THE GLUCOSE, INSULIN AND GLUTAMINE REQUIREMENTS OF SUSPENSION CULTURES OF HeLa CELLS IN A DEFINED CULTURE MEDIUM

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SUMMARY

The serum supplement in a defined medium for the growth of HeLa cells could be replaced by protamine-zinc-insulin (0.2 u./ml). Insulin (0.4 u./ml) replaced the growth-stimulatory properties of protamine-zinc-insulin, whilst protamine sulphate (5 µg/ml) was found to be toxic to the cells. The addition of insulin to cultures depleted of insulin increased both cell growth rates and maximum cell populations. In the defined medium, HeLa cells could only utilize glutamate when a small amount of glutamine was included. Glucose, at a level of 2 mg/ml, was shown to limit maximum cell populations. The growth yield from glucose was 295 µg cell dry weight/mg glucose. When the medium glucose concentration was increased to 4 mg/ml, HeLa cell populations in excess of 16 x 10⁵ cells (i.e. 640 µg dry weight)/ml were routinely achieved in the defined medium supplemented with insulin. Growth is then limited by the amino acid supply. Increasing the amino acid concentration of the medium by 50% raised the maximum cell population to 23.5 x 10⁵ cells (i.e. 940 µg dry weight)/ml.

INTRODUCTION

Whilst the mouse L cell has been successfully propagated in a chemically defined medium, there are very few reports describing growth of HeLa cells in defined media, without the need for long periods of cell adaptation. Although Rappaport, Poole & Rappaport (1960) were able to grow monolayer HeLa cell cultures in protein-free medium on specially prepared glass surfaces, cells cultured in suspension had a requirement for serum, indicating a role for serum other than that of mere attachment. Higuchi (1963) and Tribble & Higuchi (1963) reported growth of HeLa cells in a serum-free medium supplemented with protamine-zinc-insulin, but had to resort to frequent medium changes to achieve high cell populations. In the present study, the factors limiting maximum cell populations and growth rates of HeLa cells in a chemically defined medium were investigated. This led to the development of a medium which supported HeLa cell growth to high populations, without medium changes. This medium was employed to determine the quantitative glucose and insulin requirements of HeLa cells.
MATERIALS AND METHODS

Cell line

The cell line employed was the HeLa cell, described by Gey, Coffman & Kubicek (1952), and obtained from Flow Laboratories (Scotland). Tests previously described (Birch & Pirt, 1969) failed to show any evidence of mycoplasma infection.

Culture medium

The basic defined medium, designated medium A, was that of Birch & Pirt (1970), except that L-glutamine (12 mg/ml) was substituted for the monosodium glutamate, and, for monolayer culture, the calcium chloride concentration was increased to 185 μg/ml. The keto acids and methylcellulose were included, but polyvinylpyrrolidone was excluded from the medium. Modifications were made to this basic medium during the course of this study, and the medium tonicity was maintained constant by adjustment of the sodium chloride concentration. When the medium was supplemented with serum, Pluronic F68 0·1 % (w/v) (Jacobson Van Den Berg & Co. (U.K.) Ltd.) was added to prevent precipitation of serum protein (Swim & Parker, 1960). Crystalline insulin and protamine sulphate (ex herring) were obtained from Sigma London Chemical Co. Ltd.; protamine-zinc-insulin was obtained from British Drug Houses Ltd. These preparations were diluted with phosphate-buffered saline, and, when required, were added to the culture medium, just prior to inoculation.

Culture procedure

Monolayer cultures were maintained in 8-oz. (200-ml) medical flat bottles containing 10 ml of medium. Suspension cultures were grown in magnetically stirred 500-ml screw-cap bottles containing 100 ml of medium, or in 250-ml screw-cap Erlenmeyer flasks, containing 20 ml of medium, shaken at 200 rev/min on a Gallenkamp orbital shaker. Glassware for suspension cell culture was coated with silicone. Growth was measured by cell counts in a Fuchs-Rosenthal counting chamber, using trypan blue dye exclusion to distinguish viable cells.

Preparation of inocula

Inocula of 1 to 2 × 10⁵ cells/ml were used in all experiments and were taken from exponentially growing cultures. Cells for inoculation into medium containing serum were suspended from monolayer cultures using a trypsin-EDTA method (Pirt & Thackeray, 1964). For inoculation into defined medium, it was necessary to use a glass bead method to suspend cells from monolayers (Nagle, 1960).

Cells were harvested by centrifugation at 170 g for 5 min. They were washed and resuspended in phosphate-buffered saline containing 0·5 % (w/v) methylcellulose.

Analytical methods

Glucose and lactate were estimated enzymically, using Biochemica Test Combinations (Boehringer Mannheim GmbH). Keto acids were estimated as pyruvate equivalents, using the method of Friedmann & Haugen (1943).

RESULTS

The effects of serum, protamine-zinc-insulin, protamine and insulin on growth

The effects of serum and insulin on the growth of HeLa cells in medium A are shown in Table 1. In medium A, by itself, cell growth was poorly reproducible, and maximum cell populations and growth rates were low, compared with the serum-supplemented medium. In the absence of serum, growth was stimulated by protamine-
Mammalian cell nutrition

zinc-insulin (PZI), although growth rates and maximum cell populations were lower than in medium containing serum, and there was an extended lag phase. At a PZI concentration of 0.2 mIU/ml, maximum cell populations were obtained. Cells grown in the presence of PZI in monolayer culture adhered to the glass surface but flattened less than in medium containing serum.

Table 1. The effect of serum substitutes on growth of monolayer and suspension cultures in defined medium

<table>
<thead>
<tr>
<th>Addition to defined medium A</th>
<th>Duration of lag phase, h</th>
<th>Minimum population doubling time, h</th>
<th>Maximum cell population (inoculum deducted) × 10^6/ml medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% calf serum*</td>
<td>18-20</td>
<td>24-25</td>
<td>19.5-20.0</td>
</tr>
<tr>
<td>10% calf serum†</td>
<td>0</td>
<td>24-25</td>
<td>19.0-21.2</td>
</tr>
<tr>
<td>0.2 mIU PZI/ml</td>
<td>75-81</td>
<td>33-35</td>
<td>7.8-8.0</td>
</tr>
<tr>
<td>0.2 mIU PZI/ml†</td>
<td>33-46 (40)‡</td>
<td>24-36 (28)‡</td>
<td>7.8-9.9 (8.7)‡</td>
</tr>
<tr>
<td>5 μg protamine sulphate/ml†</td>
<td>—</td>
<td>—</td>
<td>0.0</td>
</tr>
<tr>
<td>0.4 mIU crystalline insulin/ml†</td>
<td>—</td>
<td>32-39 (33)‡</td>
<td>9.9-17.6 (13.6)‡</td>
</tr>
<tr>
<td>None†</td>
<td>0</td>
<td>60-180</td>
<td>0.4-7.4</td>
</tr>
</tbody>
</table>

* Monolayer culture.
† Suspension culture.
‡ Figures in parentheses represent the mean value.

Table 2. The effect of insulin concentration on growth in suspension culture in medium A

<table>
<thead>
<tr>
<th>Insulin concentration, mIU/ml</th>
<th>Minimum population doubling time, h</th>
<th>Maximum cell population (inoculum deducted) × 10^6/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 × 10^-1</td>
<td>32.0</td>
<td>15.0</td>
</tr>
<tr>
<td>4 × 10^-1</td>
<td>32.0</td>
<td>15.0</td>
</tr>
<tr>
<td>4 × 10^-2</td>
<td>37.0</td>
<td>13.2</td>
</tr>
<tr>
<td>4 × 10^-3</td>
<td>40.5</td>
<td>10.6</td>
</tr>
<tr>
<td>0</td>
<td>60.0</td>
<td>7.4</td>
</tr>
</tbody>
</table>

The effect of replacing PZI by protamine sulphate (5 μg/ml) or insulin (0.4 mIU/ml) is shown in Table 1. Protamine did not permit cell growth — indeed it showed toxicity, in that there was a 60% loss in cell viability within 18 h of inoculation. The toxicity of protamine could be the cause of the long lag phases observed in media supplemented with PZI. In suspension culture, cells grown in the presence of insulin did not exhibit detectable lag phases (Table 1). In monolayer culture, insulin was as effective as PZI in promoting the adhesion of cells to the glass surface.

Cells grown in medium A, supplemented with insulin and then transferred to medium without insulin, showed a gradual decrease in growth rate and maximum cell populations over several successive subcultures. In the absence of insulin the cells...
were smaller, as reported by Waymouth & Reed (1965) for mouse L cells; also growth was very variable and the culture viability decreased by as much as 40%.

The quantitative response of HeLa cells to insulin in suspension culture is shown in Table 2. The inoculum had been depleted of insulin through 135 population doublings in the absence of insulin. Insulin concentration had an effect on both maximum cell populations and cell doubling time. At an insulin concentration of 0.4 u/ml, maximum cell populations and culture growth rates were obtained.

There was no indication of a requirement for a period of adaptation to growth in the defined medium containing insulin. Cells previously maintained in the presence of serum, when transferred to the defined medium, grew without a lag phase and with growth kinetics characteristic of cultures grown in the presence of insulin.

**Growth on glutamate**

Griffiths & Pirt (1967) showed that, for mouse LS cell growth, glutamate could substitute for all the glutamine in a medium supplemented with serum. However, growth on glutamate was enhanced by the addition of a small amount of glutamine. Consequently the possibility of using glutamate as a substitute for glutamine for HeLa cell growth, in the serum-free medium, was examined.

Suspension cultures grown in medium A, supplemented with 0.4 u. insulin/ml and containing l-glutamine (1.20 mg/ml) or monosodium glutamate (1.53 mg/ml) plus l-glutamine (0.10 mg/ml) exhibited growth kinetics which were not significantly different. The small amount of glutamine was essential in a glutamate medium. This applied whether the inoculum had been grown in medium containing glutamine or glutamate + glutamine. The trace of glutamine could not be replaced by an equimolar amount of ammonium chloride. Eagle et al. (1956) were able to adapt HeLa cells to utilize glutamate alone in a medium containing 1% (v/v) dialysed human serum. In our experiments, however, this supplement does not overcome the glutamine requirement, and adaptation to growth in medium devoid of glutamine was not observed.

**The effect of pyruvate and \( \alpha \)-ketoglutarate on growth**

Both of these keto acids were normally included in medium A. Their omission from medium A, supplemented with 0.4 u. insulin/ml, had no effect on growth and they were not included in the media used in the carbohydrate study described below.

**Glucose utilization, lactate and keto acid production, and pH values during growth**

In medium A supplemented with 0.4 u. insulin/ml, glucose, lactate, keto acid and pH values were determined during culture, and the results are shown in Fig. 1. In this culture glucose was exhausted from the medium before the cessation of growth. After this point, a decrease in medium lactate and keto acid concentrations, and an increase in medium pH, were observed.

The quantitative requirement for glucose was determined by growing cells in an insulin-supplemented medium with a range of glucose concentrations from 0 to 4 mg/ml. Maximum cell populations (inoculum deducted) were directly proportional to glucose concentrations in the range 0–2 mg glucose/ml (Fig. 2). Hence, glucose was
growth-limiting up to the latter concentration. Glucose concentration had no effect on cell growth rate (population doubling time = 36 h). The growth yield for glucose, given by the initial slope of the graph in Fig. 2, is $7.4 \times 10^6$ stationary-phase cells (i.e. $295 \mu g$ dry weight)/mg glucose. The yield from glucose will be dependent upon the initial specific rate of glucose utilization by the cells, and the proportion of the glucose metabolized to products such as lactate. The variation in maximum cell population observed with an initial glucose concentration of 2 mg/ml (Table 1) correlated with differences in the specific rate of glucose utilization (range 2.4–9.1 $\mu g$ glucose per h per $10^5$ cells) during the first 48 h of culture.

Fig. 1. Medium glucose, lactate, keto acid and pH levels during HeLa cell growth, in suspension culture, in medium A containing 2 mg glucose/ml and supplemented with insulin (0.4 u./ml). O, Viable cell count; •, glucose; △, lactate; ▲, keto acid; □, pH.
By increasing the glucose concentration of the medium to 4 mg/ml, maximum cell populations (inocula deducted) in excess of $16 \times 10^6$ cells/ml are routinely achieved. Glucose, lactate, keto acid and pH values throughout cell culture in medium A containing 4 mg glucose/ml and supplemented with 0.4 u. insulin/ml, are shown in Fig. 3. The results show that glucose was in excess and that the additional glucose did not cause any marked increase in the lactate production during culture.

**Amino acid-limitation of growth**

In medium A containing 4 mg glucose/ml and supplemented with insulin, it was established that amino acid supply limited maximum cell populations. Increasing the amino acid concentration of this medium by 50% resulted in an increase in maximum HeLa cell population (inoculum deducted) from $16.8 \times 10^6$ to $23.5 \times 10^6$ cells/ml. This increased amino acid concentration had no effect on cell growth rate (population doubling time = 34 h) and did not introduce a culture lag phase.

**DISCUSSION**

The present study extends the application of the chemically defined medium of Birch & Pirt (1970). With certain modifications, this medium enabled maximum HeLa cell populations in excess of $16 \times 10^6$ cells (i.e. 640 µg dry weight)/ml to be routinely achieved, as compared to maximum mouse LS cell populations in excess of $26 \times 10^6$ cells (i.e. 830 µg dry weight)/ml obtained by Birch & Pirt (1970).
Suspension culture techniques and the high maximum cell populations obtained in insulin-supplemented media facilitated a quantitative determination of the glucose and insulin requirements of HeLa cells in a defined culture medium. The increase in maximum HeLa cell populations, in medium A, caused by protamine-zinc-insulin addition, is in agreement with the findings of Higuchi (1963) and Tribble & Higuchi (1963). However, in contrast to the work of these authors, protamine by itself had no growth-stimulatory activity and, at a concentration of 5 μg/ml, was growth inhibitory. The lower maximum cell populations observed with protamine-zinc-insulin cultures, compared to insulin-supplemented cultures (Table 1), probably reflects a less efficient
utilization of nutrients during the lag phase of culture, as described by Griffiths & Pirt (1967) and Munyon & Merchant (1959). The effect of insulin concentration on maximum cell populations is similar to that reported by Lieberman & Ove (1959) for human appendix cells cultured in a serum-albumin medium, though in the present study a higher range of insulin concentrations was employed. The present work shows that insulin also increases cell growth rates by a factor of 2 to 5 (Table 1). The graded responses of HeLa cells to insulin concentrations in the defined medium makes this an attractive system as a means of studying the mode of action of insulin.

Previous reports of the adaptation of HeLa cells to utilize glutamate in the absence of glutamine concerned cell growth in media supplemented with serum (Eagle et al. 1956). However, in the present study, a low level of glutamine was essential for growth of HeLa cells in defined glutamate media supplemented with insulin. This indicates a requirement for glutamine itself, or degradation products of glutamine, other than ammonium ions.

In contrast to the reports of Nagle, Tribble, Anderson & Gary (1963) and Higuchi (1963), omission of pyruvate from medium supplemented with insulin had no effect on cell growth. Since neither of these authors added iron to their media, this difference might reflect the bypassing, by keto acids, of blocks in metabolism caused by iron deficiency, as suggested by Birch & Pirt (1970).

The variability in the initial specific rate of utilization of glucose probably reflects variability in parameters such as the physiological state and density of the inoculum and the conditions of culture. Substantial utilization of lactate by the cells was observed when glucose was exhausted from the medium. This, coupled with the possible deamination of amino acids, might account for the rise in medium pH observed during the later phases of culture (Figs. 1, 3). A growth yield for glucose estimated from the total glucose uptake during culture will depend upon the proportion of glucose converted to products such as lactate. An estimation of a growth yield from glucose from a limitation experiment gives a maximum value for the growth yield, which will, itself, probably be dependent upon the growth rates of the cultures employed in the experiment. From a growth-limitation experiment, the growth yield of HeLa cells from glucose was estimated as 205 μg dry weight/mg glucose. Thus, the HeLa cell requires considerably more glucose than the mouse LS cell, for which the growth yield is 755 μg dry weight/mg glucose (Birch & Pirt, 1971). However, since insulin is known to stimulate the rate of glycolysis of cultured cells (Paul & Pearson, 1960), the difference in the HeLa and L cell growth yields from glucose may reflect the addition of insulin to the HeLa culture medium.

In medium A containing 4 mg glucose/ml and supplemented with 0.4 μg insulin/ml, it was established that maximum HeLa cell populations were limited by the amino acid supply.

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REFERENCES


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