lows an initial increase repeatedly observed when the cycling organism was transferred to salts. The need for free radical defenses during spherulation is also suggested by changes in the nature of oxygen consumption by the differentiating plasmodium. The rate of aerobic respiration was higher in homogenates of plasmodia prepared at the time of transfer to the salts medium (0-h starvation) than after 24 h of starvation, when the rate of oxygen consumption approached zero (fig., starvation B).

When oxygen consumption by the homogenates was measured in a medium containing KCN in buffered glucose (pH 7.1) an increase in rate was observed after 6 h of starvation; logarithmically growing plasmodia exhibited only a slight capacity for cyanide-resistant respiration at the time of transfer to the differentiation medium. After 24 h in salts the cyanide-resistant respiration of the homogenates approximately doubled under the assay conditions employed (fig., starvation A). An increase in the generation of O_2^- would be expected to require an increase in the rate of cyanide-resistant oxygen consumption; more oxygen would be univalently reduced to O_2^- and less reduced to water under conditions favoring this form of oxygen utilization. The increase observed is therefore consistent with the changes observed in MnSOD, H_2O_2 and GSH. The relationship between respiration, cyanide-insensitive respiration and O_2^- production has been described by Hassan[11]. We have previously reported that coupled mitochondria from *P. polyephalum* exhibit qualitatively typical respiratory patterns when provided with succinate, malate, isocitrate and α-glycerophosphate as substrates[17]. We presume that the MnSOD measured in this study is also mitochondrial. This presumption will be tested in future experiments directed specifically toward an evaluation of the role of *Physarum* mitochondria in free radical production.

Our observations suggest that the process of spherulation in *P. polycephalum* involves a cell state that is characterized by oxidative stress. It has previously been speculated that this form of differentiation is triggered by anoxic conditions[18]. This suggestion has never been seriously tested. We have observed that when *Physarum* microplasmodia are cultured in salts in an atmosphere of nitrogen, the microplasmodia do not spherulate; instead, they form a plasmodial collar on the flask within 24 h. They ultimately die without exhibiting any signs of differentiation. We have also observed that the rate at which microplasmodia spherulate tends to parallel the increase in MnSOD activity. A white mutant strain of *Physarum* (LU887 × LU897) that does not spherulate in salts also does not exhibit an increased MnSOD activity in salts; a closely related yellow strain (LU897 × LU863) spherulates more slowly than does the M_3cVII strain of this study and it also exhibits a more gradual increase in MnSOD activity. When the closely related yellow and white strains are fused the resulting heterokaryon spherulates at a rate intermediate between the rates of its parent strains; the increase in MnSOD activity of the heterokaryon is also intermediate between the increases observed in the parent strains[3]. Based on these findings and the results of the present study we conclude that sharp increases in MnSOD activity are characteristic of starvation-induced spherulation and that these increases are not observed at other times during the diploid phase of the life cycle of *P. polycephalum*. We surmise that either oxy-free radicals or the antioxidant defenses against these free radicals play a causative role in spherulation.

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Synthetic activities of mass cultures and clones of human gingival fibroblasts[1]

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Summary. The percentage of synthesis dedicated to collagen is elevated in low-density cultures of human gingival fibroblasts, as is per-cell total protein synthetic activity and glycosaminoglycan accumulation. These observations can be explained, in part, by a decrease in membrane transport of precursor substance in high-density cultures. Synthetic activity by human fibroblasts can be reliably assayed in vitro using as few as 500 cells sparsely seeded. Such low-cell number assay is essential for study of single-cell clones, where replicative life span is limited.
Key words. Gingiva; collagen; fibroblast; single-cell clones.

Study of the synthetic activities of cultured fibroblast-like cells from various donor sites may aid in the elucidation of normal cell biology, as well as the pathogenesis of connective tissue disorders. It has been demonstrated, however, that varying the conditions of culture can lead to widely disparate experimental results[2-4]. For example, differences in in vitro cell density may greatly alter the data obtained in experiments designed to evaluate cellular biochemical functions. Furthermore, since normal diploid fibroblasts routinely exhibit finite replicative life spans and synthetic capacities in vitro, the question of culture 'age' becomes critical. This is particularly important in experiments utilizing clones derived from single cells. For example, it is generally accepted that fibroblast senescence ensues after circa 40 population doublings[5]; thus, by the time only 1000 cells have

been generated from a single fibroblast, fully 25% of the replicative life span has been traversed; for 10^6 cells, 50%. Experiments designed to evaluate the synthetic activities of such clones must therefore use low cell numbers to preclude evaluating culture age rather than clonal peculiarities.

The present investigations were designed to ascertain the mechanism responsible for cell density-related variation in the synthetic activities of cultured human cells, to demonstrate the feasibility of using very low cell numbers in assays for protein, collagen and glycosaminoglycan synthesis by mass cultures and single-cell clones, and to evaluate the effect of clonal age on the results of such assays.

Materials and methods. Mass cultures of diploid human fibroblasts were derived from biopsies of healthy gingiva[9] by emigration under glass in Leighton tubes, using Eagle's minimum essential medium (MEM, Gibco no. 410–1100) supplemented with sodium pyruvate (0.11 g/l), L-serine (10.5 mg/l), L-asparagine (15 mg/l), L-glutamine (1.16 g/l), streptomycin sulfate (100 mg/l), penicillin G (100,000 U/l), sodium bicarbonate (2.25 g/l) and 10% fetal bovine serum. Incubation was at 37 °C in an atmosphere of 5% CO_2–95% air, 100% humidity. Only young mass cultures (passage no. 3) were used. From a mass culture of 4×10^6 cells, actively-proliferating triplicate cultures of 100,000, 25,000, 10,000, 5000, 2500, 1000 and 500 cells each were seeded into 1-cm Linbro® test wells (Flow Laboratories, no. 76-033-05) and the synthesis assays (see below) performed. Identical assays were also performed on such cultures that had been rendered quiescent and synchronous in G_0 by means of serum-deprivation for 48 h[7,8].

Single-cell clones of gingival fibroblasts were derived from first passage primary cultures, using the dilute-plating technique of Martin[6] in microtest wells (Falcon no. 3072). Clones were evaluated for synthetic activities as soon as a rapidly-proliferating clone of 2000 or 50,000 cells was obtained, as determined by direct observation and Coulter Counter®. Single-cell clones from two young females were evaluated at 500 and 10,000 cells per Linbro® well.

Total protein synthetic activity was measured as nondialyzable radioactivity after a 24-h ^{3}H-proline pulse in serum-free medium, as described in detail previously[9]. Collagen production was estimated as collagenase-digestible radioactive protein[9–11]. Accumulation of sulfated glycosaminoglycans was ascertained via ^{35}S-incorporation, using the method of Bauer et al.[12], as modified by Kantor and Hassell[13]. Proline transport across the cell membrane was determined as described by Ko et al.[8], utilizing a brief (15 min) pulse with the radiolabeled amino acid (^{3}H-proline) followed by quantitation of intracellular radioactivity by means of scintillation counting.

Results. Figure 1 depicts total protein synthetic activity and production of collagenase-digestible protein by actively-proliferating fibroblast mass cultures seeded at varying cell numbers. The results were reproducible at all seeding densities, with a dramatic inverse relationship between cell number and per-cell synthesis. A similar but more linear pattern was observed for synthetic activity by quiescent cells (fig. 2). In these two studies of actively proliferating and quiescent cultures, two different cell strains were used. In each experiment, the strain used to study quiescent cells produced more protein and collagen than the strain that was used to study actively-dividing cells. This raised the question of whether quiescent cells, in general, produce more protein and collagen than active cells. To test this, the synthetic activity of an additional strain of normal human gingival fibroblasts was subsequently evaluated in both quiescent and actively-proliferating states. This experiment revealed that actively-dividing cells in fact produce more protein and collagen than those which had been rendered quiescent[34], a finding supported be previous work[10,35,36].

Table 1 presents collagenase-digestible protein data expressed as a percentage of the total synthetic activity of proliferating and quiescent fibroblasts at various seeding densities. In active cultures, this percentage ranged from 5.4 to 33.0, with a clear trend toward elevated collagen synthesis with lower cell numbers. In quiescent cells, a similar trend was observed but the range was not as dramatic (15.2–25.1%). The observed percentages of synthetic activity dedicated to collagen production are within the normally expected range under our experimental conditions[9,10].

In figure 3, sulfated glycosaminoglycan accumulation by normal diploid human gingival fibroblasts may be observed. The pattern of elevated synthesis in low-cell number cultures is again evident. The sensitivity of synthetic activity culture density is even more dramatic in the case of radiolabeled sulfate accumulation.

The results of the membrane transport experiments in mass cultures are presented in table 2. There was an approximately linear decrease in per-cell proline transport across cell membrane as culture density increased.

Table 3 presents protein synthesis by single-cell clones from two donors. The magnitude of synthesis in the 500-cell assays is similar to the 500-cell assays from mass cultures (cf. fig. 1). However, the 10,000-cell assays from clones exhibit significant reduction in protein and collagen synthesis compared to 10,000-cell assays from mass cultures, and compared to the 500-cell clone experiments.

Table 1. Collagen production by fibroblast mass cultures seeded at high and low cell numbers, expressed as a percentage of total synthetic activity

Number of cells per Linbro® well	Collagen as percent of total protein	
	Active cultures	Quiescent cultures
500	33.0	25.1
1000	28.6	26.6
2500	25.2	18.6
5000	14.2	17.4
10000	29.8	17.1
25000	8.7	18.2
100000	5.4	15.2

Table 2. Membrane transport in human fibroblast cultures of varying cell density

Seeding density**	Intracellular radiolabel*	
	^{3}H-proline	^{35}S-(H_2SO_4)
0.5	***	310160 (139858)
1	175752 (38899)	164102 (46526)
2.5	***	66701 (6914)
5	71261 (10474)	60128 (1415)
10	55531 (8130)	61540 (5727)
25	36307 (11536)	56272 (9189)
50	25041 (1922)	52153 (2580)
100	18644 (1503)	55411 (4934)

* Reported as $\bar{x}$ CPM/10^6 cells ± SD for triplicate; ** cell number $\times 10^{-3}$; *** assay not performed.

Table 3. Protein synthetic activity by single-cell clones of human gingival fibroblasts

Clone No.	Cell number seeded	Synthetic activity*
RAF-7	10000	6.2 (0.5)
RAF-8	10000	9.1 (1.1)
RAF-9	10000	12.2 (0.9)
RAF-10	10000	16.4 (1.7)
KGJ-2	500	2231.3 (230)
KGJ-3	500	1733.9 (151)
KGJ-5	500	1157.2 (97)
KGJ-10	500	2036.1 (218)
KGJ-12	500	851.6 (90)
KGJ-14	500	1409.5 (123)
KGJ-16	500	18.3 **
KGJ-17	500	951.7 (103)

* Reported as nondialyzable cpm $\times 10^{-3}/10^6$ cells (± SD) for triplicate cultures; ** cells were nonproliferating and bizarre in appearance.

Discussion. In the present study, protein, collagen and glycosaminoglycan synthesis by normal human diploid gingival fibroblasts were measured after seeding cells over a range of cell numbers. Differences among production of protein, collagen and glycosaminoglycans were most striking with low-cell numbers; for example, nearly 100% more protein was produced by cells seeded at 500 cells/well as compared to cells seeded at 1000 cells/well. However, between 5000 cells/well and 10,000 cells/well the difference was about 25%.

In all of the experiments with mass cultures, an inverse relationship between cell number (density) and magnitude of synthesis was observed, corroborating previous studies[14,15]. The proline transport data offer an explanation for this observation, in that membrane transport was depressed in a linear fashion as cultures became more confluent. Since proline is an essential amino acid, an in vitro situation precluding its transfer from the extracellular milieu into the cytoplast would result in de facto inhibition of normal protein synthetic activity. Also, Ahn et al. demonstrated that intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) increases sharply as soon as cell-cell contact occurs in human diploid fibroblasts but that there is no additional cAMP increase as culture density continues to increase toward confluency[23]. A cAMP increase would elicit a decrease in protein synthetic activity[8], but the decrease would not be linear with increasing cell density, as our data reflect. This and other cell cycle phenomena[27], as well as changes associated with intercellular communication as a culture reaches confluence may also be reflected in the constituents of the cell membrane[28–30].

The present study revealed a selective increase of collagenous versus non-collagenous proteins in low-cell number cultures, in contrast to the report by Muller et al.[16]. Our findings do, however, agree with Abe et al.[17] and Steinmann et al.[18], who reported a two-fold increase in the production of type I over type III collagen in the low-versus high-cell number cultures of human (skin) fibroblasts. Furthermore, it is well established that collagen synthesis is more rapid in proliferating cultures than in stationary (quiescent) cultures[25], and that cell proliferation slows, then ceases, as cell density increases[26]. In the present study, it was possible to estimate cell proliferation rate from Coulter counts of cell numbers at the time of seeding and again at the time of harvest. These counts (data not shown), as expected, revealed rapid proliferation by cells seeded at low density, and slow or no proliferation by high cell-number cultures. This would account, at least in part, for the observed synthesis

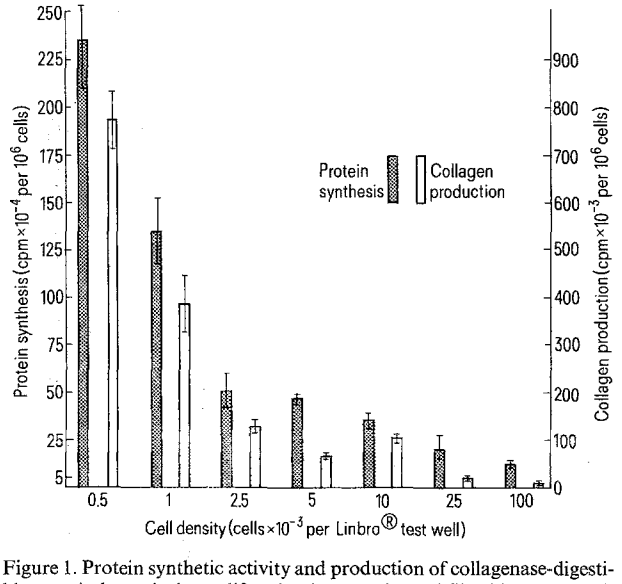

Figure 1. Protein synthetic activity and production of collagenase-digestible protein by actively-proliferating human gingival fibroblast mass cultures.

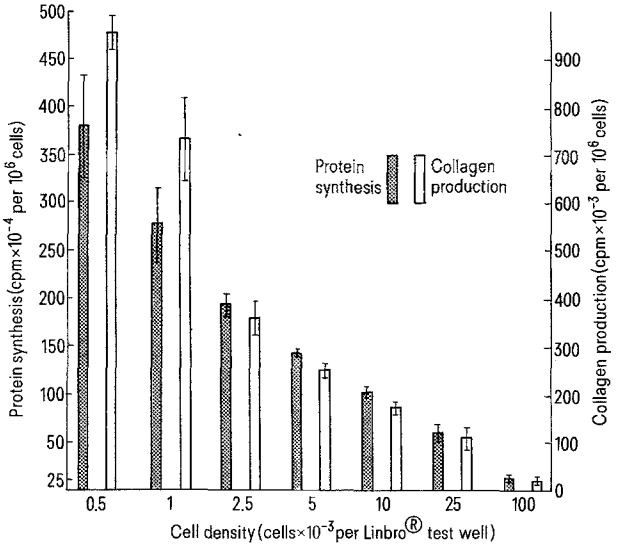

Figure 2. Protein synthetic activity and production of collagenase-digestible protein by serum-deprived quiescent human gingival fibroblast mass cultures.

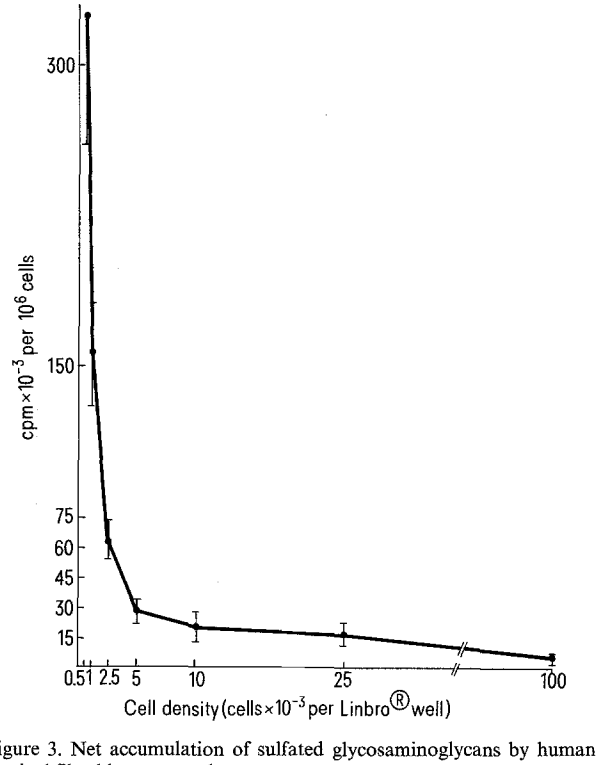

Figure 3. Net accumulation of sulfated glycosaminoglycans by human gingival fibroblast mass cultures.

data. The concentration of serum factors in the culture medium can also alter the collagen type I/type III ratio[2].

The recent work by Buddecke and his coworkers must be mentioned in this context as well. For example, using a ^{35}S label, Prinz et al. showed that endocytosis of proteoglycans is significantly lower in sparse cultures than after confluency[31], suggesting that endocytosis is decreased during the log phase of growth. Also, Sudhakaran et al. have suggested that glycosaminoglycans regulate extracellular transport by a mechanism involving molecular sieving, steric exclusion and ionic interaction with lipoproteins[32]. Human gingival fibroblasts synthesize and secrete significant quantities of dermatan sulfate (H. Solomon and T.

Hassell, unpublished observation, 1985), and dermatan sulfate binds to lipoproteins at physiological ionic strength[33].

Fibroblast-like cells generally have a limited life span of about 40 doublings[5]. The presence of a limit suggests that changes occur as a culture ages. Such aging becomes particularly important when experiments are performed using clones derived from single cells. As cell numbers in a developing clone increase, the number of potential doublings remaining in the lifespan decreases. At very high numbers, the cells are approaching senescence and are probably not functioning at normal levels of activity. If results from studies of clones are to be used to understand normal cell biology or the pathogenesis of connective tissue disorders, it would be advantageous to use cells early in their lifespan, and this necessitates using lower cell numbers per assay. Russell et al. performed a study of collagen synthesis by clones of human dermal fibroblasts and noted considerable variation among control groups[19]; we would suggest that this was due to differences in the population density or the age of these clones. Russell et al. advised that the limited proliferative potential of normal cells may make it impossible to design cloning experiments in which culture age is not a confounding factor. However, the data reported in the present study, demonstrating the feasibility of examining cellular synthetic activities using very small numbers of sparsely seeded cells of young age, support the use of single-cell clones to study normal cell functions as well as disorder. Recent work in our laboratory and elsewhere supports this finding[20-22,24].

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Detection of pyrimidine 5′-nucleotidase deficiency using ^{1}H- or ^{31}P-nuclear magnetic resonance

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Summary. We describe here a further Japanese family with pyrimidine 5′-nucleotidase (P5′N) deficiency diagnosed using a nuclear magnetic resonance (NMR) spectrum, in Kumamoto prefecture where two families having the disease have been reported before. The specific spectra in ^{1}H-NMR of P5′N deficient erythrocytes were due to three methyl protons of CDP-choline at 3.22 ppm and to H-2, H-8 and ribose-1′ of pyrimidine nucleotide phosphate(s) in the lower fields (at 5.82 and 8.00 ppm). The other specificities in ^{31}P-NMR spectra were due to CDP-choline, CDP-ethanolamine and UDP-glucose. Those spectra were not detected in other types of hemolytic anemia.

Key words. Pyrimidine 5′-nucleotidase (P5′N); nuclear magnetic resonance.

Nuclear magnetic resonance (NMR) studies of whole cell biology, especially the non-invasive study of whole cell biochemistry, has been pioneered by investigations of energy metabolism with ^{31}P-NMR spectrometry[1,2]. Furthermore, ^{13}C-NMR instrument has demonstrated its potential power for the investigation of ^{13}C-enriched metabolites in cell suspensions[2,3]. It is well known that ^{1}H-NMR can detect a wide range of metabolic compounds simultaneously and at higher sensitivity than ^{13}C- or ^{31}P-NMR. Thus, ^{1}H-NMR has been used in recent years to reveal many intracellular events[4-7].

Pyrimidine 5′-nucleotidase deficiency associated with hereditary hemolytic anemia was first described by Valentine et al.[8], and about 35 cases[9] of the deficiency have been reported from other laboratories[10-19]. The enzyme deficiency was first detected by a somewhat laborious assay in which the Fiske-Subarow method was used to measure the quantity of inorganic phosphate released from CMP or UMP during a 2-h incubation. Torrance et al. developed a new method for the assay of this enzyme using ^{14}C-CMP as substrate[20]. However, the method can be useful only for diagnosis and not for metabolic analysis of P5N deficient