detailed examination of its properties and permit us to manipulate its genome with the intent of developing attenuated mutants for use in the prevention of a serious diarrheal disease of human infants.

Note added in proof: After submission of this manuscript, we developed a plaque assay for the Wa strain of human rotavirus. The relationship of the Wa strain to the cultivable animal rotaviruses shown in Table 1 was confirmed with this assay for measurement of plaque reduction antibody.

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23 July 1979; revised 19 September 1979

Retesting the Commitment Theory of Cellular Aging

Abstract. *The commitment theory of human fibroblast aging predicts that 55 percent of cells will be nondividing at the middle to late stages of the replicative life-span; in the present study, however, fewer than 10 percent were nondividing. The fact that no immortal diploid cells have yet been reported is also at odds with the theory. Available data on the variable life-span of clones and mass cultures, the dependence of longevity on population size, and the predominance of certain cell types at termination of a culture are compatible with simpler theories, which support the idea that the limited replicative life-span of diploid fibroblasts is a valid model for organismic aging.*

Kirkwood and Holliday (*1*) and Holliday *et al.* (*2*) proposed a commitment theory to explain the finite life-span in culture of human diploid fibroblasts (HDF). They asserted that all cells initially belong to a class of uncommitted or immortal cells. At each division a fraction of the cells enters the committed or mortal class, which, after additional divisions, ceases to divide. As the culture expands, the uncommitted population accounts for a diminishing fraction of the total, and by passage 20 there is a good chance of losing the uncommitted population altogether if the number of cells retained at each subcultivation does not greatly exceed 10^6 (*2*). They concluded that the finite replicative life-span of HDF is an artifact of the relatively small number of cells in culture. We argue that the commitment theory of cellular aging is untenable as proposed; even if it were correct in principle, the finite life-span of HDF would still reflect the age-dependent decline of cellular function in vivo.

We emphasize that we have tested the Kirkwood-Holliday model and its assumptions only and are proposing neither an altered version of their commitment theory nor a new model of our own. The main assumptions of their model, then, are (i) initially there is one uncommitted cell (U), and at each generation (g) the probability (P) that an uncommitted cell becomes committed is .275; (ii) all cells attach after each subcultivation and double at a uniform rate; and (iii) committed cells (C) become nondividers (D) after an additional 60 divisions (M).

Equations for the fraction of the population in each class have been derived for the case in which uncommitted cells are lost by dilution at generation 43 (*3*). Solutions to these equations were determined and the theoretical fractions of dividing and nondividing cells were plotted as a function of the percentage of life-span completed (Fig. 1). Kirkwood and Holliday (*1*) determined the relationship between generations and passage level in vitro based on 20 to 25 cell doublings in vivo and during the phase of explantation. This assumption is tenuous, since little is known about the history of cells in vivo or during emigration from the explant. The relationship between passage and generation is further complicated by asynchronous cell division and plating efficiencies of less than 100 percent (*4*, *5*). Nonetheless, the striking feature of the theoretical curve (Fig. 1b) is the sharp rise in the fraction of nondividing cells at $g = 60$ (50 percent of the life-span completed) from 0 to 55 percent. This change must correspond to the 45 percent decline in growth rate reported by Kirkwood and Holliday (*1*). Although the abrupt transition is an artifact of a simplified model wherein cells divide synchronously, it is fundamental to the commitment theory, since it was used to determine P and M (*2*).

Rather than measure growth rate, which is influenced by several variables, we measured the fraction of nondividing cells directly by labeling with thymidine (*6*) to determine whether the proposed transition existed. The fraction of labeled nuclei was 80 to 90 percent throughout the middle to late interval of passage (Fig. 1a); the commitment theory predicts only 45 percent for MRC-5 cells, which is clearly inconsistent with our data or data on any of the 12 fibroblast strains tested so far (*6*, *7*). The decline in growth rate at passage 42, during what was ostensibly a single experiment (*1*), must have been caused by other factors, such as a serum change or alteration in culture conditions.

Since the fraction of nondividing cells is less than 10 percent for MRC-5 and other cells (including WI-38 and IMR-90)

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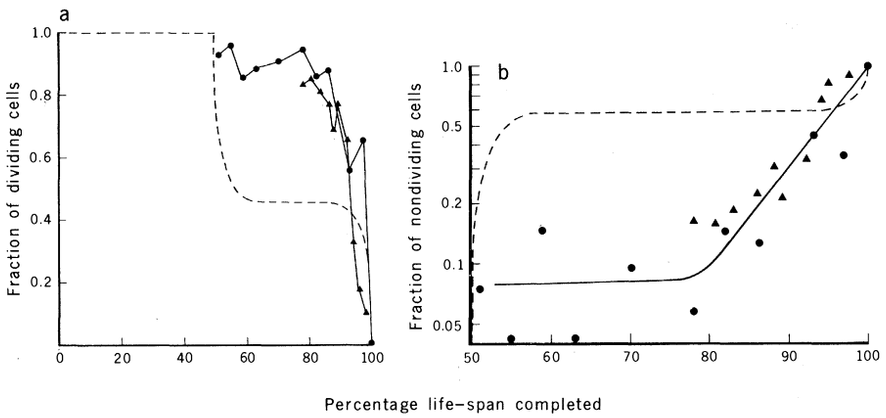

Fig. 1. Predicted (dashed line) and observed (solid line) decrement in the fraction of dividing cells during the replicative life-span of MRC-5 fibroblasts. The theoretical line shows a transition when the oldest committed cells cease division at passage 40 (*2*). Since these MRC-5 cultures achieved 78 (●) and 82 (▲) population doublings, we set the transition at 50 percent of the life-span completed. Experimental data were obtained at intervals by harvesting confluent cultures and inoculating 10^4 cells into dishes containing 20 by 20 mm^2 cover slips. After 1 to 2 days, ^{3}H-labeled thymidine was added (6.7 Ci/mmole; final concentration, 0.5 μCi/ml) and the cultures were incubated a further 30 to 40 hours. Cover slips were then treated as described in (*6*). Data represent the mean fraction of (a) labeled nuclei and (b) unlabeled nuclei among 400 to 600 nuclei at each passage of cultures from two separately reconstituted ampules of MRC-5.

until 80 to 85 percent of the life-span is completed (*6–8*), the commitment theory would predict $P < .05$. However, if $P < .05$, then M would have to be greater than 269 doublings for the immortal cells to decline in number to less than one in 10^6 before the transition to stage 2 (*9*). To reach terminal stages after this transition, the culture would require at least 269 additional generations. However, the most generous estimates for the maximum number of cell generations accruing to HDF prior to termination do not exceed 180 (*4, 10*). Therefore, our data cannot be reconciled with the model—even after adjusting various parameters.

According to the commitment theory, replicative life-span depends on the size of the culture. With $P = .275$, each tenfold reduction in the number of cells in culture should reduce the life-span by about seven generations (*9*). This was not tested directly; instead, the effect of a single large reduction in the culture size ("bottleneck") was studied at various passages (*1, 2*). If a bottleneck of 2×10^3 cells happened to contain an uncommitted cell and was then expanded to 2×10^6 cells, the life-span would, theoretically, increase due to the relative increase in the fraction of uncommitted cells. However, all 34 bottlenecks reported at passages 8, 13, and 21 showed reduced mean life-spans (*2, 11*), which can be explained by other models postulating a low frequency of young or early-generation cells in culture (*5*).

According to the commitment theory, if cultures contain more than 2×10^8 cells, uncommitted cells would still be present at the transition to stage 2 and an immortal cell strain would result (*9*). A direct test of this prediction is not feasible, since propagation of more than 10^8 cells in replicate vessels is technically prohibitive. However, the probability of observing an immortal culture is > .98 if 500 cultures of 2×10^6 cells are carried to senescence, and approaches certainty thereafter (*12*). Hayflick (*13*) has estimated that at least 175 ampules of WI-38 cells at passage 8 have been reconstituted, expanded, and carried to phase III, representing more than 10^4 senescent cultures. We estimate that the number of other cultures carried to senescence in other laboratories is well over 1000 (*14*). The fact that no culture of HDF has ever been reported to yield an immortal diploid strain spontaneously argues against the commitment theory. In fact, when cultures from lower forms of life transform spontaneously, or when human cells are transformed by viruses to immortal lines, there is invariably a change in karyotype and in cellular metabolism (*15*). This suggests that the mechanism of transformation involves more than the mere selection of normal uncommitted cells.

Holliday *et al.* (*2*) marshal four main points as evidence for the commitment theory: (i) individual fibroblasts produce clones with variable growth potential; (ii) replicate populations of one cell strain vary considerably in their longevity; (iii) longevity is related to population size; and (*4*) when uncloned (mass) cultures contain equal numbers of electrophoretically identifiable allelic variants of glucose-6-phosphate dehydrogenase (G6PD), one of these two Lyonized cell types frequently becomes dominant at the end of the life-span (*16*).

We argue that these observations are equally consistent with a distribution of generation levels among the cells in mass culture and that it is unnecessary to postulate the presence of immortal cells. Such a distribution can arise by a variety of mechanisms based on asynchronous division (*5*). The random nature of cell selection during cloning and passage guarantees that the life-span of clones and replicate mass cultures will show considerable variation. Furthermore, a sharp reduction in culture size, especially during mid-life (when the distribution is widest), will truncate the tail of the distribution corresponding to cells at early generation levels and shorten the life-span accordingly. In cultures consisting of two distinguishable cell types, each with a minor fraction of early generation cells, random selection may by chance lead to dominance of one type of cell at termination. However, we know of no evidence for this type of random selection. Zavala *et al.* (*16*) reported that in mass cultures derived from 29 individual donors who were heterozygous for G6PD type A or B alleles, some were predominantly type A whereas others were predominantly type B at termination. But these data are invalid as evidence for the commitment theory, since its predictions apply exclusively to replicate cultures from a single cell strain. In fact, the data for a limited number of replicate cultures are contrary to the theory: the prevailing G6PD phenotype at termination appears to be consistent for a given strain rather than determined by stochastic processes (*16*).

Other data are also in conflict. Whereas the theory would predict an abrupt change in size distributions of fibroblast colonies at the time that nondividing cells first appear, Smith *et al.* (*17*) observed a gradual change throughout the life-span of the culture. The prediction (*2*) that the life-span of bottleneck cultures is reduced is supported by some studies in which WI-38 cells were used (*18*), but is at odds with other reports (*10, 19*) that the life-spans of cultures plated at clonal densities are essentially the same as those of cultures plated at high density.

In conclusion, the Kirkwood-Holliday model of uncommitted cells is novel but untenable. Even if an altered version were valid, the statement (*2*) that the limited life-span of diploid fibroblasts is an

artifact of normal culturing procedures is dubious. If a cell population stabilized in the immortal state, the proportion of uncommitted cells would s...l be extremely small. Thus, if function depends on the presence of healthy cells (*20*), then senescence could be a result of the increasing fraction of old and terminally committed cells (*21*). The idea that the replicative limit is related to terminal differentiation (*1, 2, 22*) is in accord with the more general hypothesis of programmed aging (*23*), and the culture of human fibroblasts provides the best model for experimentation.

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3. For $M = 60$, there are 62 classes of cells (U, C_1, C_2, C_3, . . . C_{60}, D). The following expressions apply to the fraction of cells in each class for $g > 60$ when uncommitted cells are lost at $g = 43$.

$$U = 0$$

$$C_i = 2^{g-60}(0.725)^{g-i}(0.275)/N$$

$$D = \frac{0.275}{0.725N}\sum_{j=1}^{g-60}(1.45)^j$$

where

$$g = 61, 62, \ldots 103$$
$$i = g - 43, g - 42, \ldots 60$$

$$N = 2^{g-60}\sum_{i=g-43}^{60}(0.725)^{g-i}(0.275) + \frac{0.275}{0.725}\sum_{j=1}^{g-60}(1.45)^j$$

For all other i, $C_i = 0$.
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8. Since some dividing cells may not utilize exogenous thymidine for DNA synthesis (*6*) or may cycle more slowly than the labeling period of 30 to 40 hours, the fraction of unlabeled cells may actually overestimate the fraction of nondividing cells.
9. The fraction of uncommitted cells = $U/N = (1 - P)^g$ or $g = \log (U/N)/\log (1 - P)$. When $U/N < 10^{-6}$ and $P < .05$, $g > 269$. When $P = .275$ and $U = 1$, $g = 7 \log N$. When $N > 2 \times 10^8$, $g > 60$. See also figure 3 in (*1*).
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11. Data from these bottleneck experiments (*2*) were not analyzed statistically for goodness of fit to the predictions of the commitment theory.
12. The probability of finding at least one uncommitted cell in a culture of 2×10^6 cells is $1 - (1 - P^{60})^{2 \times 10^6} = .00831$. Thus in N' cultures of 2×10^6 cells, the probability that at least one culture contains an uncommitted cell is $1 - (1 - .00831)^{N'}$.
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25 September 1979

Doridosine: A New Hypotensive *N*-Methylpurine Riboside from the Nudibranch *Anisodoris nobilis*

Abstract. *A new* N-*methylpurine riboside (doridosine), probably* N^1-*methylisoguanosine, was isolated from the digestive glands of a nudibranch. Doridosine produces prolonged hypotension and bradycardia in anesthetized rats, decreases the rate and the amplitude of contraction of guinea pig atria in vitro, and causes the heart rate in anesthetized mice to be reduced by 50 percent for many hours after which the animals recover completely.*

We recently reported that some common California dorid nudibranchs contain several pharmacologically active substances (*1*). We now report the isolation, chemical characterization, and pharmacological properties of one of these, a new *N*-methylpurine riboside named doridosine, isolated from aqueous extracts of the digestive glands of *Anisodoris nobilis*.

Specimens of *Anisodoris nobilis* were collected subtidally and intertidally in Monterey Bay, California. The progress of purification was assayed on spontaneously beating guinea pig atria (*1*). The activity was determined from the amplitude of contraction after 1 minute as a percentage of the control. The logarithmic dose-response relationship was linear over a 15-fold range.

Pooled digestive glands (128 g, wet weight) were homogenized and dialyzed (Spectrapor 1 tubing; molecular weight cutoff, 6000 to 8000) at 5°C against one volume of distilled water changed five to eight times. The combined dialyzates were concentrated and lyophilized, and the residue was extracted with methanol. The syrup resulting from vacuum evaporation of the methanol was chromatographed on a column (Bio-Gel P-2) by elution with distilled water. The lyophilized combined active fractions were extracted with methanol, and the concentrate was chromatographed on silica gel (Woelm, 0.03 to 0.06 mm, 2.2 by 22 cm) with a mixture of tertiary butyl alcohol, ethyl acetate, water, and acetic acid (40:10:2:1). The active fractions (400 through 700 ml) were concentrated, and the residue was crystallized from a mixture of tertiary butyl alcohol, methanol, and water (2:1:1) to give 31 mg (about 1 mg of doridosine per gram of dry digestive gland). These crystals were further crystallized from a mixture of methanol and tertiary butyl alcohol and twice washed with 0.25 and 0.10 ml of acetic acid

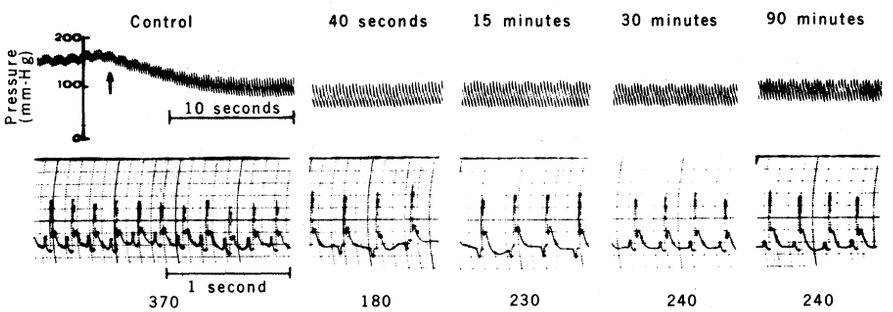

Fig. 1. Arterial pressure (upper tracing) and electrocardiogram (lower tracing) of a rat anesthetized with sodium pentobarbital. Arterial pressure was recorded from one carotid artery on a Grass polygraph by means of a Statham model P23 AC transducer. Electrocardiograms from lead 2 were recorded simultaneously on a separate polygraph at higher chart speed. Doridosine (2 mg/kg) was injected into the saphenous vein beginning at the arrow; the injection (0.52 ml) lasted 140 seconds. Figures below the electrocardiograms are heart rates per minute.

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