Desert Tortoise Gopherus agassizii: Cutaneous Water Loss

Abstract. *Evaporative water loss from the integument of the desert tortoise* Gopherus agassizii *constitutes a major proportion of the water loss, but is far less than in tortoises from wetter regions. Respiratory water loss also is less.*

We have shown that, contrary to previous assumptions, the skin of reptiles is a major avenue for water loss (*1*). We also found that evaporation from the skin was less in certain species that live in dry rather than in wetter areas. Thus the lizard *Iguana iguana*, a dweller in tropical forests, loses water about five times as rapidly as the lizard *Sauromalus obesus*, which lives in hot desert regions. This finding suggests that in lizards there may be adaptation of the integument, associated with aridity of the habitat. Lizards and tortoises have evolved separately for a very long period, and so it was of interest to learn whether water losses differ similarly in tortoises. We shall show that comparison of the desert tortoise *Gopherus agassizii* with the box turtle, *Terrapene carolina*, suggests that such differences do indeed exist in the Chelonia.

Total water loss was determined (*1*) from changes in weight, after correction for metabolic loss of carbon. Water loss from the respiratory tract (head) was determined separately by enclosing the trunk and limbs in a thick plastic bag containing anhydrous $CaSO_4$ (Drierite) to absorb water lost from the skin. The surface area was calculated according to the formula of Benedict (*2*); oxygen consumption was determined with a Beckman paramagnetic oxygen analyzer.

Evaporative water losses from the desert tortoise were measured at 23° and 35°C, the latter temperature probably being more "normal" (Table 1). Evaporation from the desert tortoise was far less than from the two other chelonians previously examined (*1*). Thus the cutaneous water loss at 23°C was 12.2 mg cm^{-2} day^{-1} in the aquatic species *Pseudemys scripta*, 5.3 mg in the forest dweller *T. carolina*, and only 1.5 mg in *Gopherus*. The respiratory water losses in *Gopherus* were also less. The integument, however, remained a major avenue of water loss at both 23° and 35°C.

In previous tests the lizard *S. obesus* lost water less rapidly than the other reptiles, but it was the only representative from a desert. It is interesting that water losses from the integument and respiratory tract of *Gopherus* are similar to those in *Sauromalus*; the former has a far lower rate of oxygen consumption than *Sauromalus* but loses more water relative to the amount of oxygen consumed, and the similarity in respiratory loss of water is therefore fortuitous. The difference is probably caused by a greater extraction of oxygen by *Sauromalus* from the inspired air. We have shown (*3*) that *Sauromalus* may have periodic or discontinuous breathing and may extract oxygen from the inspired air at levels as low as 5 percent; such a mechanism may contribute to the above differences.

Table 1. Evaporative water loss from the desert tortoise *Gopherus agassizii* in dry air at 23°C (6 animals) and 35°C (5 animals); means ± S.E. Animals weighed 725 to 2600 g; mean, 1770 g. BW, body weight.

T (°C)	Oxygen consumption (ml g^{-1}BW day^{-1})	Water loss: Total: Percentage BW (per day)	Water loss: Total: Weight (mg cm^{-2} day^{-1})	Water loss: Respiratory: Weight (mg g^{-1}BW day^{-1})	Water loss: Respiratory: Relation (mg H_2O: ml O_2)	Water loss: Cutaneous: Weight (mg cm^{-2} day^{-1})	Water loss: Cutaneous: Percentage of total loss
23°C	0.26 ± 0.04	0.17 ± 0.03	2.0 ± 0.22	0.4 ± 0.12	1.5 ± 0.42	1.5 ± 0.21	76 ± 3.8
35°C	.47 ± .05	.34 ± .05	3.8 ± .54	1.6 ± .15	4.0 ± .65	2.1 ± .36	52 ± 4.5

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Nucleotide Formation as a Determinant of 5-Fluorouracil Response in Mouse Leukemias

Abstract. *Survival of mice bearing different transplantable leukemias and treated with 5-fluorouracil was compared with accumulation of drug nucleotides in vitro. There was significant correlation, suggesting that cellular capacity for conversion of the drug to nucleotides is a major determinant of inherent drug sensitivity of these leukemias.*

Transplantable mouse leukemias show various responses to the drug, 5-fluorouracil, ranging from almost complete resistance to "cures" (*1*). We incubated suspensions of 15 lines of murine leukemia with radioactive 5-fluorouracil and examined the intracellular disposition of the drug in order to find a basis for the variation in response observed. The rate of uptake of radioactive 5-fluorouracil in vitro was roughly similar in all cells regardless of tumor line, and the uptake occurred by passive, temperature-insensitive diffusion (*2*). Some of the radioactivity which had accumulated failed to diffuse from the cells during their subsequent washing at 37°C in drug-free medium. The relative amount of this nondiffusable fraction varied considerably among the groups tested. A correlation (Fig. 1) was found between the extent of drug conversion into nondiffusable metabolites in vitro and drug response in vivo. These metabolites were identified as a mixture of 5-fluorouracil nucleotides and RNA that contained 5-fluorouracil.

The methods we used for collection of ascitic fluid from tumor-bearing mice, isolation of cells, and incubations have been described (*3*). The uptake of the drug was measured by incubating portions of cell suspensions containing 7.5 mg of cells (wet weight) in 150 μl of buffer (*4*) with 5-fluorouracil-2-C^{14} (10 μg/ml) (*5*) for 30 minutes at 37°C, then collecting cells by centrifugation. To measure drug conversion to nucleotides, the centrifuged cells were suspended in 300 μl of buffer and incubated for 15 minutes at

37°C. After collection by centrifugation as before, the pellets were washed once in buffer at 0°C and centrifugated again, and then they were dissolved in 250 μl of NCS reagent (*6*). This solution was diluted with 10 ml of a scintillation mixture (*7*), and radioactivity was measured with a Nuclear-Chicago counter. Data thus obtained are expressed as micrograms of 5-fluorouracil converted to micrograms of nucleotides and RNA per gram of wet cells, during the standard incubation and washing procedure described.

The addition of 2,4-dinitrophenol (0.1 mmole/liter) to the medium prevented the formation of nondiffusable drug components, and efflux of intracellular radioactivity was rapid and complete during the second incubation period for all 15 cell lines. Jacquez has reported that 2,4-dinitrophenol inhibited phosphorylation of 5-fluorouracil (*8*). This, together with studies by Heidelberger *et al.* (*9*) of 5-fluorouracil metabolism by Ehrlich tumor cells, suggested that the nondiffusable drug metabolites observed probably consisted of nucleotides of 5-fluorouracil. To test this hypothesis, drug metabolites were extracted from the cells and examined by paper chromatography.

After incubation in medium containing labeled drug, cells were collected by centrifugation, and the collected cells were extracted with ten volumes of 4 percent perchloric acid at 0°C for 15 minutes; the resulting suspensions were clarified by centrifugation and adjusted to *p*H 6 with potassium hydroxide. The precipitate of potassium perchlorate was removed by centrifugation, and the supernatant was concentrated by lyophilization. Approximately 60 percent of the total radioactivity present in the cell pellets was extracted by the perchloric acid. This fraction did not vary significantly in all 15 cell lines. The lyophilized extracts were dissolved in water, then chromatographed on Whatman No. 1 paper with two different solvents (*10*). The paper chromatograms were scanned by placing small sections in vials and moistening with scintillator solution. This method detects C^{14} radioactivity efficiently enough to locate regions containing as little as 500 to 750 disintegrations per minute. Most of the radioactivity always migrated just behind uridine triphosphate. The remainder was divided into two fractions which migrated behind uridine diphosphate and uridine monophosphate. Elution from the paper and subsequent acid hydrolysis yielded material which behaved chromatographically like 5-fluorouracil. From this observation, and previous reports on cellular handling of 5-fluorouracil, the three fractions were judged to be the mono-, di-, and triphosphate nucleosides of 5-fluorouracil.

The 40 percent radioactivity, residual in the cell pellet after cold perchloric acid extraction, was digested in 1*M* KOH for 16 hours at 24°C. This solution was neutralized with perchloric acid, the precipitate of potassium perchlorate was removed by centrifugation, and the resulting fluid was chromatographically examined. One radioactive spot was found which migrated slightly behind uridine monophosphate. This was judged to represent 5-fluorouridine monophosphate liberated from cellular RNA.

This study, and work cited (*9*), has shown that the principal products of 5-fluorouracil metabolism in animal leukemia cells are ribonucleotides, with considerable incorporation of drug into cellular RNA. The enzymes responsible for conversion of 5-fluorouracil into nucleotides are apparently the same enzymes that carry out analogous reactions of uracil (*11*). The deoxyribotide of 5-fluorouracil is formed to only a minor extent and could not be detected. It is believed to be the most potent antitumor drug metabolite (*9*). This compound inhibits thymidylate synthetase (inhibition constant, $K_i = 5 \times 10^{-9} M$) leading to interference with DNA synthesis (*12*). Our studies on total nucleotide formation may reflect the relative amount of the deoxyribotide formed, and this may account for the correlation between nucleotide formed and drug response. The therapeutic effect of 5-fluorouracil may, however, be related to the total amount of all drug nucleotides formed. The relative contribution of each to response is still unknown.

Our studies indicate that the cellular capacity for nucleotide formation from 5-fluorouracil is an important determinant of inherent sensitivity to the drug. The rate-limiting step in the formation of the nondiffusable pool of drug metabolites is not precisely known, since several enzymes are involved in nucleotide formation. In human leukemic cells, 5-fluorouracil nucleotide formation occurred to only a minor extent, three- to fourfold less than the lowest rate shown in Fig. 1 (*13*). Hence, these

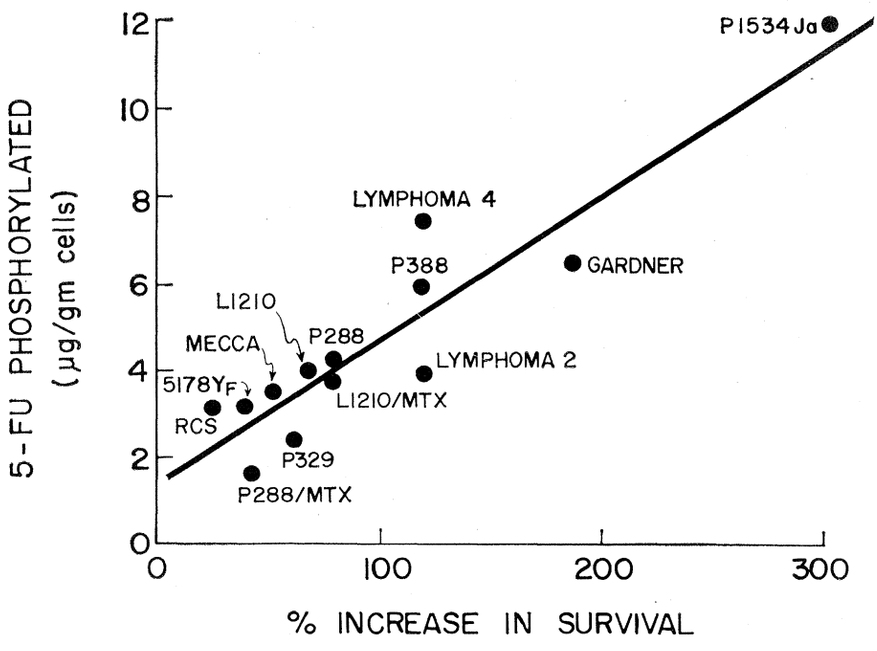

Fig. 1. Correlation between 5-fluorouracil metabolism in vitro and drug-promoted survival of tumor-bearing animals. Metabolism was determined by measurement of drug conversion to ribotides plus RNA as described in the text; results are shown as the number of micrograms of drug metabolized per gram of wet cells per standard incubation period. For survival data, see (*1*). Each circle represents cells from the tumor strain indicated.

cells might be described as "naturally resistant" to 5-fluorouracil. Response to 5-fluorouracil therapy in human leukemia has not been impressive. The extent of the conversion of 5-fluorouracil to nucleotides may offer a suitable predictive index of drug response in man, and may be an aid in selecting patients for 5-fluorouracil therapy.

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References and Notes

1. To obtain survival data, intraperitoneal injections (30 mg/kg) were given, from day 1 to day 10, to animals after inoculation with 10^6 tumor cells. Survival increase = 100 $(T-C)/C$, where T is the mean survival time (in days) of tumor-bearing animals receiving the drug and C is the mean survival time of the untreated (control) animals. Each point on the figure represents the data obtained from at least ten mice.
2. Rapid diffusion of 5-fluorouracil into Ehrlich ascites cells was reported by J. A. Jacquez, *Proc. Soc. Exp. Biol. Med.* **109**, 133 (1962).
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4. This buffer contained 62 m*M* tris, *p*H 7.2; 65 m*M* NaCl, 15 m*M* KCl, and 8m*M* $CaCl_2$.
5. 5-Fluorouracil-2-C^{14} (20 mc/mmole) was obtained from Calbiochem Corp.
6. Nuclear-Chicago Solubilizer (NCS) was furnished by the Nuclear-Chicago Corporation for solubilizing proteins for scintillation counting. Substitution of similar products, such as hyamine or ethanolamine, was satisfactory.
7. This was prepared by mixing 600 ml of toluene, 400 ml of pure methyl cellosolve, 4 g of 2,5-bis-[2(5-*tert*- butylbenzoxazolyl)] thiophene (BBOT) (Packard Instrument Co.), and 60 g of naphthalene.
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Cilia Regeneration in the Sea Urchin Embryo: Evidence for a Pool of Ciliary Proteins

Abstract. *Late gastrulae of* Paracentrotus lividus *regenerated cilia after being deciliated in hypertonic sea water. Regeneration was not affected by actinomycin D or puromycin. Actinomycin D also did not affect ciliary protein synthesis during regeneration, although overall embryonic synthesis was depressed. Puromycin inhibited both total embryonic and ciliary protein synthesis. The data indicate that ciliary protein synthesis is controlled by a stable template and that the regenerating cilia are formed from a pool of ciliary proteins. It is suggested that the proteins of the mitotic apparatus and of the cilia may be related.*

There is fairly general agreement that the template- or messenger-RNA requisite for protein synthesis during early development is present in an inactive state in the unfertilized egg of the sea urchin and that fertilization or parthenogenetic treatment of the egg is required for it to become active (*1, 2*). Although RNA, some or all of which may be informational, is synthesized early in development, it appears to remain in a masked form until later (*2, 3*).

The overall pattern of early protein synthesis has been examined by Spiegel *et al.* (*4*) and by Terman and Gross (*5*) who used gel-electrophoretic procedures on soluble proteins isolated from embryos in various stages of development. In agreement with the work of Pfohl and Monroy (*6*), the former group found no marked qualitative difference during early development, either in the presence or absence of actinomycin D, a compound which has been shown by Gross *et al.* (*2*) to inhibit RNA synthesis during this period. Terman and Gross (*5*) found systematic changes in the pattern of protein synthesis, either in the kinds of proteins made or in the changing rate of synthesis of one particular protein, but these changes were not affected by actinomycin D.

Some of the specific proteins made during early development appear to be associated with the mitotic figure (*7*) and, more specifically, with the microtubular elements of the spindle region (*8*).

Previous study of ciliary protein synthesis associated with the initial formation of cilia during the blastula stage of *Arbacia punctulata* has indicated that ciliary proteins are made during early development and that they form a reserve necessary for ciliary development (*9*). Actinomycin D partially reduced the incorporation of precursors into ciliary proteins, but cilia still developed.

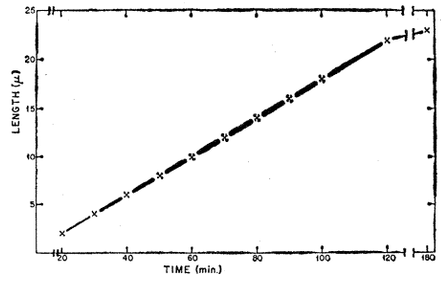

Fig. 1. Growth rate of regenerating cilia of 24-hour-old gastrulae of *Paracentrotus lividus* at 20°C.

To determine whether these conditions also hold later in development, 18- to 24-hour-old embryos of the sea urchin, *Paracentrotus lividus*, in the late gastrula stage were used for the regeneration experiments. Adults collected from the Bay of Naples region shed their gametes when 0.5*M* KCl was injected into their peritoneal cavities. The eggs were fertilized and placed overnight in continuously stirred, Millipore-filtered sea water at 20°C. Deciliation was induced by short exposure of the embryos to sea water whose osmolarity was doubled by the addition of 29.2 g of NaCl per liter of filtered sea water. The embryos were rapidly returned to continuously agitated, normal sea water at 20°C.

The regenerating cilia at the embryo surface were measured with oil immersion-phase contrast optics. Measurable cilia were first seen 20 minutes after deciliation as stiff rods 2 μ long. The rate of growth was 1 μ/5 min for nearly 2 hours; the rate then decreased over the next 2 to 3 hours until the cilia reached a final length, 24 to 25 μ, equal to that of the original cilia (Fig. 1). The cilia of the apical tuft do not regenerate to their original length of 70 to 75 μ. Extrapolation of the curve in Fig. 1 to the abcissa indicates that there is a 10-minute lag period between deciliation and the beginning of ciliary regeneration if one assumes a constant rate of growth. This may be caused by disturbing influences