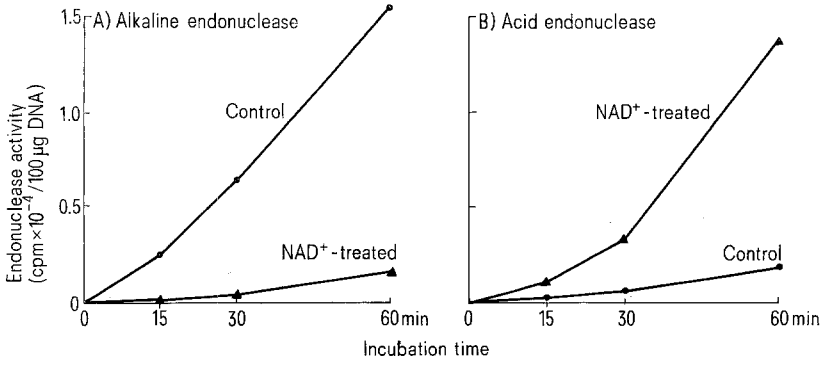


Fig. 2. Effect of ADP-ribosylation on the activities of $Ca^{2+}$, $Mg^{2+}$-dependent (A) and $Mg^{2+}$-dependent endonuclease (B) of adult rat testis nuclei.

of rat liver nuclei and chromatin treated with $NAD^+$ was inhibited[4], the effect of poly(ADP-Rib) on the template activity was investigated. $Ca^{2+}$ was added to the reaction mixture to activate the template (Figure 1). The template of chromatin incubated with $NAD^+$ was markedly suppressed compared to that of untreated chromatin (Figure 1). This finding is in agreement with the result obtained with rat liver nuclei[4]. However, the template activity of immature rat testis chromatin incubated with $NAD^+$ was not suppressed (Figure 1). The basis for the observed differences in template activity between adult and immature rat testis chromatin treated with $NAD^+$ is not known.

Since it was demonstrated that $Ca^{2+}$, $Mg^{2+}$-dependent endonuclease can activate the template of rat liver nuclei for DNA synthesis in vitro[4], the influence of poly(ADP-Rib) on endonuclease activities of rat testis nuclei was investigated. The isolated adult rat testis nuclei possessed high acid and alkaline endonuclease activities (Figure 2). Preincubation of the isolated testis nuclei with $NAD^+$ affected an inhibition of the $Ca^{2+}$, $Mg^{2+}$-dependent endonuclease activity (Figure 2). The finding is in agreement with the result obtained with rat liver nuclei[7,11]. In contrast, preincubation of isolated adult rat testis nuclei with $NAD^+$ resulted in a stimulation of $Mg^{2+}$-dependent acid endonuclease activity (Figure 2).

To establish that the observed stimulation of the acid endonuclease activity by $NAD^+$ treatment was due to poly(ADP-Rib) formation and not to a direct effect of the nucleotide on the enzymic activity, other nucleotides were tested for their capacity to influence endonuclease activity (Table II). Only $NAD^+$ which formed poly(ADP-Rib) was stimulatory while nucleotides which were not substrates for poly(ADP-Rib) synthetase did not influence acid endonuclease activity (Table II). The present results suggest that the stimulatory effect of $NAD^+$ is dependent upon its conversion to poly(ADP-Rib).

In a previous study it was demonstrated that the DNA polymerase bound to rat liver chromatin was easily dissociated on incubation of chromatin with $NAD^+$[5]. Although the molecular mechanism of the interaction of enzymes with chromatin is not known, poly(ADP-Rib) formation might alter the structure and/or electrostatic charge of chromatin and nuclei and influence the interaction of acid endonuclease and DNA polymerase with nucleoproteins. In a previous study it was demonstrated that ADP-ribosylation of $Mg^{2+}$-dependent endonuclease of rat liver did not occur, suggesting that the effect of poly(ADP-Rib) formation on the enzyme is probably indirect[11].

*Zusammenfassung.* Inkubation von isolierten Rattenhodenzellkernen mit Nikotinamidadenindinukleotid induzierte die Bildung von Polyadenosindiphosphatribose, eine Hemmung der $Ca^{2+}$, $Mg^{2+}$-abhängigen, alkalischen Endonuklease und paradoxerweise eine Stimulation der $Mg^{2+}$-abhängigen, sauren Endonuklease.

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[13] Acknowledgments. This work was supported by NICHHD grant No. PO1 HD05671. The authors are grateful to Ms. D. Breger for technical assistance.

## Glucose Consumption by Early and Late-Passage Diploid Human Fibroblasts During Growth and Stationary Phase

Cultured human fibroblasts are now used extensively to study hereditary disorders[1,2] and since these cells have a finite replicative capacity, they are also useful for research on biological aging[3–7]. Earlier work on carbohydrate metabolism has shown that while human fibroblasts can utilize a variety of hexoses, they have a preference for glucose[8] which is degraded predominantly to lactate[9,10] and $CO_2$, the latter occurring mainly via the pentose pathway[11,12]. In previous studies on glucose oxidation[11] we were unable to find differences between normal strains at early-passage and those derived from individuals with diabetes mellitus, an age-dependent disorder of carbohydrate metabolism[13]. However, since diabetic cultures have a decreased growth capacity[4,14–16]

it seemed important to determine whether glucose metabolism was altered during aging in vitro. We now report that late-passage cultures of skin fibroblasts derived from a normal adult male utilize significantly more glucose per cell than the same strain at early passage during growth and in stationary phase.

*Materials and methods.* Human fibroblasts. The cell strain J-004 was used throughout these studies and was derived from a normal 22-year-old male with repeatedly normal tests of glucose tolerance and a negative family history for diabetes mellitus[4]. Cells were derived and grown as described previously[4,11] in Eagle's medium supplemented with non-essential amino acids, 15% fetal calf serum and glucose to a final level of 15.5 m*M*. Incubation was carried out in a humidified atmosphere of 95% air — 5% $CO_2$.

Culture techniques. Cells were subcultured at a 1:8 split ratio and utilized 3 mean population doublings each time they attained confluence[3-5]. This cell strain could be subcultured in this manner about 25 times, or through 75 mean population doublings, prior to complete cessation of growth. Early-passage cells were studied within 5-10 subcultures of the primary explant stage while late-passage fibroblasts were used within 3–5 subcultures of termination.

For these experiments, cells at early and late-passage levels were thawed from storage in liquid nitrogen into the regular growth medium and allowed to grow to confluence twice before use. Cells were then harvested by routine methods[3-5,11], and split at a 1:8 ratio with cell counting on a Cytograph® electronic counter (Biophysics Inc., Mahopac, N.Y.). Starting on day 1 and at intervals thereafter (see below), triplicate dishes of early and late-passage cultures were removed from the incubator followed by decanting and freezing of the medium and harvesting and counting of the adherent cells. Beginning on day 2, in addition to the regular triplicate dishes for early and late-passage cultures, another triplicate set was refed with fresh growth medium followed 2 days later by determination of cell counts and the concentration of glucose remaining in the medium. In this way, cell proliferation and glucose consumption were monitored under two conditions: during continuous incubation in the original growth medium, and starting on day 4, following refeeding with fresh medium at day 2 and subsequent 2 day intervals. Determinations of the pH of the medium were made at random intervals during growth and stationary phase and were found to be within the range of 7.2–7.4.

Glucose determination. Assays were performed on aliquots of zero time and spent medium following deproteinization with 0.5 *N* perchloric acid, neutralization with 6 *N* KOH and an enzymatic assay[17] using hexokinase and glucose-6-phosphate dehydrogenase (Boehringer-

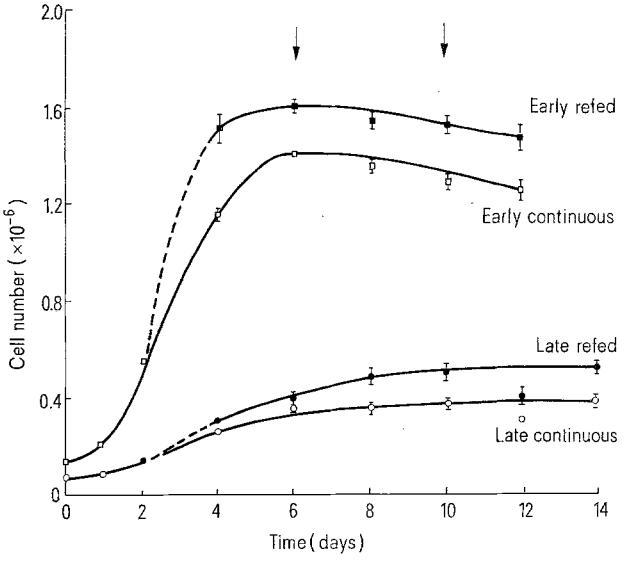


Fig. 1. Growth of early and late-passage fibroblasts under conditions of continuous incubation and refeeding. Cells were subcultured at a 1:8 split ratio at time 0. Triplicate dishes were harvested on the day indicated after continuous incubation conditions or 2 days following refeeding. Refeeding involved total replacement of growth medium with fresh medium in triplicate dishes beginning at day 2 and at 2 day intervals thereafter. Arrows at days 6 and 10 indicate visual confluence in early and late-passage cultures, respectively. Values plotted are the means ± S.E.M. When not shown S.E.M. was smaller than the symbol.

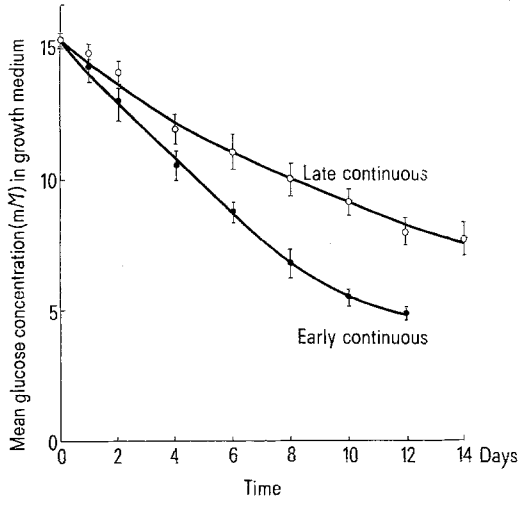


Fig. 2. Mean glucose concentration of growth medium as a function of time after subculturing early and late-passage fibroblasts at a 1:8 split ratio.


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Mannheim Co.) to convert glucose to 6-phosphogluconate and NADP to NADPH followed by measurement of absorbance at 340 nm in a Gilford spectrophotometer.

*Results.* Figure 1 shows that under continuous incubation, early-passage cells began to increase logarithmically about 1 day after subculture with 1 population doubling occurring approximately every 24 h. Cell number reached a maximum at day 6 at which time cells appeared visually confluent on microscopic examination. Thereafter, cell counts may have decreased slightly to day 12. Refed cells at early passage proliferated more rapidly, reached a plateau phase earlier and at a 10–15% higher saturation density compared to non-refed cells. Late-passage fibroblasts under continuous incubation grew more slowly after subculture with a mean doubling time of approximately 48–60 h. Plateau phase was achieved between days 8 and 12. Refed late-passage cells, as at early passage, appeared to grow more rapidly and to reach a plateau somewhat earlier and at higher saturation density than non-refed cohorts.

Figure 2 indicates that per dish, under continuous incubation, total glucose consumption was greater in rate and extent in early compared to late-passage cultures. At both passage levels, glucose consumption was greater during the period of most rapid growth, diminishing later in stationary phase, but this was more pronounced in early-passage cells. Figure 3 shows that in refed cultures as well, early-passage cells used more glucose per dish compared to late passage. However, the amount of glucose consumed under these conditions appeared to be constant, that is, independent of whether cells were actively dividing or in stationary phase. Using the data from Figures 1–3, calculations were made for the glucose consumption per cell (Table). Under continuous incubation, both young and old cultures consumed more glucose during early stages of growth with glucose consumption dropping significantly in stationary phase. With refeeding, this effect was less pronounced for late-passage cells and was marginally apparent at early passage. However, in both cases refeeding was clearly associated with augmented glucose utilization. Under continuous incubation, late-passage cells consumed 2.0–3.3 times more glucose than early-passage cells and no consistent trends were seen in this ratio at different stages of growth or stationary phase. With refeeding, these ratios ranged from 1.4–2.3.

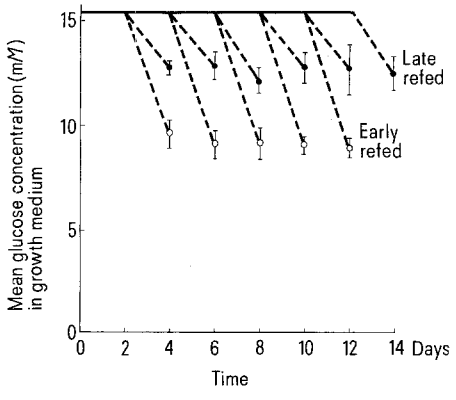


Fig. 3. Mean glucose concentration of growth medium 2 days after refeeding beginning on day 2 and at 2 day intervals in early and late-passage cultures. Horizontal continuous line indicates glucose concentration of fresh, unused medium. Oblique interrupted lines represent theoretical glucose depletion during 2 day intervals.

Glucose consumed during 2 day intervals in early and late-passage cultures[a] (ng per cell)

| | Continuous incubation Passage level | | | Refeeding Passage level | | |
|---|---|---|---|---|---|---|
| Days | Early | Late | Ratio L/E | Early | Late | Ratio L/E |
| 0–2 | 6.1 | 16.0 | 2.6 | — | — | — |
| 2–4 | 2.3 | 6.0 | 2.6 | 5.0 | 11.3 | 2.3 |
| 4–6 | 1.5 | 3.6 | 2.4 | 4.1 | 7.0 | 1.7 |
| 6–8 | 1.3 | 2.6 | 2.0 | 3.8 | 7.0 | 1.8 |
| 8–10 | 0.9 | 2.3 | 2.6 | 4.1 | 5.6 | 1.4 |
| 10–12 | 0.6 | 2.0 | 3.3 | 4.1 | 6.2 | 1.5 |
| 12–14 | — | 1.5 | — | — | 6.1 | — |

[a] Calculated from data on glucose consumption in Figures 2 and 3 and the cell count at mid interval in Figure 1.

*Discussion.* The data confirm earlier reports that glucose consumption is highest during intervals of rapid cell proliferation[8, 18]. This is the case in both the rapidly growing early-passage cells and the more slowly growing cells at late-passage. During aging in vitro, late-passage fibroblasts utilize about 1.5–3 times more glucose per cell than early-passage cells at comparable stages. While these results appear in conflict with those of CRISTOFALO and KRITCHEVSKY[10] for WI-38 cells, it may be that certain methodological and computational differences are important. Thus, their results were obtained on cell suspensions incubated for 1 h with 7.7 m*M* glucose in a minimal salt solution. Additionally, glucose metabolism was studied in cells harvested during late log growth, presumably after microscopic visualization, and it may have been difficult to consistently obtain cells at comparable stages of confluency, particularly as growth began to decelerate in old cultures. Furthermore, since their results were expressed per mg dry weight, a parameter which increases during senescence[19], it may be that differences between early and late-passage cultures were masked. Even then, it appeared that cells beyond 30 subcultures were in fact consuming somewhat more glucose. Nevertheless, it is recognized that the choice of a denominator from among the several that are available often presents a problem. Indeed, if we had chosen to express the data per unit of protein or volume the differences in this report would have been abolished because the same diploid strain used here doubles its protein content and triples its volume during aging in vitro[20]. However, since the cell is the smallest intact functional unit, it seemed reasonable to present data on a cellular basis. Finally, it is also noteworthly that WI-38 was derived from the lung of an embryo of unknown parentage which raises questions of tissue specific, fetal versus postnatal and genetic differences in glucose metabolism[11–16, 21, 22]. It is essential, therefore, for valid com-

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parisons of glucose metabolism, that cultures are carefully matched for age in vivo and in vitro, preferably derived from skin (the best tissue for routine biopsy) from a single anatomical site and from postnatal donors of known predisposition to diabetes.

[23] We thank Mrs. ELENA MOERMAN for expert technical assistance. Supported by grants from the Medical Research Council of Canada and the Canadian Diabetic Association Foundation Fund during the tenure of a scholarship from the M.R.C. (SG).

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*Résumé.* Les fibroblastes humains utilisent une quantité de glucose plus grande pendant leur croissance logarithmique que dans leur phase stationnaire. Les cultures à passage tardif consomment plus de glucose par cellule que les cultures à passage hâtif sans égard à la phase. Ce système doit être utile pour rechercher les altérations métaboliques au cours du vieillissement cellulaire et pour découvrir la base biochimique de certaines maladies dépendant de l'âge.

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## Biometric Analysis of Incipient Speciation in the Ringed Snake, *Natrix natrix* (L.)

*N. natrix* shows a considerable amount of infrsapecific variation and a large number of subspecies have been recognized[1–4] based on only a few characters, an inadequate number of specimens and without proper consideration of the non-geographic infraspecific variation.

This paper is an attempt to make a more objective and comprehensive assessment of the status of the various populations by using univariate and multivariate biometric methods to analyze the complicated patterns of geographic variation and univariate methods to detect and negate the effect of the non-geographic infraspecific variation such as allometric growth and sexual dimorphism.

750 specimens representing the entire range of the species were studied and the range of the species was then divided into approximately 50 small relatively homogeneous geographic units (on the basis of collecting gaps, physical isolation, and the overall similarity of specimens as assessed by cluster analyses) so that the geographic variation between the geographic units and the non-geographic infraspecific variation within the units could be analyzed.

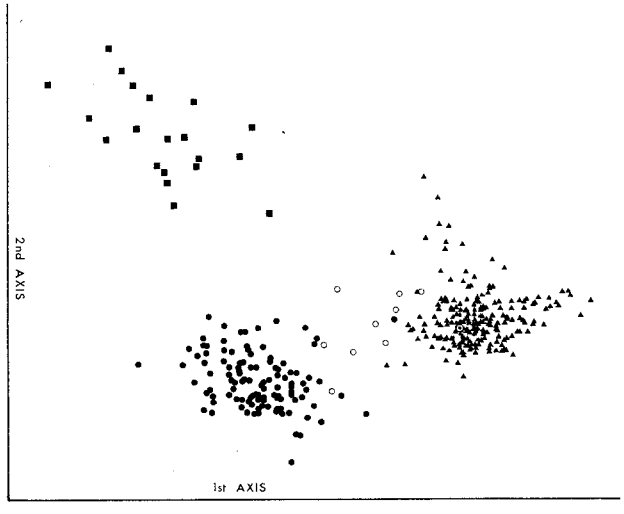


Fig. 1. Canonical analysis. Female specimens, 48 characters. First axis has 33.3% discrimination. Second axis has 16.5% discrimination. Solid circles, western specimens; triangles, eastern specimens; squares, Tyrrhenian (Corsica and Sardinia) specimens; open circles, Bologna area specimens.

Of the 160 characters[5] originally recorded from the scalation, colour pattern, internal anatomy, dentition, dermal sense organs and body proportions, 52 (50♀) characters were chosen for the canonical analyses; the rest being discarded because they did not show significant geographic variation (as assessed by a one-way analysis of variance), did not contribute original information (i.e. had a high pooled within-group correlation to other included characters) or for other taxonomic or statistical reasons.

Canonical analysis simultaneously takes into account the variation and covariation of all of the characters and computes discriminant axes so as to produce the minimum overlap between the geographic units. This method, together with the related $D^2$ analyses, clearly indicates that the geographic units largely cluster into 3 groups, i.e., an eastern cluster, a western cluster and a Tyrrhenian cluster (Figures 1 and 2). However, the specimens from the Bologna area of north Italy are phenetically and geographically intermediate between the eastern and western specimens and the Coriscan specimens are phenetically intermediate between the Sardinian and western mainland specimens.

It should be noted that a detailed investigation of all the available canonical axes, together with further multivariate analyses of the variation within the eastern and western clusters indicate that these 2 groups are not homogeneous but show a considerable amount of complex geographic variation.

The question arises as to how the 2 mainland populations could have diverged from one another because the eastern and western population are adjacent to one another in Central Europe and are not separated by any physiographic barrier. The contact zone between these 2 populations does not completely coincide with the River Rhine or mountains to the east as previously implied[1,2] and, moreover, since *N. natrix* is both fairly aquatic and found in mountainous regions the Rhine and associated mountains cannot be considered as effective barriers to gene flow.

It is suggested that during the extension of the Pleistocene ice caps the population of *N. natrix* would have been split so that separate south western and south eastern

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