or the amphibian cell line FT, in keeping with the phylogenetic position of the reptiles. Representatives of the poxvirus, herpesvirus, myxovirus, arbovirus, and rhabdovirus groups have been shown to be cytopathic for various reptilian cell types. Certain of the reptilian cell types are incapable of multiplying at the 37° temperature employed for growth of mammalian and avian viruses. At such temperatures cellular macromolecular synthesis may be sharply reduced and if so, virus synthetic processes in reptilian cells may be studied in a milieu uncomplicated by host cell synthetic activity (23). The metabolism of poikilothermic cells at high temperatures, which are nonpermissive for growth, merits further study.

An added feature of the virological investigations was the finding that several reptile cell types supported the replication of avian laryngotracheitis virus. As only certain types of avian cells are susceptible, this virus could not previously be propagated in continuously cultivated cells. It is suggested here, that in the absence of avian cell lines, cell lines derived from the closely related reptiles may provide an expedient tool for the study of avian viruses *in vitro*.

*Summary.* Cell lines were established from several species of turtles and lizards, but not from snakes, crocodilians, or amphisbaenids. Growth was obtained in mammalian cell medium at incubation temperatures from 23 to 36°, but cell lines were most often successfully established at 30°. Cell morphology was variable, including fibroblastic, epithelioid, and some aberrant types. Karyoptic analysis of several cell lines derived from *Iguana iguana* revealed in each case a diploid karyotype at early passage levels followed by acquisition of an added marker chromosome in later (25th to 73rd) passages. On the basis of this and previously published data it was concluded that reptilian cell lines typically emerge by early (5th to 10th passage) selection of one morphological cell type followed by a later (10th to 40th passage) appearance of usually minor chromosomal changes. Reptilian cell lines supported the replication with cytopathic effect of homeothermic vertebrate viruses of the poxvirus, herpesvirus, myxovirus, rhabdovirus, and arbovirus groups at an incu-

bation temperature of 36°. Reptile cells *in vitro* apparently support a wider spectrum of mammalian and avian viruses than do fish or amphibian cells.

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