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THE EFFECT OF MEDIUM CHANGES ON THE GROWTH AND METABOLISM OF THE HUMAN DIPLOID CELL, *WI-38*

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SUMMARY

It has been established that although an inhibitory interaction occurs when a culture of human diploid cells become crowded together (contact inhibition of growth), multiple-layered cell sheets are obtained by using a continuous medium perfusion culture. A similar effect is obtained when the culture medium is changed at frequent intervals, and this paper reports the effects of a medium change on cell growth and metabolism.

A direct relationship was found between cell yield and the number of medium changes given to a culture. This was an unexpected result because normally when a culture is prolonged by additional feeding the cell yield shows a diminishing return. The amino acid and glucose uptakes and growth yields (the ratio of the amount of cell dry weight produced to substrate used) were determined and they also showed that a unit amount of growth occurred per medium change, and that cessation of growth was accompanied by cessation of nutrient uptake and metabolism. Medium changes had a profound affect on cellular metabolism, especially on DNA and protein synthesis. As a culture approached confluency, DNA, RNA and protein synthesis were sequentially inhibited. After a medium change there was a sequential stimulation of DNA, RNA and protein synthesis in the same order as they were inhibited. The inhibitory mechanism that is affected by cell crowding is obviously reversed by a medium change.

The results presented in this paper suggest that contact inhibition of growth primarily affects DNA synthesis and that if the cell is able to take up a sufficient supply of nutrients in a crowded culture then this inhibition can be overcome.

INTRODUCTION

Human diploid cells exhibit contact inhibition of growth and stop growing at relatively low population densities. This phenomenon is believed to be an intrinsic property of the cells and regulated by a cellular control mechanism. The possibility that nutrient exhaustion affects the yields of diploid cells has not been seriously considered but observations by Kruse & Miedema (1965), Kruse, Whittle & Miedema (1969) and Baugh & Tytell (1967) have shown that medium composition and culture conditions do have some effect on yields of these cells. In a previous report a quantitative study of amino acid and glucose utilization by *WI-38* cells showed that although nutritionally enriched media supported more cell growth than normal media, no nutrient was depleted enough to be considered growth-limiting (Griffiths, 1970*a*). When the medium was changed at daily intervals over a period of 1 week the cell yield was increased several-fold. Medium changes had a similar, although not so dramatic an effect as continuous medium perfusion (Kruse & Miedema, 1965) and demonstrated that diploid cells are capable of growing in multilayered sheets. The term contact

inhibition of growth as a characteristic of diploid cells, therefore, needs further qualification.

This report describes further studies on the effect of medium changes on cell growth and metabolism with the aim of determining their precise effect on the cell and consequently on the mechanism of contact inhibition of growth.

MATERIAL AND METHODS

Cell culture

The human diploid line, *WI-38*, derived by Hayflick from foetal lung tissue was used. This line was cultured by the recommended procedure of Hayflick & Moorhead (1961) and only cells between passages 21 and 29 were used. The cells were at least 98% diploid as revealed by chromosome analysis. Cells were grown in Eagles' Minimal Essential Medium (MEM) (Gibco) supplemented with 10% foetal calf serum (Flow Laboratories), 300 μg/ml glutamine and 500 μg/ml glucose. Experimental cultures were grown in 5-cm plastic dishes (with 5 ml medium) or 4-oz (approx. 100 ml) medical flats (with 10 ml medium) inoculated at $5–10 \times 10^5$ cells/culture. Growth was measured by nuclei counts and the cultures were considered to be confluent when the cell density reached 1×10^5 cells/cm^2.

Analyses

The measurement of cell protein, cell dry weight, glucose, lactic acid, amino acids and ammonia has been described elsewhere (Griffiths, 1970*a*, *b*).

Isotope labelling experiments

Radiochemicals were obtained from the Radiochemical Centre, Amersham. The following were used: L-Leucine-C^{14} (U) (10 mCi/mM), 1 μCi/culture; Thymidine-6-T(n) (2 Ci/mM), 10 μCi/culture; Uridine-5-T (5 Ci/mM), 10 μCi/culture. Experimental details were as described previously (Griffiths, 1970*b*).

Growth yield (Y)

This expresses the relationship between cell growth and the utilization of the amino acids and glucose and is defined as

$$Y = \triangle x / \triangle s,$$

where $\triangle x$ is the cell dry weight produced (μg) and $\triangle s$ is the quantity of amino acid or glucose utilized (μg).

RESULTS

The effect of medium changes on cell yield

The response of cell growth to medium changes is shown in Table 1. A yield of nearly 6×10^4/cm^2 was obtained after 96 h and prolonging the incubation time did not significantly increase the cell yield. However, when the medium was changed for fresh growth medium at 72 or 96 h then further growth occurred. After 4 medium changes the cell yield was equivalent to a 2–3 cell thick monolayer which is more a characteristic of heteroploid than diploid cell cultures. Normally when a culture is prolonged by additional feeding a diminishing return in cell yield is obtained but in this system the cell yield increased exponentially with the number of medium changes (Fig. 1). Curve

A was plotted from the data in Table 1 and curve *B* from the experiments described later (see Fig. 2, p. 46). The slopes of the curves for both sets of results are similar but the values for *B* are higher, presumably because this experiment was carried out in plates which contained 17% more medium per unit area of the culture and a far larger volume of air to equilibrate with the medium.

Table 1. *The effect of medium changes on the yield of WI-38 cells*

(Cultures were grown in 4-oz medical flats containing 10 ml MEM(E) for 168 h, the inoculum was $2 \times 10^4/cm^2$.)

Number of medium changes	Time of medium changes, h	Cell yield $\times 10^4/cm^2$	Increase on yield from control, %
0	—	6·0	Control
1	96	7·8	30
2	72,120	10·3	72
4	48,72,96,120	21·0	250

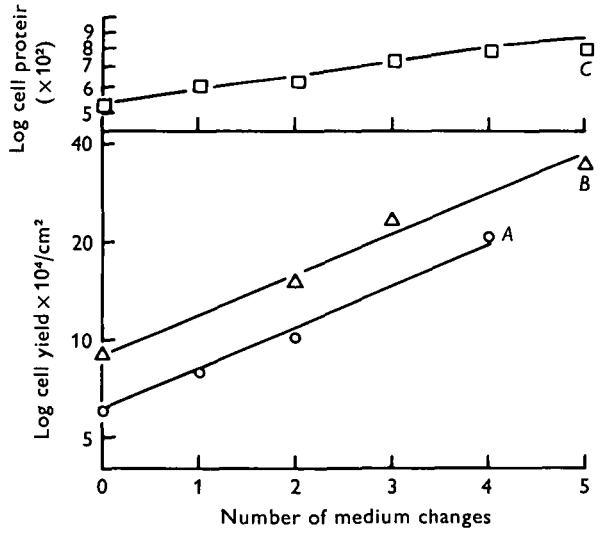

Fig. 1. The relationship between cell yield and the number of medium changes. *A* and *B*, cell numbers in a 4-oz medical flat and 5-cm plastic dish, respectively. *C*, cell protein.

Cell protein was measured as an alternative parameter of growth to cell counting as previous work had shown that insulin stimulated post-confluent protein synthesis but not division, resulting in larger cells (Griffiths, 1970*b*). There was also a direct relationship between protein formation and medium changes (Fig. 1, curve *C*) but apparently only for the first 4 medium changes.

The effect of medium changes on cell metabolism

The effects of various numbers of medium changes on the rates of protein, DNA and RNA synthesis during a culture cycle were found. The measurements, shown in

Fig. 2, demonstrate the significant effect medium changes have on cell metabolism. Protein synthesis reached its peak when the culture became confluent but this value was attained only if a medium change was given. Subsequent medium changes proportionally prolonged the period during which protein synthesis continued at its maximum rate. DNA synthesis followed a similar pattern to protein synthesis except that without a medium change the rate of synthesis continued to fall from 72 h onwards. The maximum rate of synthesis was attained before confluency and medium changes prolonged the period of maximum synthesis, but there was no significant

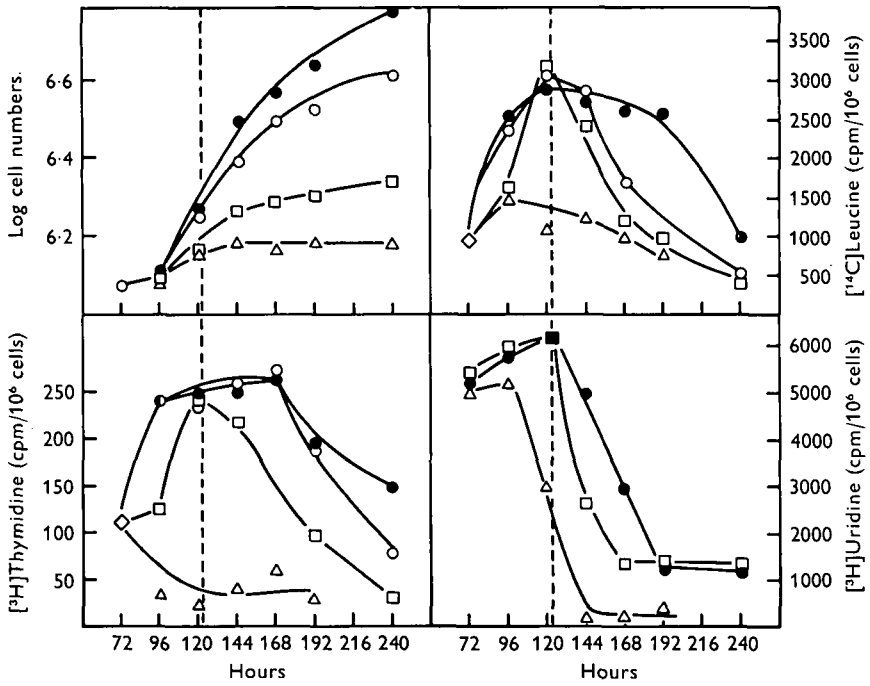

Fig. 2. The effect of medium changes on cell growth and the rates of protein, DNA and RNA synthesis. △—△, no medium change; □—□, 1 medium change (96 h); ○—○, 3 medium changes (72, 96, 120 h); ●—●, 5 medium changes (72, 96, 120, 144, 168 h). The vertical dotted lines represent the stage at which the cultures became confluent.

difference between 3 and 5 medium changes. The rate of RNA synthesis fell very sharply at confluency, the rate of fall being greater with 1 medium change than 5. The rates of macromolecular synthesis correlate with the growth curve (Fig. 2) as the fall off in the growth rate was concurrent with changes in the metabolic rates.

The data in Fig. 2 indicate that without a medium change sequential changes in the rates of macromolecular synthesis occurred when the culture approached confluency. DNA synthesis was affected first, followed by RNA synthesis which declined very rapidly, and protein synthesis which declined steadily but slowly. Medium changes had a more marked effect on DNA and protein than on RNA synthesis, but as the data in Fig. 2 give only a general indication of metabolism (because the determinations were

at 24-h intervals) it is difficult to decide whether a medium change also has a sequential effect on cell metabolism. Accordingly, the rates of protein, DNA and RNA synthesis were measured at much shorter intervals following a medium change to determine the time sequence of events (Fig. 3). Again DNA synthesis was the first to be affected reaching a new peak of activity after 12 h, followed by RNA after 24 h and protein synthesis between 30 and 48 h. The medium change caused an initial decrease in the rates of DNA and RNA synthesis whereas protein synthesis was unaffected. This was probably due to the leakage from cells of precursors for nucleic acid synthesis into the fresh medium.

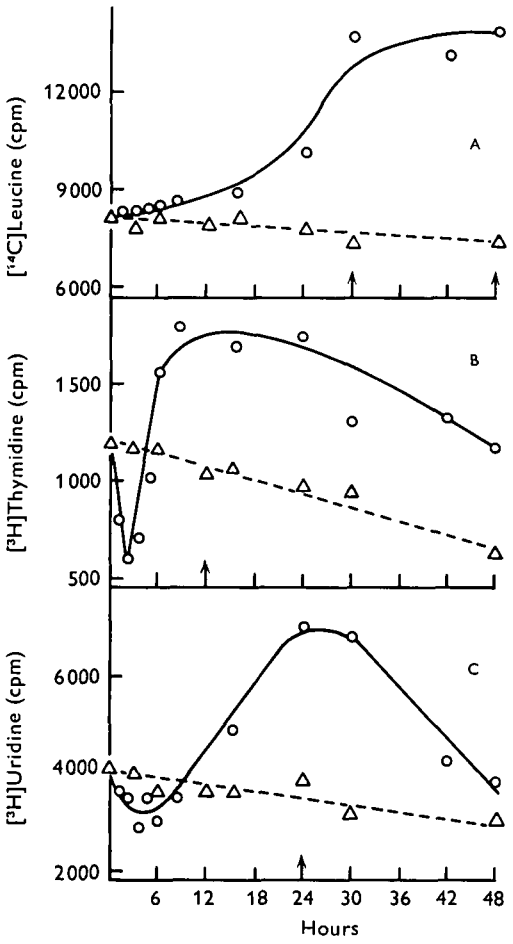

Fig. 3. The time sequence of changes in the rates of synthesis of A, protein, B, DNA, and C, RNA following a medium change; O—O, culture with a medium change; △—△, culture without medium change. Arrows indicate times of peak activity.

The effect of medium changes on nutrient utilization

Usually a medium change stimulates growth because a nutrient has become growth-limiting, but this has been shown to be unlikely in cultures of *WI-38* cells (Griffiths, 1970*a*). A medium change stimulates DNA and protein synthesis indicating that a

missing nutrient has been supplied but in the absence of an exhausted nutrient the presence of a concentration-dependent reaction must be suspected. The media from cultures subjected to various regimes of medium changes were analysed in the anticipation that this could elucidate the mechanism by which a medium change enhances growth (Tables 2, 3).

Table 2. *Amino acid and glucose utilization by WI-38 cells during various regimes of medium changes (M/C)*

	Cell count $\times 10^6$	Amino acid utilization (μg)†	*Y* amino acids†	Glucose utilization (μg)	*Y* glucose	Conversion of glucose to lactic acid %
Daily M/C*						
72–96	1·38	63	3·5	420	0·38	50
96–120	1·55	84	2·7	730	0·30	45
120–144	1·85	115	2·4	930	0·27	39
144–168	2·30	112	2·4	1170	0·26	44
168–192	3·15	120	2·8	1250	0·29	39
192–240	4·12	96	3·3	1550	0·31	31
Total 0–240 (5 M/C)		677		7020		42
Periodic M/C						
0–96	1·25	102	4·5	1170	0·40	36
96–120	1·45	84	3·1	730	0·31	45
120–168	1·95	138	2·7	1650(E)‡	0·25	29
168–240 (3 M/C)	2·55	166	2·6	1650(E)†	0·24	42
168–192	2·35	104	2·6	1250	0·23	39
192–240 (4 M/C)	3·21	118	3·0	1650(E)‡	0·26	40
Total 0–240 (4 M/C)		546		6450		36
No M/C						
0–72	1·18	87	4·7	970	0·43	59
0–96	1·28	101	4·7	1170	0·40	36
0–168	1·55	123	5·0	1470	0·41	27
0–192	1·60	146	4·6	1550	0·43	20
0–240	1·60	160	4·2	1650(E)‡	0·41	13

*Data taken from 2 separate experiments: daily M/C from 72–168 h; and daily M/C 96–192 h.
†These values are for a total of 10 amino acids (arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine and valine).
‡(E) signifies that glucose was completely exhausted.

The main utilization of amino acids occurred within the first 24 h following a medium change and prolonging the time between medium changes from 24 to 48 h or more did not result in very much more utilization, or growth. This result means that amino acids are chiefly used when their concentration is high and although no amino acid was used sufficiently to become exhausted, the ones that were utilized 50% or more (Table 3) are of the greatest interest if a concentration-dependent effect is present.

The consistency of the growth yields (*Y*) is compatible with the earlier observation that growth is proportional to the number to medium changes and that there is no

diminishing return with additional feeding. However, although the Y values are similar there is a relationship with the number of medium changes as Y is highest in cultures with no medium changes and gets progressively lower from 5 medium changes down to 1 (e.g. Y is 3·3, 3·0 and 2·6 after 5, 4 and 3 medium changes, respectively). The reason for this anomaly is that products of metabolism, for example glycine and ammonia, are reutilized in the later stages of the culture cycle.

Table 3. *Amino acid uptake and production during various regimes of medium changes (M/C)*

(Only the amino acids that were at least 50% utilized at some stage are shown. When the utilization exceeds 50% the value is in bold type.)

	Amino acids utilized (μg)				Nutrients produced (μg)		
	Cys (24)*	Ile (53)*	Leu (53)*	Met (15)*	Ala	Gly	Ammonia
Daily M/C							
72–96	7	18	17	4	22	9	120
96–120	5	22	18	5	23	9	136
120–144	9	28	25	4	32	7	135
144–168	8	23	25	7	33	6	133
168–192	10	22	25	7	37	9	134
192–240	8	21	24	4	44	6	130
Total 0–240 (5 M/C)	**56**	**154**	**145**	**35**	231	56	920
Periodic M/C							
0–96	9	19	14	8	40	11	140
120–168	10	**30**	**31**	7	48	6	159
168–240 (3 M/C)	**13**	**36**	**35**	**9**	102	9	208
192–240 (4 M/C)	9	**30**	**26**	4	99	10	171
0–240 (4 M/C)	**43**	**123**	**114**	**31**	247	45	740
No M/C							
0–72	9	20	11	4	40	10	132
0–96	9	19	14	**8**	40	11	140
0–168	11	23	18	**8**	54	13	146
0–192	**12**	**29**	23	**8**	67	11	147
0–240	**13**	**30**	25	**8**	78	10	158

*( ) Initial concentration in μg.

Glucose utilization, unlike that of the amino acids, is not confined mainly to the first 24-h period following a medium change but continues steadily until exhaustion occurs. Glucose poses a special problem in cell nutrition as it is utilized by the cell according to the law of mass action and, therefore, the higher the initial concentration the more is metabolized. This is demonstrated by the data in Fig. 2 which show that Y for glucose is lower when the medium is changed daily and also that lactic acid is reutilized in the later stages of a culture when the glucose concentration is low (conversion of glucose to lactic acid is 59% after 72 h but only 13% after 240 h). Daily medium changes prevent glucose exhaustion but the data in Table 4 indicate

that the glucose concentration does not affect growth until it falls below 500 μg/ml. The effect of a medium change, therefore, can be partly explained as preventing glucose exhaustion but it must be surmised from all the available data that this is not the only factor involved.

Table 4. *The effect of glucose concentration on the growth of WI–38 cells*

Initial glucose concentration, μg/ml	Cell yield (× 10^6/culture) 96 h	168 h
100	0·89	1·03
250	0·97	1·03
500	1·20	1·30
750	1·20	1·25
1000	1·25	1·35

DISCUSSION

Continuous medium perfusion is a very successful method for growing diploid cells in multiple-layered sheets. However, as this method is inconvenient for large-scale batch production of cells, it would be more advantageous to use medium changes if they have the same effect as medium perfusion. Higher cell densities have been attained using perfusion techniques but the total medium replacement has exceeded that used in medium-change experiments to date. The real value of perfusion cultures has been to demonstrate that contact inhibition of growth is not inevitable and the fact that it can be suppressed encourages further studies on the cell mechanisms involved.

Although nutrient exhaustion does not occur it has been suggested that diploid cells may not be able to take up nutrients in sufficient quantity once the cell sheet becomes confluent (Griffiths, 1970*a*, *b*). The 2 factors that could be involved are, first, a reduced nutrient uptake capacity due to the structure of diploid cell membranes, which differ markedly from those of heteroploid cells (Abercrombie & Ambrose, 1962; Defendi & Gasic, 1963; Kraemer, 1966) and secondly, the large reduction in cell membrane area exposed to the environment in a confluent culture (Castor, 1969). The results from the present investigation are in accordance with these ideas as most of the amino acids that are utilized in between medium changes are used in the first 24 h. It is usual for the amino acid uptake to be high in the first 24 h of a culture as this is the lag phase during which the cells actively establish themselves and come into equilibrium with the environment (Griffiths & Pirt, 1967) but the *WI–38* cell shows this activity at every medium change, however frequent. The nutrients, therefore, are mainly utilized when their concentration is high.

The direct relationship between cell yield and the number of medium changes establishes a new concept in cell culture and must be considered a characteristic of diploid cells. When successive additions of nutrients are given to a culture it is usual for the growth yield to become progressively lower (Griffiths & Pirt, 1967). This is due to an increased turnover of nutrients as the cell number increases and also to nutrients being utilized for maintenance functions (Pirt, 1965), especially when the

overall growth rate is slow. However, diploid cells show a unit amount of growth per medium change and cessation of growth is accompanied by a virtual cessation of nutrient uptake and metabolism which means that no nutrients are used for maintenance functions. The data collected on nutrient utilization support this conclusion and the constancy of the growth yield values confirms that there is no diminishing return with additional feeding.

Contact inhibition of growth affects the synthesis of all macromolecules (Levine, Becker, Boone & Eagle, 1965) but it has been suggested that it is DNA synthesis which is primarily inhibited, and that RNA and protein synthesis are affected as a consequence of DNA inhibition (Griffiths, 1970*b*). This was proposed after finding that insulin, which acts on a process between RNA and DNA synthesis, released the inhibition on protein synthesis but not on DNA synthesis in a confluent culture. It was found in this present work that as the cell sheet became confluent, DNA synthesis was inhibited first, followed by RNA and protein synthesis respectively (Fig. 2). It was also found that when the medium was changed, DNA synthesis was the first to be affected followed by RNA and protein synthesis (Fig. 3). This evidence strongly supports the thesis that DNA synthesis is the prime target of contact inhibition of growth and shows that protein synthesis is not affected as much as nucleic acid synthesis by both culture confluency and a medium change. In view of the data suggesting that the inability of the cell to take up sufficient nutrients in a confluent culture is a major factor in contact inhibition of growth it is interesting to note that amino acid starvation markedly interferes with DNA synthesis but has very little affect on RNA synthesis (Sköld & Zetterberg, 1969).

It has been established that a regular supply of fresh medium to a culture of human diploid cells permits growth to extend beyond the limits thought to be imposed upon the cell by contact inhibition of growth. Neither the mechanism of this inhibition nor the way in which it is affected by fresh medium is really known, but DNA synthesis appears to be the principal metabolic step which is affected and inhibition can probably be avoided if the cell is able to take up an adequate supply of nutrients in a crowded culture.

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