In Vitro And *In Vivo* Aging In Cultured Human Diploid Fibroblasts: The Effect Of Insulin And Hydrocortisone On DNA Synthesis

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ABSTRACT

In Vitro and *In Vivo* Aging in Cultured Human Diploid Fibroblasts: The Effect of Insulin and Hydrocortisone on DNA Synthesis

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Changes in DNA synthesis with age were studied in human diploid fibroblasts from young and old human donors (*in vivo* aging) as well as in early and late passage human fibroblasts (*in vitro* aging). In serum-free cultures, insulin, at concentrations as low as 1 mU/ml, was shown to stimulate DNA synthesis in both *in vitro*- and *in vivo*-aged cells. No age-related differences in insulin-stimulated DNA synthesis were observed in these cultures. Further, no differences in specific insulin binding were observed in either *in vitro*- or *in vivo*-aged cells. The glucocorticoid hydrocortisone (5×10^{-7}M), was found to increase serum-stimulated cell growth in adult human fibroblasts. In addition, hydrocortisone enhanced insulin-stimulated DNA synthesis in serum-free cultures of both young and old *in vitro*-aged cells. While hydrocortisone enhanced insulin-stimulated DNA synthesis in young *in vivo*-aged cells, this was not observed in old *in vivo*-aged cells. Hydrocortisone exposure led to elevated specific insulin binding in cultured human fibroblasts. This was seen in both the young and old *in vitro*- and *in vivo*-aged cells without demonstrating any age-related differences.

The data indicate that loss of replicative capacity (i.e. *in vitro* aging), does not result in an inability of insulin to stimulate DNA synthesis or for hydrocortisone to modulate the insulin response. In *in vivo*-aged cells, which have a relatively similar replicative capacity, an age-related difference was observed during the hydrocortisone-modulated insulin response.

In Loving Memory

of

my Father

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INTRODUCTION

Fibroblasts as a Model System for Studying Cellular Aging

It has been well documented that cultured human diploid fibroblasts show a limited replicative lifespan *in vitro* and have been used extensively as a model for cellular aging (Hayflick and Moorhead, 1961; Goldstein, 1971; Cristofalo, 1972; Hayflick, 1975). After a period of active proliferation, the cells demonstrate an increased generation time, a gradual loss of mitotic activity, accumulation of cellular debris and finally, a total degeneration or senescence of the culture (Hayflick, 1965).

Support for the hypothesis that the changes occurring in these cells *in vitro* reflect human cellular aging, comes from the following observations:

1) The lifespan of skin fibroblast cultures *in vitro* is inversely correlated with the age of the donor (Goldstein *et al.*, 1969; Martin *et al.*, 1970; Schneider, 1979; Hayflick, 1980a).

2) Cells taken from patients with diseases associated with premature aging (Progeria or Werners' syndrome) display a reduced ability to grow and divide as compared to control cells from normal donors of the same age (Goldstein, 1969).

3) There is a direct correlation between the lifespan of a species and the replicative potential of its normal fibroblasts *in vitro* (Goldstein, 1974; Hayflick, 1974b).

When looking for changes *in vitro* which may reflect human cellular aging, it is important to look at both early and late passage

cell cultures (in vitro aging) as well as cell cultures derived from young and old human donors (in vivo aging). Evidence to support the use of both in vitro- and in vivo-aged cells comes from the work of Schneider and Mitsui (1976), who showed that differences in the parameters studied in in vivo-aged cells varied considerably from what was observed in cells of early and late in vitro passage. Also, they showed that some parameters (eg. protein and RNA content) which were significantly increased with in vitro age were not altered at all as a function of in vivo donor age.

The objectives of this study were to examine insulin-stimulated DNA synthesis in early and late passage cell cultures and to see how it compared in cell cultures derived from young and old human donors. The cells derived from young and old human donors were used at early in vitro passages.

Insulin Action in Cell Culture

Binding of hormones to specific receptors is believed to initiate biochemical responses in target cells (Cuatrecasas, 1971; Rousseau et al., 1972; Roth and Adelman, 1975). Much of the early work in hormone receptor interactions has employed target tissues, and it has only been within the past ten years that considerable interest has been focused on the binding of hormones to other cell types. It has been shown that cultured human fibroblasts, although not a major target tissue in vivo, have specific insulin receptors (Hollenberg and Cuatrecasas, 1975; Gelehrter et al., 1981; Hidaka et al., 1981). Furthermore, it has been reported that insulin stimulates DNA

synthesis in human fibroblasts (Rechler et al., 1974; Hollenberg and Schneider, 1979). Together these findings allow one to correlate binding measurements with specific biological responsiveness in cells.

There are few documented studies involving changes which occur in insulin binding and responsiveness in cultured human cells with increasing in vitro and in vivo age. In one study (Ito, 1979), a decrease in the number of cellular insulin binding sites was obtained from aged donors as compared to young donors. Another observation by Hollenberg and Schneider (1979) demonstrated that there was no significant change in either the insulin receptor number or the affinity of the receptor for insulin with respect to donor age. In a third study (Rosenbloom et al., 1976), it was shown that there was a clear correlation between the affinity of the insulin receptor and increasing in vivo age. Despite these conflicting observations, one factor which all three studies mention is the large variation observed from experiment to experiment in any given strain as well as from one sample to another within an individual experiment.

Insulin is known to affect many metabolic processes in vivo and has been shown to stimulate growth and DNA synthesis in vitro in cell types such as : mouse 3T3 cells (Holley and Kiernan, 1974), Chinese hamster ovary cells (Aidells et al., 1979), mouse embryo fibroblasts (Straus and Williamson, 1978) and human fibroblasts (Hollenberg and Cuatrecasas, 1975). In human fibroblasts, insulin has been found to stimulate DNA synthesis by as much as two and a half-fold over that of controls (Rechler et al., 1974). Rechler, however, utilized an insulin concentration (500 mU/ml) far exceeding the normal circulating

levels of the hormone present in serum (0.01 - 0.5 mU/ml). Although demonstration of biological activity at physiological concentrations of insulin in fibroblast cultures has been difficult, there have been several studies where insulin at physiological concentrations was shown to stimulate DNA synthesis in rat liver cells (Richman et al., 1976), H35 rat hepatoma cells (Koontz and Iwahashi, 1981), carcinoma cell lines (Nagarjan and Anderson, 1982) and cultured human fibroblasts (Germinario et al., 1983). The majority of investigations concerning the effect of insulin on DNA synthesis in human cells used higher than physiological levels of insulin ranging from 500-4000 mU/ml (Griffiths, 1972; Hollenberg and Cuatrecasas, 1973; Goldberg et al., 1974; Rechler et al., 1974). Why such high concentrations of insulin are necessary to stimulate growth and proliferation in cultured human cells is still unknown (Hollenberg and Cuatrecasas, 1975). It may be that insulin-stimulated DNA synthesis is mediated via a growth factor receptor rather than by the insulin receptor (Hollenberg and Cuatrecasas, 1975; Rechler et al., 1981).

Hydrocortisone-Phosphate (HP) Effects on Cultured Cells

The multiple effects of glucocorticoid hormones in cell culture have been widely studied and documented. Glucocorticoids are often called pleiotropic hormones because they are known to affect many different responses depending on the cell type. These responses include differences in cell proliferation and cell density at confluence (Smith et al., 1973; Grove et al., 1977; Rowe et al., 1977), as well as a marked increase in the overall in vitro lifespan

of several cell types including human embryonic lung cells (Macieira - Coelho, 1966; Rosner and Cristofalo, 1979). This enhancement of cellular responsiveness to HP and other glucocorticoids is not a universal phenomenon. On the contrary, there appears to be both species-and age-specific differences involved in the cell's response to the hormone (Rowe et al., 1977). Although HP was observed to consistently stimulate fetal human lung cell growth, it was also found to inhibit the virus-transformed cell lines as well as all of the cell lines derived from other vertebrate species (Rosner and Cristofalo, 1979). In the same study, it was also demonstrated that cells derived from human tissues at varying developmental stages did not all respond uniformly to the hormone. The largest degree of variability was observed in the human skin-derived cultures ranging in age from a fetus to an 82-year-old adult. In the fetal cultures studied, there was an inhibition of growth even though the lung-derived cultures from the same fetus were stimulated. As for the other non-fetal tissue, the results varied from stimulation to inhibition and, in some cases, non-responsiveness. The reasons behind this varying response to HP remain unclear.

Cristofalo (1975) found that HP enhanced serum-stimulated DNA synthesis in both early and late passage fetal human lung cells. Since there was a significant increase in the percent labelled nuclei after a 48-hour exposure to HP in both the young and the old cells, he postulated that the observed increase in DNA synthesis with HP must be partly due to an increase in the number of cells in the proliferating pool. Thus, HP somehow amplifies the stimulus for proliferation in

some cell types.

Dexamethasone (a potent synthetic glucocorticoid) was also shown to enhance the mitogenic action of epidermal growth factor in human diploid foreskin cells (Baker et al., 1978) and of insulin in chick embryo fibroblasts (Baker et al., 1979) and human fibroblasts (Germinario et al., 1983).

Objectives and Questions Asked:

In this study, cultured human fibroblasts were used as a model for investigating aging at the cellular level. In recent years, with the increased interest in the field of cytogerontology, investigators have found that there are a multitude of changes which are expressed during the process of in vitro aging in normal human cells in the presence of serum (Hayflick, 1980b). Of all the changes observed, the loss of replicative ability was a characteristic of all normal cells in culture. Hence, it was of particular interest to study several parameters which affect DNA synthesis, as normal cells age in vitro and in vivo.

In order to pursue this goal, the following questions were asked:

1) How does insulin affect DNA synthesis in cultured human fibroblasts with increasing in vitro and in vivo donor age?

2) To what extent does HP modify the insulin-responsiveness of these cells as they age in vitro and in vivo?

3) Are there any changes in the specific binding of insulin to cultured human fibroblasts as they age in vitro and in vivo?

4) Does HP alter the specific binding of insulin to these cells as they age *in vitro* and *in vivo*?

MATERIALS AND METHODS

Cell Culture

All studies were performed with human diploid fibroblasts which were established from deltoid or foreskin biopsies. Several normal skin fibroblast cell strains were purchased from the Mutant Cell Repository (Camden, N.J.). The cell strains used in this study are listed in Table I.

All cells were cultured in antibiotic-free Eagle's minimal essential medium (Eagle, 1959) supplemented with 1 mM pyruvate and 10% (v/v) fetal calf serum (10% MEM) (Microbiological Associates, Bethesda, Maryland). Cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air and the medium was changed three times weekly. When the cells reached confluence they were harvested from the culture vessels following an incubation of 3 minutes with 0.25% (w/v) trypsin and 5 minutes with 0.2% (w/v) EDTA at 37°C. The cells were counted with a hemocytometer and subcultured into 75 cm^2 plastic flasks (1:3 split ratio). The cell number at confluence was used in order to determine the number of population doublings (PD) the cells had undergone. When calculating PD, we made the assumption that 50% of the cells will not attach and grow after subcultivation (Good, 1972). That is, if there were 2 x 10^6 cells present at confluence and only 5.0 x 10^5 cells were placed in that flask to begin with, the number of PD would be equal to 3, as calculated from the equation:

[log final number of cells - (log initial 2)] / log 2.

Table I. Sex, Age and Origin of Various Cell Strains

Cell strain designation	Sex	Age (years)	Origin of tissue
GM-730*	F	45	skin biopsy
TUF	M	neonate	foreskin
PUF	M	neonate	foreskin
JSD	M	34	skin biopsy from deltoid region
GM-498*	M	3	skin biopsy
GM-500*	M	10	skin biopsy
GM-288*	M	64	skin biopsy
GM-3525*	F	80	skin biopsy from abdomen
GM-2674*	F	29	skin biopsy
GM-1681*	M	70	skin biopsy from forearm
GM-1706*	F	82	skin biopsy from forearm

All donors were apparently normal with no known history of genetic disease.

*Obtained from the Human Genetic Mutant Cell Repository, Camdon N.J.

For *in vitro* aging studies the culture was considered senescent (i.e. showed no mitotic figures after serum feeding, cells appeared large and granular) if it failed to reach confluence after 3 weeks in culture. At this point, the *in vitro* lifespan in terms of PD accrued for a particular cell strain was recorded. In this study, the *in vitro* age of the cells was often expressed as the percent lifespan completed (% LSC). This value was determined by taking the number of PD accrued at the time of the experiment, dividing it by the maximum number of PD at senescence and multiplying by 100.

General Experimental Procedure

Cells were plated at a density of approximately 1.0×10^5 cells per 35 mm diameter petri dish (Corning Co.) and grown to confluence (usually 1 week). When confluent, the medium was removed from the plates and the monolayers were rinsed once with serum-free medium (0% MEM) containing 1 mg/ml bovine serum albumin (BSA, Sigma Chemical Co.) and 4 mg (22.2 mM) glucose per ml. For the HP (Sigma Chemical Co.)-insulin interaction experiments, the monolayers were incubated in 0% MEM for a period of 48 hours with or without HP prior to insulin exposure. It has been previously shown that a 48-hour exposure to HP gives optimal results (Germinario *et al.*, 1983). Following this period of serum starvation, the medium was removed and varying concentrations of insulin (bovine pancreas, crystalline, Sigma Chemical Co.) were added to the experimental plates for 18-24 hours. The monolayers were rinsed twice with phosphate buffered saline (PBS) at pH 7.4 (37°C) and then exposed to (^{3}H-methyl) thymidine

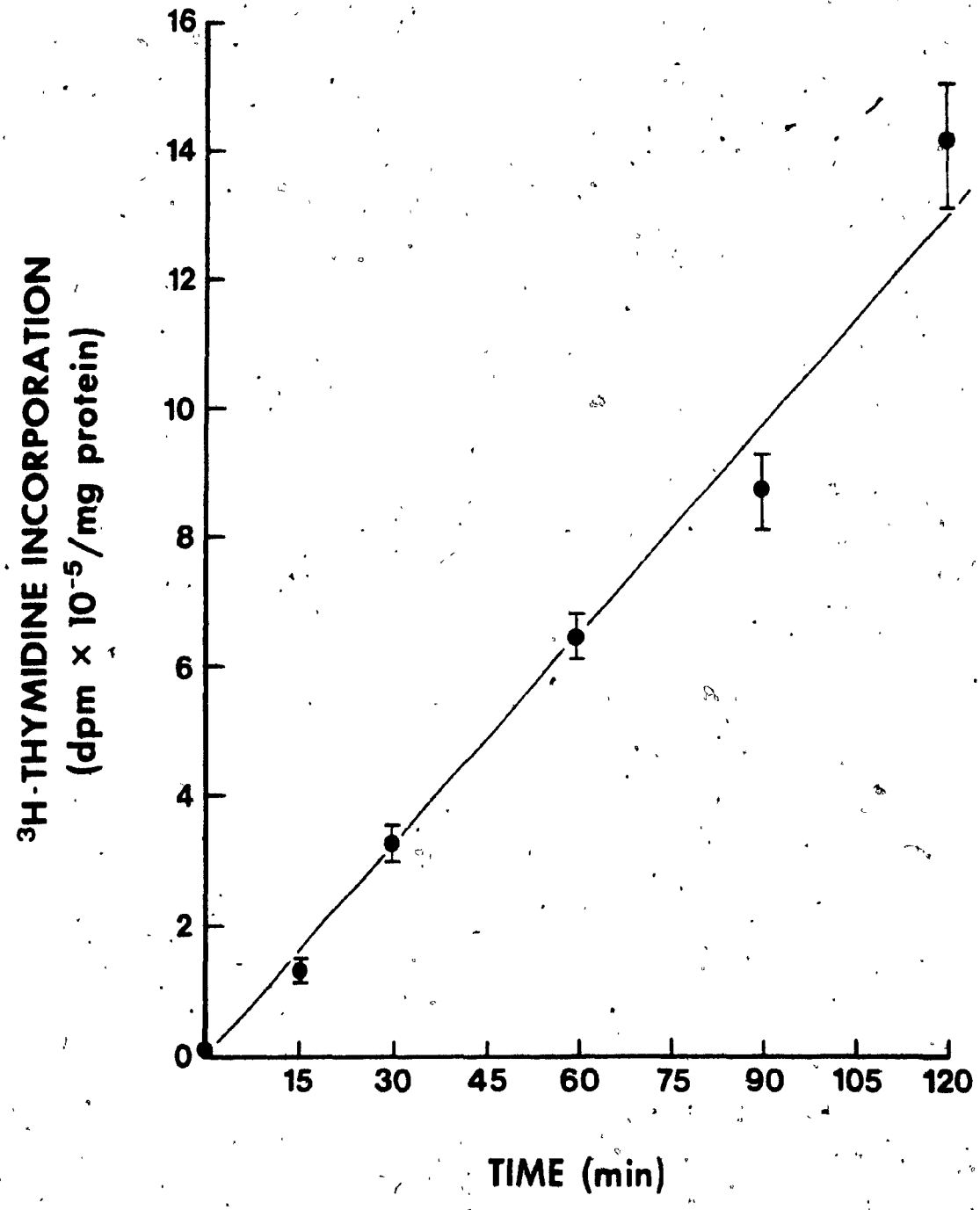
(0.25 nmoles/ml, 5 μCi/ml, New England Nuclear Corp., Boston, Mass.) in PBS for 1 hour at 37°C. Incorporation of (^{3}H-methyl) thymidine was linear for up to 2 hours (Fig. 1). Following the incubation, the cells were washed four times with cold (4°C) PBS and then exposed to cold 10% (w/v) trichloroacetic acid (TCA) for 1 hour. DNA synthesis was measured as the amount of (^{3}H-methyl) thymidine incorporated into TCA precipitable material. The monolayers were rinsed twice with cold TCA and solubilized with 1 ml of 1N NaOH for 1 hour. Aliquots from each plate were taken for liquid scintillation counting and protein determination (Lowry et al., 1951).

Insulin Binding Studies

The procedure followed in these studies was modified from that previously described by Fukushima et al., (1981). Briefly, ^{125}I-insulin binding was determined following a 48-hour period of serum-starvation either in the presence or absence of HP. The medium was removed and the confluent monolayers were washed twice with 3 ml of room temperature (22°C) Hank's-HEPES (20 mM) buffer, pH 7.4, containing 0.2% (w/v) BSA. Cells were incubated with 1 ml per plate of the same buffer containing 1.66 x 10^{-10} M ^{125}I-insulin (SA = 80-90 μCi/μg) (New England Nuclear Corp., Boston, Mass.) alone or with 40 μg of unlabelled insulin (Insulin-Toronto, 100 U/cc, Connaught Laboratories Ltd., Ontario, Canada) in order to correct for nonspecific binding.

The binding assay was performed at 22°C with gentle shaking. Preliminary experiments indicated that binding achieved its maximum level after 90 to 120 minutes. Therefore, a 120-minute incubation

Fig. 1 Time course of ^{3}H-thymidine incorporation into TCA precipitable material.


16
14
12
10
8
6
4
2
0
15
30
45
60
75
90
105
120
TIME (min)
^{3}H-THYMIDINE INCORPORATION
(dpm × 10^{-5}/mg protein)

time was used in all experiments. The equilibrium reaction was terminated by washing the monolayers with cold Hanks-HEPES buffer and solubilized in 1.2 ml of 1N NaOH. Specific binding was determined by subtracting the nonspecific binding from the total binding.

Autoradiography

The method of (^{3}H-methyl) thymidine incorporation and preparation of an autogram was modified from that previously described by Cristofalo and Sharf (1973). Cells were plated on tissue culture chamber/slides (Lab Tek No. 4802, 2 chamber) at a density of 10,000 cells per cm^2. Twenty hours after seeding, (^{3}H-methyl) thymidine was added to the cultures at a final concentration of 0.1 μCi/ml (SA = 2 Ci/mmole). After a 30-hour incubation, the slides were rinsed 3 times with cold PBS (pH 7.4) and fixed in cold formalin (3.6% in PBS at pH 7.4) for 30 minutes. Slides were then stained with Harris' hematoxylin for 30 minutes at 22°C and the staining was enhanced with lithium carbonate (1%) for 1 minute.

The slides were air dried and coated with photographic emulsion (Kodak NTB-2) and stored dessicated in the dark for 7 days at 4°C. Slides were then developed for 5 minutes (Kodak D-19 developer, pH 10), fixed for 5 minutes (Kodak Fixer pH 6.7) and allowed to air dry. Autoradiographs were analyzed by counting a total of 200 nuclei per slide in random fields. A labelled nucleus was considered to be one which had 5 grains or more. Background labelling was determined on control slides and experimental slides were corrected accordingly.

Scintillation Counting

Samples were added to 5 ml of scintillation cocktail [0.4% w/v Omnifluor (New England Nuclear Corp.), 8% w/v naphthalene, 30% v/v 1,4 dioxane and 30% v/v of methyl cellosolve and toluene] (Kahlenberg, 1969). Vials were counted in a liquid scintillation spectrometer (Intertechnique Model SL-30). Counting efficiency was determined by automatic external standardization (approximately 25% for ^{3}H). The data was processed on a Wang 700A/701 programmable calculator.

Gamma Counting

Samples were counted in a gamma counter (Nuclear Chicago, Model 1085). The machine efficiency was 64% as determined by an ^{129}I standard. This information was used to convert counts per minute (cpm) to disintegrations per minute (dpm).

Statistics

Levels of significance were determined by the Student's t-test for paired and unpaired data, with 5% being the level chosen for significance.

RESULTS

The data shown in Fig. 2 illustrate that there was an inverse relationship between confluent cell density and *in vitro* age in the cell strain GM-730. When confluent, the growth of cultured cells becomes density dependent inhibited (eg. after Day 4). In *in vitro* old cells this occurs at a lower cell density than in *in vitro* young cells. The results in Figure 3 suggest that there is also an inverse relationship between confluent cell density and the chronological age of the donor.

Another way to examine the proliferative capacity was to determine the percentage of labelled nuclei in cells which were incorporating ^{3}H-thymidine into DNA. The data in Table II show that there was clearly more than an 80% decrease in the percent labelled nuclei (PLN) as a function of *in vitro* age (a drop from 60% to 11%). It was also evident from the data (Table II), that there was less of a difference in the PLN as a function of *in vivo* age (80% vs 62%). The cells obtained from the young and old *in vivo*-aged donors had less then 40% of their *in vitro* lifespans completed. One final observation on the data (Table II) is that the cell population doubling rate was prolonged in both the old *in vitro*-aged cells as well as in the cells from old *in vivo*-aged donors.

It is important to note that the reductions in proliferative capacity and PLN, which were reported by Schneider and Mitsui (1976) were seen in response to serum, which contains a mixture of growth factors. In my study, insulin (a pure "growth factor") was used to

Fig. 2 Growth curve of the *in vitro*-aged cell strain GM-730

●= *in vitro* young (25% LSC) Ó= *in vitro* old (80% LSC).

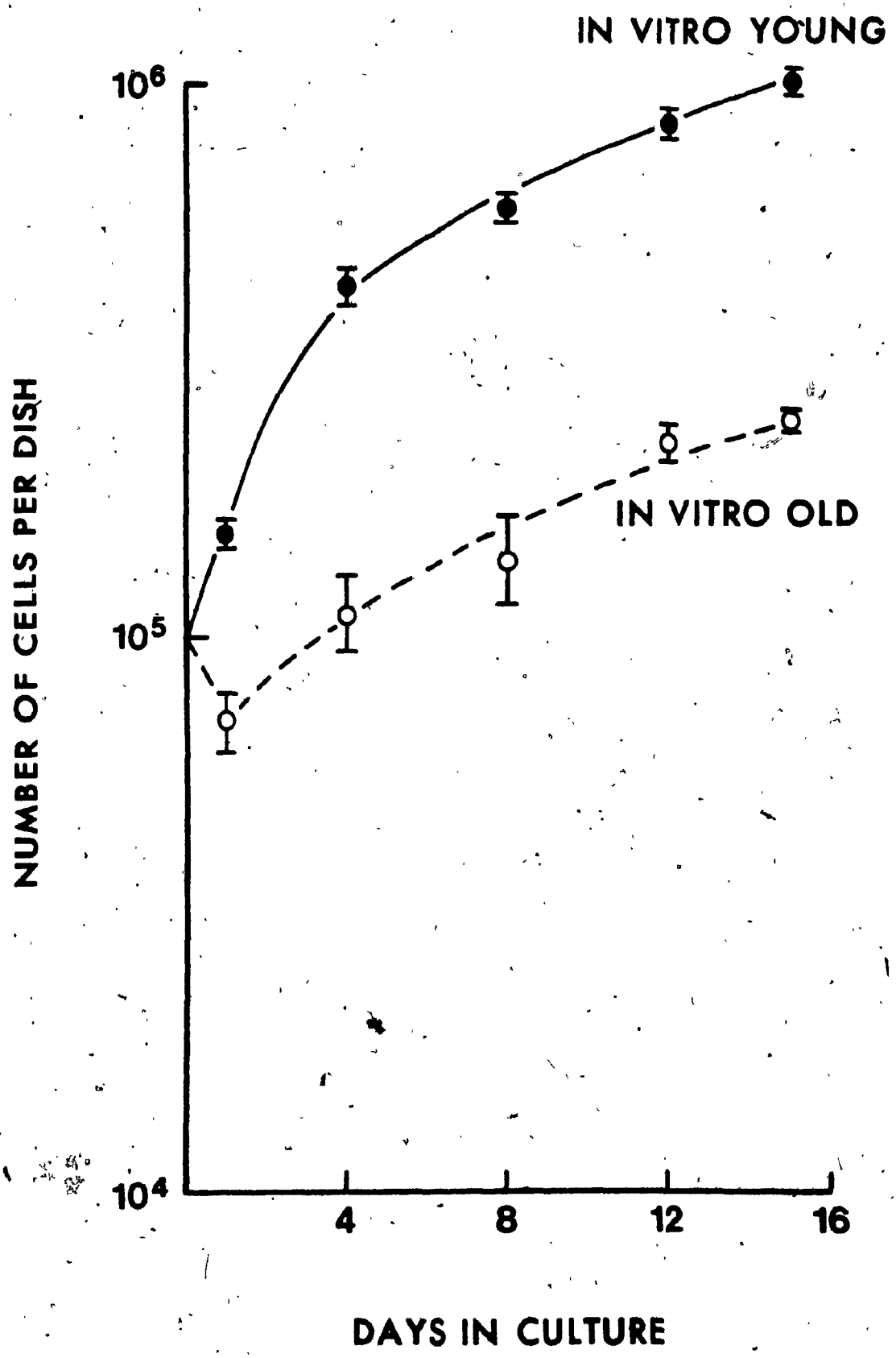

IN VITRO YOUNG
IN VITRO OLD
10^6
10^5
10^4
NUMBER OF CELLS PER DISH
4
8
12
16
DAYS IN CULTURE

Fig. 3 Growth curves of the *in vivo*-aged cell strains GM-2674 (● Age 29) and GM-3525 (○ Age 80).

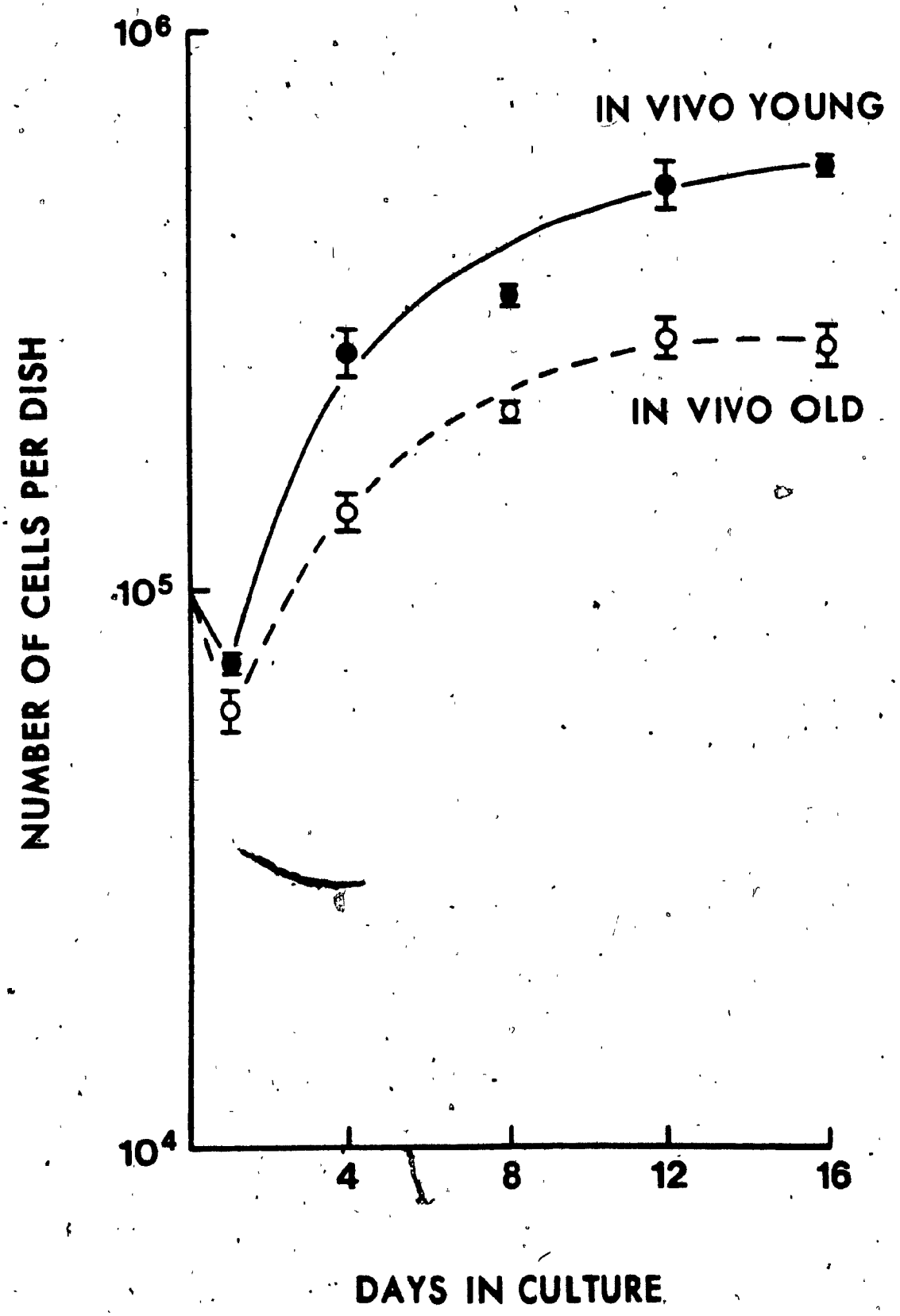

IN VIVO YOUNG
IN VIVO OLD
NUMBER OF CELLS PER DISH
10^6
10^5
10^4
4
8
12
16
DAYS IN CULTURE

Table II. Parameters of *In Vitro* Aging in Early and Late Passage GM-730 Cells as Compared to Cell Cultures Derived from Young and Old Human Donors.

Parameter measured	*In Vitro* Age % LSC[a]		*In Vivo* Age (years)	
	33	94	29	78[b]
Percent labelled nuclei	60	11	80	62
Doubling Time (h)	49	113	39	61
Confluent Density ($\times 10^5$)	10.0 ± 1.2	2.4 ± 1.0	5.7 ± 0.06	2.7 ± 0.19

Cell strains used: GM-730, GM-2674, GM-1681, GM-1706, GM-3525.

Values for the percent labelled nuclei represent the mean of 3 experiments with duplicate slides in each and an average of 200 nuclei counted randomly per slide.

Values for doubling time and confluent density represent the average of triplicate plates from one experiment.

[a] % LSC = percent lifespan completed.
[b] average of *in vivo* ages ranging from 70-82 years.

see if there were any age-related changes in insulin-stimulated DNA synthesis as cultured human cells age *in vitro* and *in vivo*. This was achieved by removing serum from the culture medium 48 hours prior to each experiment. This period of serum starvation allowed the cells to reach a quiescent state where they were able to perform basic metabolism but not grow or divide (Hill, 1977).

The data in Table III show the effect of increasing insulin concentrations on the stimulation of DNA synthesis in cells of different *in vitro* age. There was clearly a concentration dependent increase in insulin-stimulated DNA synthesis in both the young and the old *in vitro*-aged cells. A comparison of the young and old *in vitro*-aged cells indicated that there was no significant difference in insulin-stimulated DNA synthesis at concentrations which are above maximal (i.e. > 5 mU/ml). Interestingly, at a very high insulin concentration (100 mU/ml) there was a significant difference between the young and the old cells, the old cells actually responding better than the young cells. This concentration is 10,000 times greater than physiological levels of insulin normally present in the body. At this high concentration, insulin, in the old cells, could be stimulating DNA synthesis via some related growth factor receptor (eg. MSA/IGF receptor) (Rechler *et al.*, 1981).

With regard to the cells of different *in vivo* age (Table IV), it is apparent that significant concentration-dependent increases in insulin-stimulated DNA synthesis were observed in both young and old *in vivo*-aged cells. However, there were no significant age-related differences in the ability of insulin to stimulate DNA synthesis at

Table III. The Effect of *In Vitro* Age on Insulin-Stimulated DNA Synthesis.

Insulin Conc. (mU/ml)	I/C ratio[a] 37.8% LSC	81.6% LSC	p[b]
0	1.00	1.00	>0.05
1.0	1.14 ± 0.07	1.18 ± 0.05	>0.05
2.5	1.34 ± 0.08	1.44 ± 0.08	>0.05
5.0	1.35 ± 0.12	1.43 ± 0.09	>0.05
10.0	1.35 ± 0.08	1.63 ± 0.13	>0.05
100.0	1.24 ± 0.07	1.73 ± 0.05	<0.001

Cells strain used: GM-730

[a] The insulin/control ratios were determined by dividing the dpm/mg protein/h in each of the insulin exposed groups by the non-insulin exposed group. Data represent the averages of 9 different experiments, triplicate plates in each experiment ± SEM.

[b] Significance was determined by the Student's t-test for unpaired observations.

Table IV. The Effect of *In Vivo* Age on Insulin-Stimulated DNA Synthesis.

Insulin Conc. (mU/ml)	I/C ratio[a] 29 years	78 years[b]	P[c]
0	1.00	1.00	
1	1.31 ± 0.04	1.64 ± 0.20	>0.05
5	1.97 ± 0.13	1.51 ± 0.18	>0.05
20	1.85 ± 0.10	2.81 ± 0.69	>0.05

Cell strains used: GM-2674, GM-1681, GM-1706, GM-3525.

[a] Insulin/control ratio as previously explained in Table III. All data represent the averages of 3 different experiments, triplicate plates in each experiment ± SEM.

[b] Average of *in vivo* ages ranging from 70-82 years.

[c] Significance was determined by the Student's t-test for unpaired observations.

any of the insulin concentrations tested (up to 20 mU/ml). Since insulin alone was not able to induce any significant age-related differences in the stimulation of DNA synthesis, it was postulated that by amplifying the insulin response with the glucocorticoid, hydrocortisone (HP), it may be possible to pick up any age-related changes which may be occurring in these cells.

Hydrocortisone has been shown to have many effects on cells in culture. These include the ability to (1) extend the lifespan of some types of cells (Maciera-Coelho, 1966; Rosner and Cristofalo, 1979); (2) increase the confluent density of cells over a short time interval (Cristofalo, 1975; Grove et al., 1977; Rowe et al., 1977; Germinario et al., 1983); (3) enhance the response of cells to insulin-stimulated DNA synthesis (Baker et al., 1979; Germinario et al., 1983).

The present study showed that HP did not appear to extend the lifespan of the cell strain GM-730 (Table V), as the population doublings at senescence in the HP treated group (66 PD) were virtually the same as those of the control group (69 PD).

The results in Table VI clearly showed that HP significantly elevated the confluent cell density of cultured human fibroblasts over a seven-day period. The fact that HP was able to increase confluent cell density over a short time-interval, yet was not able to extend the overall lifespan of GM-730, may have something to do with some type of an age-related change in the glucocorticoid receptor (Roth, 1975).

In the present study, HP was found to enhance insulin-stimulated DNA synthesis in both young (Fig. 4) and old (Fig. 5) in vitro-aged

Table V. The Effect of Hydrocortisone on the Extension of In Vitro Lifespan in the Cell Strain GM-730.

Cell Strain	Population Doublings at Senescence Control	+ HP
GM-730	69	66

Cells were grown from Day 1 in either 10% MEM (Control) or in 10% MEM containing 5 x 10^{-7}M hydrocortisone (+HP). During the initial passages, cells were subcultured weekly when the monolayer reached confluence. If cultures failed to reach confluence after 1 week, they were retained (MEM being changed every 2 days) until such time as they did become confluent. If, after 3 weeks in culture, the cells were still unable to reach confluence the cell strain was considered to have reached senescence and the in vitro lifespan (expressed in population doublings) was recorded.

Table VI. The Effect of Hydrocortisone on Cell Growth in Cultured Human Fibroblasts.

Cell Strain	% LSC[a]	Cell No. ($\times 10^{-4}$)/cm^2 Control	+ HP		P[c]
TUF (n = 3)	33 ± 9	4.99 ± 0.81	7.12 ± 0.62	(46)[b]	< 0.025
GM-730 (n = 4)	48 ± 5	6.72 ± 0.94	10.94 ± 0.87	(71)[b]	< 0.025

Cells were plated at 1×10^4 cells/cm^2 in 60 mm plastic petri plates in 10% MEM with or without 10^{-7}M HP and refed every 2 days. After 7 days in culture, cell counts were performed in duplicate for all experiments. All data ± SEM.

[a] %LSC = %lifespan completed.
[b] Numbers in parentheses represent the average % increase of the individual experiments.
[c] Significance was determined by the Student's t-test for paired data.

Fig. 4 Effect of hydrocortisone (HP) on insulin-stimulated DNA synthesis in young *in vitro*-aged cells. Serum starved confluent monolayers of cells were exposed to 5 x 10^{-7}M HP (O) or no HP (●) for 48 hours. The cells were then exposed to varying concentrations of insulin for 18-24 hours. Data represent the averages of 4 different experiments, triplicate plates in each experiment ± SEM. Cell strain used: GM-730 (33% LSC).

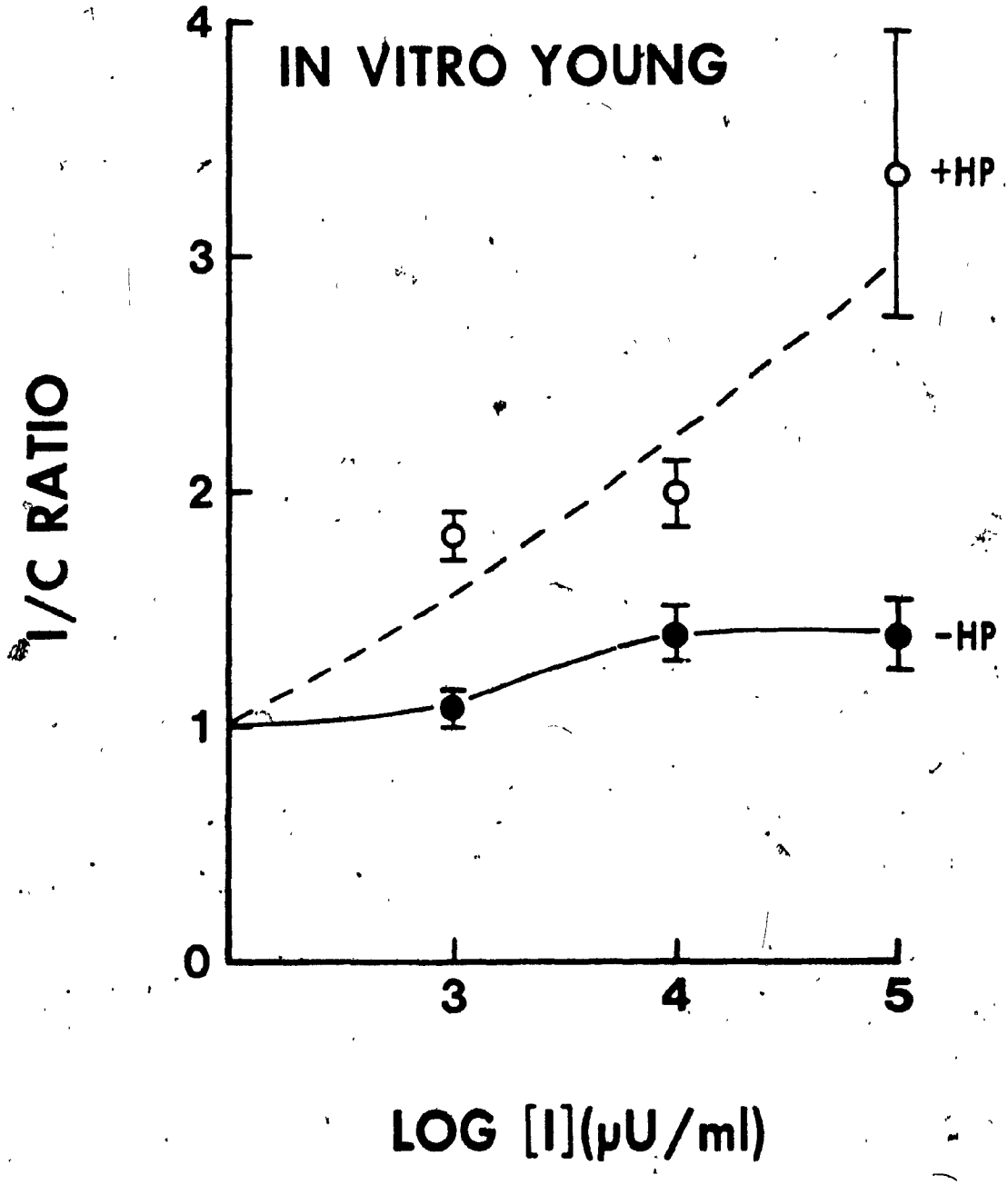
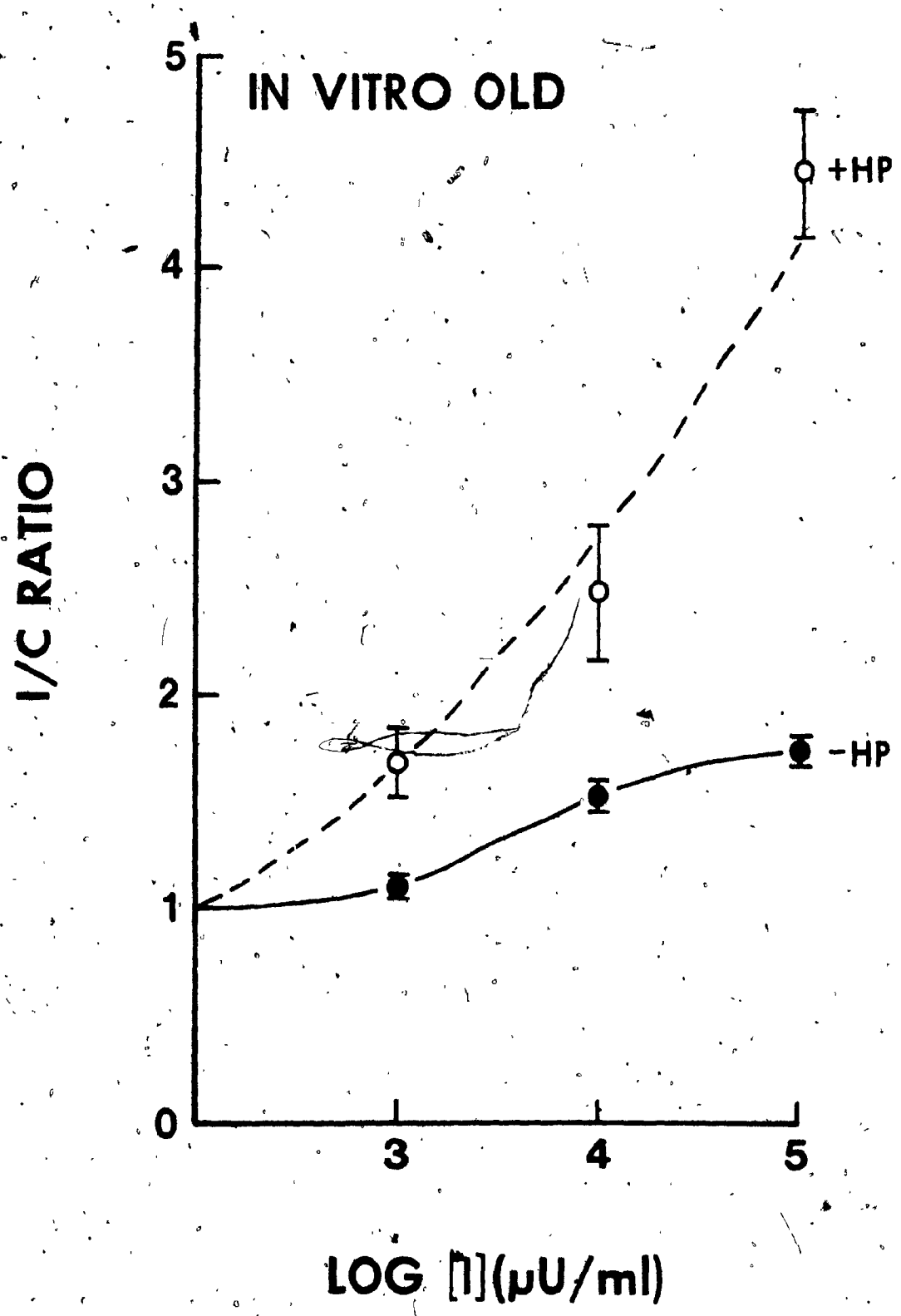

IN VITRO YOUNG
I/C RATIO
4
3
2
1
0
+HP
-HP
3
4
5
LOG [I](μU/ml)

Fig. 5 Effect of hydrocortisone (HP) on insulin-stimulated DNA synthesis in old *in vitro*-aged cells. Serum starved confluent monolayers of cells were exposed to 5×10^{-7}M HP (O) or no HP (●) for 48 hours. The cells were then exposed to varying concentrations of insulin for 18-24 hours. Data represent the averages of 4 different experiments, triplicate plates in each experiment ± SEM. Cell strain used: GM-730 (80% LSC).


IN VITRO OLD
+HP
-HP
I/C RATIO
LOG [I](μU/ml)
0
1
2
3
4
5
3
4
5

cells. The data clearly indicate that (1) there was a significant effect of HP on insulin-stimulated DNA synthesis at all insulin concentrations in both the young and old *in vitro*-aged cells; (2) there was no significant difference between *in vitro* age and the ability of HP to enhance insulin-stimulated DNA synthesis. A summary of the statistical analyses on this data can be found in Table VII (A and B).

When cells derived from young (Fig. 6) and old (Fig. 7) human donors were exposed to HP, it was evident that (1) there was a significant effect of HP on insulin-stimulated DNA synthesis only in the young *in vivo*-aged cells; (2) HP enhanced insulin-stimulated DNA synthesis differentially in young and old *in vivo*-aged cells; (3) the greater than four-fold increase in DNA synthesis in the young cells was due solely to HP. A summary of the statistical analyses performed on this data is summarized in Table VIII (A and B).

In an attempt to determine the site(s) of any age-associated changes in the biological action of insulin, the binding of the hormone was studied in both *in vitro*- and *in vivo*-aged cells with and without HP. The importance of this study was to determine not only if there was a correlation between insulin binding and insulin responsiveness, but also to see if specific insulin binding changed as a function of *in vitro* or *in vivo* age. The data in Tables IX and X demonstrate that specific insulin binding does not change with either *in vitro* or *in vivo* age. However, when cells were exposed to HP for 48 hours prior to the measurement of insulin binding (Table XI), the specific insulin bound was observed to be significantly elevated. The

Table VII. Statistical Analyses on Differences Between Insulin/Control Ratios from Figures 4 and 5.

A.

Insulin Conc. (mU/ml)	Significance of differences between young and old:[a] Control	+ HP
1	>0.05	>0.05
10	>0.05	>0.05
100	>0.05	>0.05

B.

Insulin Conc. (mu/ml)	Significance of differences between control and HP:[a] Young	Old
1	<0.001	<0.01
10	<0.01	<0.05
100	<0.05	<0.001

[a]Significance was determined by the Student's t-test for unpaired observations.

Fig. 6 Effect of hydrocortisone (HP) on insulin-stimulated DNA synthesis in cultured fibroblasts derived from a young human donor. Serum-starved confluent monolayers of cells were exposed to 5×10^{-7}M HP (O) or no HP (●) for 48 hours. The cells were then exposed to varying concentrations of insulin for 18-24 hours. All data represent the averages of 3 different experiments, triplicate plates in each experiment ± SEM. Cell strain used: GM-2674 (29 years old).

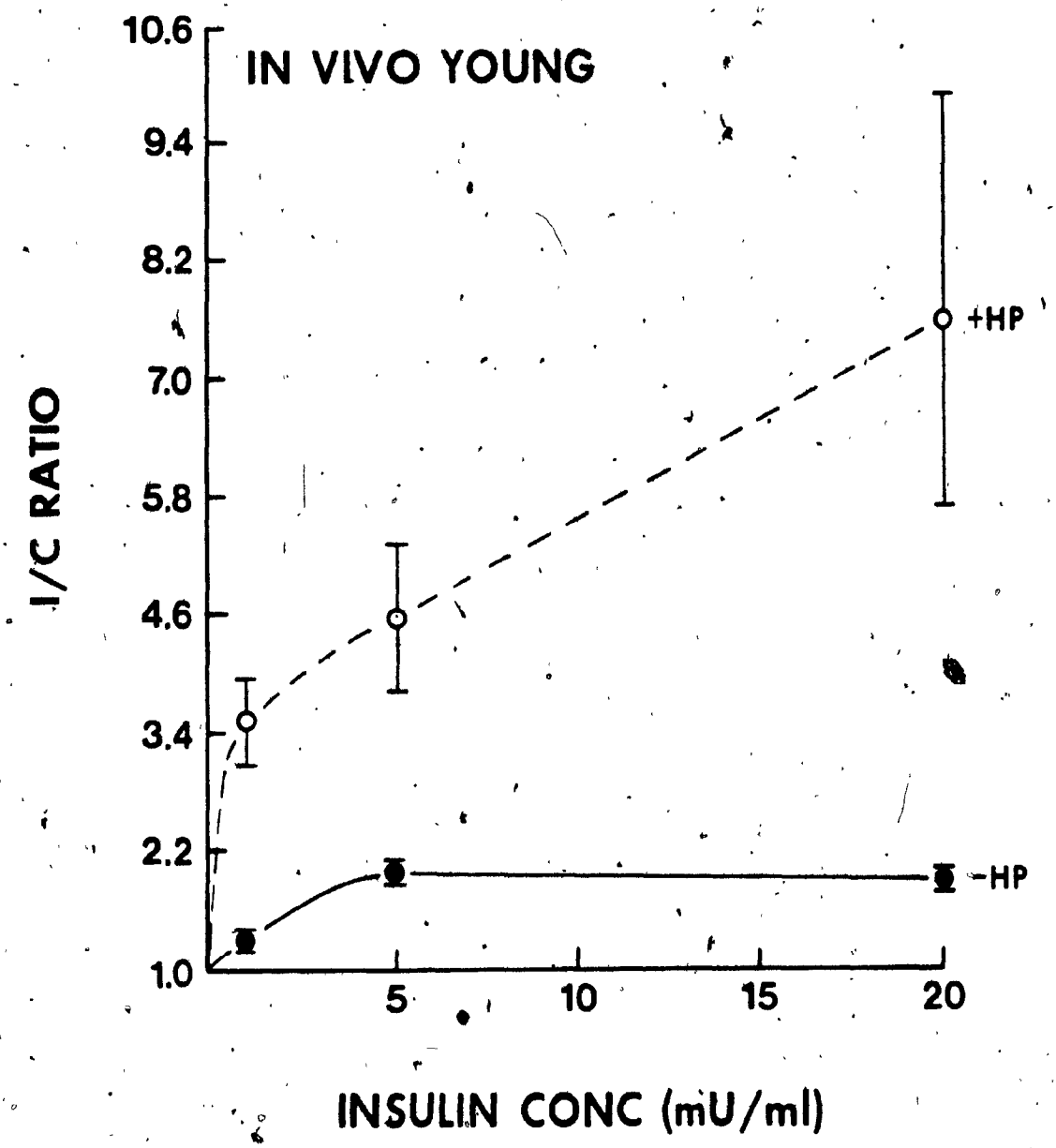

IN VIVO YOUNG
I/C RATIO
10.6
9.4
8.2
7.0
5.8
4.6
3.4
2.2
1.0
+HP
-HP
5
10
15
20
INSULIN CONC (mU/ml)

Fig. 7 Effect of hydrocortisone (HP) on insulin-stimulated DNA synthesis in cultured fibroblasts derived from old human donors. Serum-starved confluent monolayers of cells were exposed to 5×10^{-7}M HP (O) or no HP (●) for 48 hours. The cells were then exposed to varying concentrations of insulin for 18-24 hours. All data represent the averages of 3 different experiments, triplicate plates in each experiment ± SEM. Cell strains used: GM-1681 (70 years old); GM-1706 (82 years old); GM-3525 (80 years old).

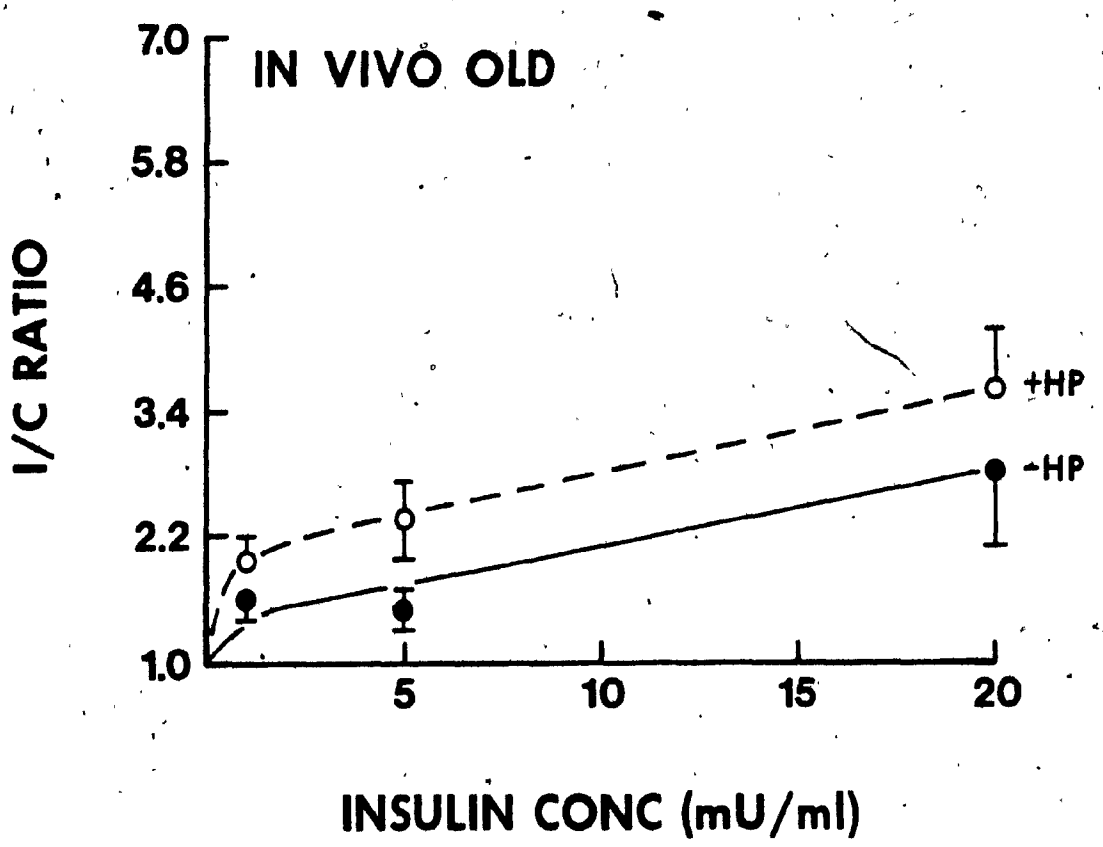

IN VIVO OLD
I/C RATIO
7.0
5.8
4.6
3.4
2.2
1.0
5
10
15
20
+HP
-HP
INSULIN CONC (mU/ml)

Table VIII. Statistical Analyses on Differences between Insulin/Control Ratios from Figures 6 and 7.

A.

Insulin Conc. (mU/ml)	Significance of differences between young and old:[a] Control	+ HP
1	> 0.05	< 0.01
5	> 0.05	< 0.02
20	> 0.05	< 0.05

B.

Insulin Conc. (mU/ml)	Significance of differences between control and HP:[a] Young	Old
1	< 0.001	> 0.05
5	< 0.01	> 0.05
20	< 0.01	> 0.05

[a]Significance was determined by the Student's t-test for unpaired observations.

Table IX. The Effect of *In Vitro* Age on Specific ^{125}I-Insulin Binding in Cultured Human Fibroblasts.

Specific Insulin Bound (fmoles/mg protein/2 h)		
Young 43.7% LSC	Old 88.8% LSC	P[a]
1.5 ± 0.2	1.7 ± 0.5	>0.05

Cell strains used: GM-730, PUF, JSD.

Confluent cell cultures were serum-starved for 48 h. Data represent the averages of 8 different experiments, triplicate plates in each experiment ± SEM.

[a] Significance was determined by the Student's t test for unpaired observations.

Table X. The Effect of *In Vivo* Age on Specific ^{125}I-Insulin Binding in Cultured Human Fibroblasts.

Specific Insulin Bound (fmoles/mg protein/2°h)		
Young 4 years[a]	Old 67 years[b]	P[c]
15.3 ± 5	14.0 ± 3	>0.05

Cell strains used: GM-498 (3 years); GM-500 (10 years); GM-288 (64 years); GM-3525 (80 years).

Confluent cell cultures were serum-starved for 48 h. All data represent the averages of 5 different experiments, triplicate plates in each experiment ± SEM.

[a] Average of *in vivo* ages ranging from 3-10 years.
[b] Average of *in vivo* ages ranging from 64-80 years.
[c] Significance was determined by the Student's t-test for unpaired observations.

Table XI. The Effect of Hydrocortisone on Specific ^{125}I-Insulin Binding in Cultured Human Fibroblasts.

Treatment (mU/ml)	Specific Insulin Bound (fmoles/mg protein/2 h)	p[a]
no HP	13.6 ± 3.7	
+ HP	19.5 ± 4.4	>0.05

Cell strain used: GM-730.

Confluent cell cultures were serum-starved in the presence and absence of HP for 48 h. The data represent the average ± SEM of 7 experiments, triplicate plates in all experiments.

[a] Significance was determined by the Student's t-test for paired observations.

enhanced insulin binding in the presence of HP can also be seen in the time-course experiment illustrated in Figure 8. When the effect of HP on insulin binding was measured in young and old *in vitro*- and *in vivo*-aged cells (Table XII), the data indicated that (1) HP elevated binding in both young and old *in vitro*- and *in vivo*-aged cells; (2) there was no significant age-related difference in the ability of HP to enhance insulin binding. The data in Table XIII show that the percent specific binding does not change as a function of *in vitro* and *in vivo* age, nor does it significantly change in the presence of HP.

Fig. 8 Time-course of specific ^{125}I-insulin binding in the presence and absence of hydrocortisone. Confluent monolayers of GM-730 (50% LSC) were serum-starved for 48 hours. Following this, they were either exposed to 5×10^{-7}M HP (O) or no HP (●) for 20 hours. The data represent the average of triplicate plates from one experiment.

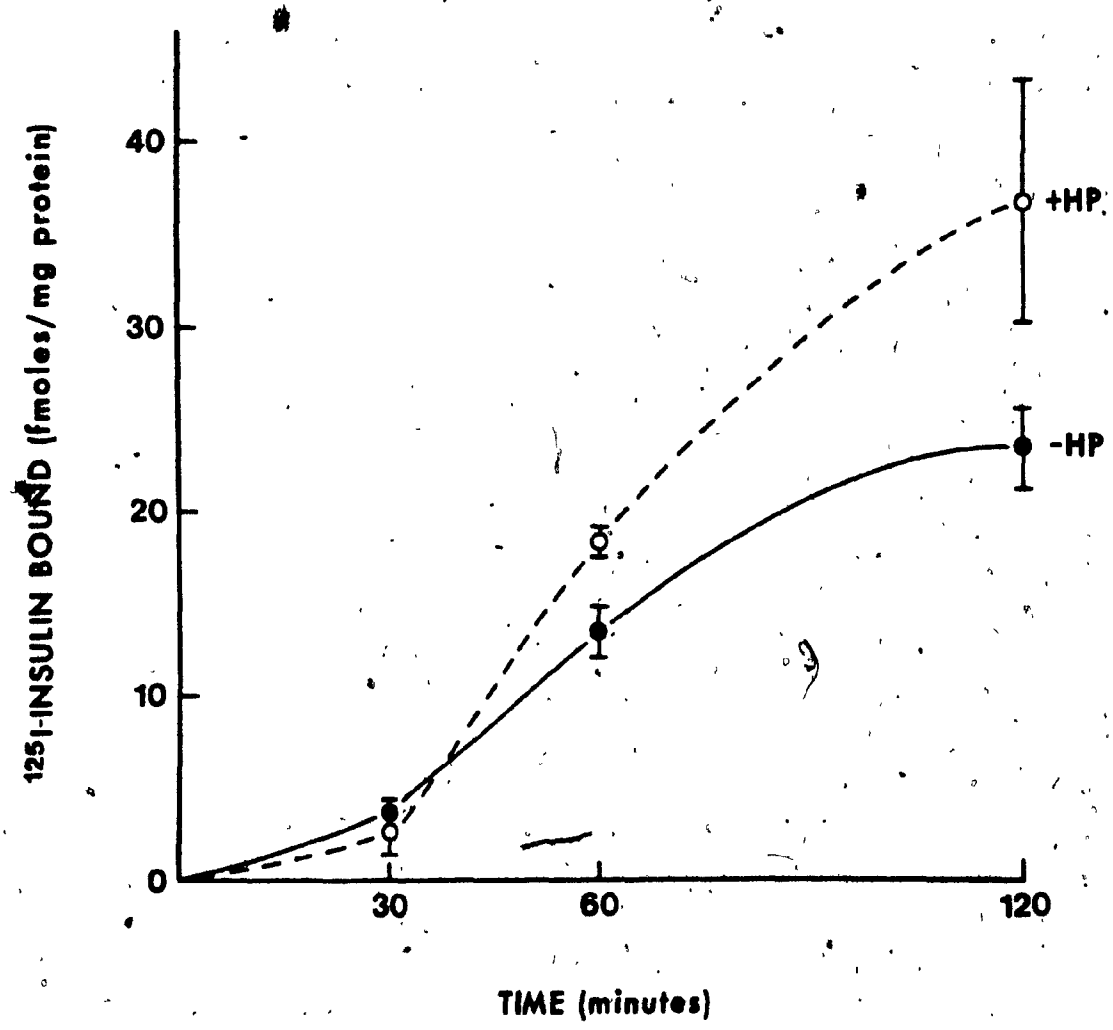

125I-INSULIN BOUND (fmoles/mg protein)
40
30
20
10
0
30
60
120
TIME (minutes)
+HP
-HP

Table XII. The Effect of Hydrocortisone on Specific ^{125}I-Insulin Binding in Cultured Human Fibroblasts as a Function of *In Vitro* and *In Vivo* Age.

Cells	HP/C ratio	
	In Vitro Age	*In Vivo* Age
Young	1.15 ± 0.08[a]	1.71 ± 0.34[b]
Old	1.24 ± 0.20[a]	1.19 ± 0.15[b]

Cell strains used: GM-730, PUF, JSD (*in vitro* age) and GM-498, GM-500, GM-288, GM-3525 (*in vivo* age).

Confluent cell cultures were serum-starved in the presence and absence of HP for 48 h. *In vitro* age data represents the averages pooled from 8 different experiments, triplicate plates in each experiment ± SEM. *In vivo* age data represents the averages of 3 different experiments, triplicate plates in each experiment ± SEM. Significance was determined by the Student's t-test for paired observations.

[a] Not significant Old vs Young *in vitro*.
[b] Not significant Old vs Young *in vivo*.

Table XIII. Percent Specific ^{125}I-Insulin Binding as a Function of *In Vitro* and *In Vivo* Age.

A. *In Vitro* Age

Treatment	% Specific Binding[a] Young	Old
No HP	44.0 ± 2.4[b]	43.2 ± 6.5
+ HP	45.1 ± 2.7	47.6 ± 8.6

B. *In Vivo* Age

Treatment	% Specific Binding[a] Young	Old
No HP	51.4 ± 5.9[b]	55.2 ± 5.8
+ HP	43.3 ± 5.0	48.4 ± 5.9

Cell strains used: GM-498, GM-500, GM-288, GM-3525.

[a] $\% \text{ Specific Binding} = \frac{\text{Total Binding} - \text{Nonspecific Binding}}{\text{Total Binding}} \times 100$

[b] ± SEM

DISCUSSION

In Vitro-Aged vs In Vivo-Aged Cultures

Cultured human cells exhibit a decreased ability to divide with advancing *in vitro* age (Fig. 2). This observation has been well documented in the literature and is one of the major factors which led to the use of human diploid fibroblasts as a model for cellular aging (Hayflick and Moorhead, 1961; Merz and Ross, 1969; Goldstein, 1971; Cristofalo, 1972). Cultured human cells derived from old human donors also showed a decreased ability to divide when compared to cells from young human aged donors (Fig. 3). This difference in proliferative potential with increasing *in vivo* donor age was also shown by Schneider and Mitsui (1976), and further supports the use of fibroblast cultures as a model for human cellular aging.

It was also of interest to see if the number of cells which were actively synthesizing DNA changed as a function of either *in vitro* or *in vivo* age. This information was determined by incubating cells from each group with (^{3}H-methyl) thymidine and performing an autoradiographic experiment to see what percentage of the cells were incorporating the labelled thymidine into their nuclei. The data (Table II) showed an 80% reduction in the PLN with *in vitro* age. This significant decrease in PLN is indicative of a marked reduction in the proliferative capacity of these cells (Cristofalo and Sharf, 1973; Yanishevsky *et al.*, 1974; Schneider and Mitsui, 1976). Furthermore, the PLN in both the young and old *in vivo*-aged cells (80% and 62% respectively) was very similar to that obtained for the young *in vitro*-

aged cells (60%). This was what might have been expected if one takes into consideration the fact that the in vivo age studies were performed with cells of early in vitro passage. These findings agree with the observations of Schneider and Mitsui (1976) who showed a 35% decrease in the PLN as a function of in vitro age (20-29 PD vs 40-49 PD) but only a 9% decrease in PLN in cultures from young (28.1 ± 1 year) and old (78.9 ± 1.7 years) human donors.

The old cells from in vivo age donors showed a small decrease in their PLN and in conjunction with this, there was an observed decrease in the cell density at confluence. This suggests that like in vitro-aged populations, cells derived from human donors of different ages express similar changes in nuclear labelling and confluent density.

The observed increase in doubling time with age and the marked decrease in confluent cell density (Table II) was in agreement with Schneider and Mitsui (1976). In the present study, although the overall picture was the same, the observed differences between young and old in vitro-aged cells were of a greater magnitude.

Hayflick (1977) states that the limited ability to divide, which is characteristic of aging human cells in culture, is probably only one of a number of changes which are occurring in these cells. In his words: "The in vitro endpoint measured by us as loss of capacity for division is simply a convenient and reproducable system but may have little to do with the actual cause of in vivo aging." (Hayflick, 1977).

The data in the present study supports Hayflick's hypothesis that a decreased ability to divide is characteristic of aging cells in

culture. It was observed that both *in vitro*- and *in vivo*-aged cells exhibited not only a decrease in their capacity for division, but also a reduction in their ability to synthesize DNA. Although an examination of the actual cause of *in vivo* aging was beyond the scope of this thesis, it has been shown by data presented here, that a reduction in division potential was characteristic of both old *in vitro*- and *in vivo*-aged fibroblasts.

Insulin-Stimulated DNA Synthesis

With regard to insulin-stimulated DNA synthesis, there was clearly a concentration dependent increase in DNA synthesis in both the young and old *in vitro*-aged cells (Table III) and the cells obtained from young and old *in vivo*-aged donors (Table IV). The data indicated that insulin had no differential effect on the stimulation of DNA synthesis as a function of either *in vitro* or *in vivo* age. This was seen at a concentration of insulin (1 mU/ml) which is only two-fold greater than physiological (0.01-0.5 mU/ml). A significant elevation in insulin-stimulated DNA synthesis was observed in the *in vitro*-aged cells at the highest insulin concentration studied (100 mu/ml). This is about 200 times above the upper limits of physiological levels of insulin.

Although no previous studies have compared insulin-stimulated DNA synthesis in *in vitro*- and *in vivo*-aged cells, it has been reported in the literature that high concentrations of insulin are normally required to enhance DNA synthesis in cultured cells. For example, Rechler *et al.*, (1974), using human skin fibroblasts of young *in vitro*

age, found that only high concentrations of insulin were effective in stimulating DNA synthesis. In most of his experiments, between 500-1000 mU/ml of insulin were required in order to give a two-fold elevation in DNA synthesis. In another study, using fetal lung fibroblasts, Griffiths (1970) used 1000 mU/ml of insulin to stimulate DNA synthesis. These concentrations of insulin are well above the normal physiological concentrations of the hormone present in the body. It is still unknown why such high concentrations of insulin are normally required to elicit biological responsiveness in cell culture. It may be that cells in culture exhibit a decreased sensitivity to the hormone, thereby necessitating its presence at a higher concentration than would be required in vivo (Ball and Sanwal, 1980). Others have suggested that a high level of insulin may be required in vitro due to its degradation in the medium (Hayashi, 1978). Additionally, it is possible that insulin at such high concentrations may be interacting with one of several insulin-like polypeptide hormone receptors whose hormones also stimulate DNA synthesis in quiescent cells (Maturo and Hollenburg, 1979; Rechler et al., 1981). This possibility will be discussed in more detail in the discussion of the insulin binding data.

Despite the fact that in the present study there appeared to be no age-related difference in the ability of insulin to stimulate DNA synthesis, there was a statistically significant elevation ($p < 0.01$) in the control groups of the old in vitro- and in vivo-aged cells [3.1 x 10^6 dpm/mg protein (n = 35) vs 4.9 x 10^6 dpm/mg protein (n = 27)]. This observed elevation could be due to differences in the intra-

cellular nucleotide pool sizes as a function of age. In other words, young cells may have larger pool sizes than old cells. Hence, when a constant amount of labelled thymidine was introduced to both groups, the label would have been diluted to a greater extent in the young cells than in the old (due to the large pool of unlabelled precursers present in the young). This kind of situation would have made it appear as if the old cells were synthesizing more DNA than the young cells, when in fact it would only have been due to the differences in their intracellular pools. When comparing the 10% serum-stimulated groups in the same set of experiments as mentioned above, there was no significant difference in the serum/control ratios between young (*in vitro* and *in vivo*) and the old cells (*in vitro* and *in vivo*) [1.6 $\pm$ 0.18 (n = 26) vs 1.8 $\pm$ 0.10 (N = 19)]. Taking into account the fact that there were no significant differences in either the insulin stimulation ratios or the serum stimulation ratios with respect to age, it was concluded that the intracellular nucleotide pool sizes were not changing with age. This is in agreement with Olashaw *et al.*, (1980, 1983) who showed that, in the presence of 10% serum, there was no appreciable difference in the nucleotide pool sizes of young and old *in vitro*-aged human lung fibroblasts (WI-38 cells).

Hydrocortisone Modulation of Cell Growth and Lifespan

The effect of HP on the extension of lifespan in the cell strain GM-730 was measured (Table V) and the results indicate that HP was unable to extend the lifespan of these adult skin fibroblasts. Other investigators have shown that although HP was consistently able to

increase the lifespan of normal fetal lung cells in culture, this observed enhancement of cell proliferation was not characteristic of all human cells (Macieira-Coelho, 1966; Grove et al., 1977; Rosner and Cristofalo, 1979). It has been shown that not all normal cell strains derived from a variety of human tissues exhibit an increased lifespan in response to HP (Rosner and Cristofalo, 1979). It should also be noted that of all the tissues studied, the highest degree of variability in the response to HP was observed in the human skin-derived cell cultures (Rosner and Cristofalo, 1979). They looked at cells ranging in age from a fetus to an 82-year-old adult and found that in some of the cultures, HP was able to extend the lifespan while in other cultures the cells were either inhibited or unresponsive. The reason for this variability is still unclear. It has been postulated that the cells derived from different human tissues may be exhibiting differential responses to HP based on their differential functions in vivo (Rosner and Cristofalo, 1979). For example, one possible explanation for the consistent extension of lifespan which has been observed in fetal human lung cells in response to HP, may somehow be related to its role in fetal lung tissue in vivo. Studies by Smith et al. (1973) have shown that the cell proliferation and differentiation which is involved in fetal human lung development in vivo is markedly enhanced by HP. On a short-term basis (i.e. 7 days), it was found that HP significantly elevated the fibroblast cell number at confluence (Table VI). Similar effects on cell proliferation and confluent cell density in cultured human cells have been reported by Cristofalo (1972); Smith et al. (1973); Maciera-Coelho (1974); Rosner

and Cristofalo (1979) and Germinario et al. (1983). The mechanism by which HP is affecting these changes is far from resolved. However, it appears that HP does not act alone but rather it seems to enhance the growth-promoting effect of serum or perhaps some component of serum (Cristofalo et al., 1979). In a series of experiments performed by Cristofalo et al. (1979) it was found that the increased confluent cell density in the presence of HP could possibly be due to HP-conditioning of the medium. They postulated that this factor could either be secreted by the cells or could be a modificaton by the cells of some pre-existing serum component in response to HP. The effects of HP on cellular proliferation and extension of lifespan are most likely related to an overall effect of serum growth factors on DNA synthesis. The data in the present study suggest that while all the factors involved in cell growth and extension of lifespan may be related, the observance of increased confluent cell density in the presence of HP does not necessarily mean extension of lifespan.

Hydrocortisone Modulation of Insulin-Stimulated DNA Synthesis

The present study is specifically concerned with insulin-stimulated DNA synthesis and its modulation by HP in both in vitro- and in vivo-aged cultures. Hydrocortisone significantly increased insulin-stimulated DNA synthesis in both the young (Fig. 4) and old (Fig. 5) in vitro-aged cells as well as the cells derived from young in vivo-aged donors (Fig. 6). Although HP yielded a slight elevation in insulin-stimulated DNA synthesis in the cells derived from old in vivo-aged donors (Fig. 7), the difference was not statistically

significant. It is interesting to note that there was a significant elevation of insulin-stimulated DNA synthesis in the HP-treated cells at the lowest insulin concentration studied (1 mU/ml). This concentration of insulin(1 mU/ml) is not abnormally high when compared to normal physiological levels of the hormone but is also much lower than what has been shown to be normally required to stimulate DNA synthesis by others (approx. 1000 mU/ml) (Griffiths, 1970; Rechler *et al.*, 1974).

Although the exact mechanism of action of HP in cell culture remains unclear, it has been proposed that HP somehow amplifies the primary stimulus for cell division. In a study performed by Cristofalo (1975), in which serum was the primary stimulus for cell division, HP was found to significantly increase the PLN in both young and old *in vitro*-aged cells of human origin. He attributed the observed increase in DNA synthesis to an overall increase in the number of cells in the proliferating pool. He also showed that although the differences between the HP-treated and control groups were larger in the old cells, the number of old cells responding to the stimulus for division was considerably lower. In other words, even though HP stimulated DNA synthesis to a greater extent in the old *in vitro*-aged cells, the fraction of old cells which went on to complete division was considerably lower than that observed in the young *in vitro*-aged cells. In the present study, hydrocortisone in the absence of insulin was found to have no effect on DNA synthesis in either *in vitro*- or *in vivo*-aged cultures (data not shown). This clearly illustrates the fact that in this system, insulin is the

primary stimulus for DNA synthesis. This is in agreement with Cristofalo (1975), who showed that in the absence of serum, HP was unable to enhance cell division. In Cristofalo's experiment, serum was the primary stimulus for cell division.

Although there was no observed difference with in vitro age in the ability of HP to enhance insulin-stimulated DNA synthesis, this was not the case in the in vivo-aged cells. There was a greater than four-fold increase in insulin-stimulated DNA synthesis in the presence of HP in the young in vivo-aged cells (Fig. 6) as compared to a slight elevation in the old in vivo-aged cells (Fig. 7). Although Cristofalo's conclusion that HP exerts its effect on DNA synthesis by increasing the number of cells in the proliferating pool may be true, the present study indicates that there were clearly other factors involved. It was found that HP significantly elevated insulin-stimulated DNA synthesis in the young but not in the old in vivo-aged cells, even though they were the same low in vitro age. Based on these findings, it is believed that the mechanism of action of HP with age in cultured human fibroblasts is somehow related to the chronological age of the donor.

Insulin Binding

Specific insulin binding was not observed to change as a function of either in vitro (Table IX) or in vivo age (Table X). It can be seen from the data that the in vivo-aged cells exhibited what appeared to be a higher level of specific binding than the in vitro-aged cells. This variability could have been observed for several reasons: (1) the experiments comparing in vitro and in vivo age were performed

at different times; (2) there may be intrinsic differences in the various cell strains used; (3) there were differences in insulin preparations from one series of experiments to the next.

No previous publications consider the effect of in vitro age on insulin binding in cultured human fibroblasts. The observation that insulin binding did not change with in vitro age (Table IX) was in agreement with that of Raizada et al., (1980) who showed that there was no change in insulin binding with increased passage number in mouse fibroblasts. With regard to the finding that insulin binding did not change as a function of in vivo donor age (Table X), a similar observation has been made by Hollenberg and Schneider (1979). They found that there was no significant change in either insulin receptor number or the affinity of the receptor for insulin in skin fibroblast cultures from young and old human donors. Others have reported that maximal specific binding was significantly decreased in cells from old donors as compared to young donors (Ito, 1979). A major criticism of this report was that the binding assay was performed for 4 hours at 37°C. Under these conditions of high temperature and extended incubation time, it has been shown that not only is binding no longer at steady state but nonspecific binding is elevated and insulin degradation extensive (Rechler and Podskalny, 1976). In yet another study, Rosenbloom et al., (1976) showed an increased affinity for insulin as a function of normal human aging. The cells used in their study were obtained from young children (5-10 years old) and from adults mostly under the age of 50. In contrast to this, in the report by Hollenberg and Schneider (1979) the young group were derived from

young adults ranging in age from 22-31 and the old cells from adults ranging in age from 65-80. It has been suggested by Hollenberg and Schneider (1979) that the observed difference in Rosenbloom's study could reflect developmental changes rather than adult aging.

The finding that HP-treated cells showed an increased ability to bind ^{125}I-insulin (Table XI) was in agreement with previous studies which demonstrated glucocorticoid enhanced binding and action of both insulin (Baker et al., 1978; Germinario et al., 1983) and epidermal growth factor in human fibroblasts (Baker et al., 1978). The glucocorticoid-enhanced binding of epidermal growth factor was also demonstrated in chicken embryo cells (Baker et al., 1979). However, no significant age-related difference in HP-enhanced specific insulin binding was observed (Table XII).

It has been postulated that glucocorticoids may exert their effects by somehow altering cell membrane receptors (Baker et al., 1978). It is interesting to note that in the present study there was a clear correlation between insulin binding and DNA synthesis in both the young and old in vitro- and in vivo-aged cells. In the presence of HP, there was also a correlation between insulin binding and DNA synthesis in young and old in vitro-aged cells and in young in vivo-aged cells, however, there was no such correlation in the old in vivo-aged cells. This fact leads one to suspect either (1) a post-receptor interaction or (2) a change in the glucocorticoid receptor (Roth, 1975) with advancing in vivo age.

It should be mentioned at this point, that there is evidence that suggests that the binding site(s) with which insulin interacts in

human fibroblasts may not represent true insulin receptors but rather receptor sites for other insulin-like polypeptide hormones (Maturo and Hollenberg, 1979). Human fibroblasts have been shown to possess multiplication stimulating activity/insulin like growth factor (MSA/IGF) receptors which can bind insulin as well as their native substrates and stimulate thymidine incorporation into DNA (Rechler et al., 1981). Rechler et al. (1981) demonstrated that the growth promoting effects of insulin in human fibroblasts were most probably mediated via the MSA/IGF receptors. This was determined by effectively blocking the insulin receptors with insulin receptor antibodies and seeing that this had no effect on the insulin-stimulated thymidine incorporation into DNA. The fact that insulin stimulates DNA synthesis at physiological concentrations (approx. 0.25 mU/ml) in these cells (Germinario et al., 1983) suggests that insulin is acting via the insulin receptor. Other studies have shown that similar low levels of insulin (i.e. 0.25 mU/ml), which stimulated DNA synthesis, were acting via the insulin receptor in both hepatoma cells (Koontz and Iwahashi, 1981) and carcinoma cell lines (Nagarjan and Anderson, 1982).

Finally, it can be noted that the percentage of the counts bound in the present study ranged from 0.2% to 2.3%. These numbers fall into the range which has been reported by other investigators for cultured human fibroblasts (Hollenberg and Cuatrecasas, 1975; Rosenbloom et al., 1976).

CONCLUSION

From this study, I can conclude that:

1) There is an inverse relationship between confluent cell density and *in vitro* and *in vivo* age.

2) There is a concentration-dependent increase in insulin-stimulated DNA synthesis in both *in vitro*-and *in vivo*-aged cells.

3) The ability of insulin to stimulate DNA synthesis is unrelated to either *in vitro* age or *in vivo* donor age.

4) Hydrocortisone increases cellular proliferation over short periods of time but does not necessarily extend lifespan in adult fibroblasts.

5) Hydrocortisone enhances insulin-stimulated DNA synthesis in both the young and old *in vitro*-aged cells.

6) There is no age-related difference in the ability of hydrocortisone to stimulate DNA synthesis with *in vitro* age.

7) Hydrocortisone elevates insulin-stimulated DNA synthesis in young but not in old *in vivo*-aged cells when both are the same *in vitro* age.

8) The expression of hydrocortisone's modulation of insulin action with age in cultured human fibroblasts is related to the chronological age of the donor.

9) Specific insulin binding does not change with respect to either *in vitro* or *in vivo* age.

10) Hydrocortisone elevates specific insulin binding in both the young and old *in vitro*-and *in vivo*-aged cells.

11) Hydrocortisone-enhanced insulin binding is similar in both *in vitro*-aged and *in vivo*-aged cells.

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