METHOD OF CONTROLLABLY RELEASING GLUCOSE TO A CELL CULTURE MEDIUM

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ABSTRACT

A cell culture medium is disclosed which contains secondary sources of glucose comprising glucose-containing oligosaccharides or polysaccharides and hydrolytic enzymes (glucosidases) to liberate free glucose. Starch, for example, can be added to a culture medium to provide the controlled release of glucose when a serum supplement such as fetal calf serum, which contains the hydrolytic enzymes amylase and maltase, is used. Amylase breaks the starch down into glucose and maltose, and maltase converts the remaining maltose to glucose. The rate of glucose release can be controlled by regulating the amount of glucosidase activity present in the medium.

23 Claims, 6 Drawing Figures
FIG. 1.

FIG. 2.
FIG. 3.

CONCENTRATION OF GLUCOSE OR STARCH (µg/ml)

TIME (days)

FIG. 4.

GLUCOSE RELEASED FROM STARCH (µg/ml)

GLUCOSE RELEASED FROM MALTOSIDE (µg/ml)

TIME (days)
METHOD OF CONTROLLABLY RELEASING GLUCOSE TO A CELL CULTURE MEDIUM

The invention herein described was made in the course of work performed with a grant from the Department of Health, Education and Welfare.

BACKGROUND OF THE INVENTION

1. Field of the Invention
The invention is in the field of biology, and more specifically in the field of cell biology.

2. Description of the Prior Art
Mammalian cells are typically grown in a culture medium to which a serum supplement is added. There are several well known culture media including Eagle’s medium, the Dulbecco-Vogt modification of Eagle’s medium, and Ham’s medium. In general, these are synthetic formulations designed to provide sources of amino acids, vitamins, glucose, etc. to the growing cells. The serum supplement is added to provide certain proteins to the culture, and typical serum supplements include calf serum, fetal calf serum, and horse serum.

It has heretofore been common to provide relatively high concentrations of glucose, e.g., greater than about 1 mg/ml, in culture media to provide a source of carbon and energy to the growing cells. Utilization by mammalian cell cultures of sugars other than glucose has, however, been studied extensively. See, for example, Harris, M. and Kutskey, P. B., J. Cell Comp. Physiol., 42, 449 (1953); Eagle, H., Barban, S., Levy, M., and Schulz, H. O., J. Biol. Chem., 233, 551 (1958); Bailey, J. M., Gey, G. O., and Gey, M. K., J. Biol. Chem., 234, 1042 (1959); Baugh, C. L. and Tytell, A. A., Life Sciences, 6, 371 (1967); Martinneau, R. Kohlbacher, M., Shaw, S. N., and Amos, H., Proc. Nat. Acad. Sci. USA, 69, 3407 (1972); Kaelkar, H. M. and Ullrey, D., Proc. Nat. Acad. Sci. USA, 70, 2596 (1973); and Paul, J., In Cells and Tissues in Culture, Vol. 1, Willmer, E. N., ed., Academic Press, New York, 239 (1965). From such studies, it has been determined that mannose and fructose are metabolized rapidly, but galactose is utilized more slowly and produces less lactate. See Eagle, H., Barban, S., Levy, M. and Schulze, H. O., J. Biol. Chem., 233, 551 (1958). In spite of these extensive studies, glucose is still the sugar mostly widely used in mammalian cell cultures, although it has been noted that cell growth could take place on maltose or glycogen after their hydrolysis by enzymes of embryo extract or serum. See Harris, M. and Kutskey, P. B., J. Cell Comp. Physiol., 42, 449 (1953).

The use of glucose in culture media does cause some problems, however. The most serious of these is that the glucose is consumed rapidly. Since cells cannot survive when their glucose supply has been exhausted, frequent refeeding to replenish the supply is necessary. Further, high concentrations of glucose are not used efficiently by growing cells because the cells ferment more glucose than they can oxidize. This results in an accumulation of lactic acid, a fermentation product, in the medium which produces a concomitant drop in pH which is deleterious to cell growth. See Cecconari, C. and Eagle, H., Proc. Nat. Acad. Sci. USA, 68, 229 (1971).

SUMMARY OF THE INVENTION
In one embodiment, the invention comprises the use of secondary sources of glucose in cell culture media, particularly those intended for mammalian cells. Suitable secondary sources of glucose include either a glucose-containing oligosaccharide or polysaccharide and a glucosidase, or enzyme capable of releasing glucose from the glucose-containing compounds. Suitable glucosidase activity can be provided by choosing serum supplement containing the desired hydrolytic enzyme or enzymes. Alternatively, commercial preparations of a glucosidase can be added to the culture medium.

The rate of glucose release to the culture medium can be controlled by adjusting the glucosidase activity present. For example, the rate can be reduced by partially inactivating the glucosidase activity in the serum supplement by heating the serum to an elevated temperature prior to its addition to the culture medium. Alternatively, different amounts or types of hydrolytic enzymes, or serum supplements containing hydrolitic enzymes, can be added to culture media to adjust the glucosidase activity to the desired level for any specific oligosaccharide or polysaccharide.

One suitable secondary source of glucose is starch together with the hydrolytic enzymes amylase and maltase. The amylase breaks starch down to predominantly maltose units and the maltase completes the hydrolysis to glucose. Another secondary source is the sugar maltose which is hydrolyzed by the enzyme maltase. Thus, glucose is released by both systems.

Providing glucose to culture media from secondary sources offers significant advantages over the direct addition of glucose. A primary advantage is that any desired rate of glucose release to the cell culture medium can be achieved for extended periods of time. Low concentrations of glucose cannot be maintained by direct addition because the growing cells consume the glucose so rapidly. In the new system, glucose continues to be available to the cells at a time when it would have been exhausted if supplied to the medium directly.

Supplying constant but relatively low levels of glucose to the cell culture from secondary sources also helps to maintain the pH of the culture medium within a beneficial range. This is primarily because little or no lactic acid is formed, since the growing cells metabolize completely the glucose liberated from the secondary source. Thus, the cells can be grown to a higher density and maintained healthier for longer periods of time than in media to which glucose is added directly, and frequent refeeding of cultures to supply glucose is eliminated.

BRIEF DESCRIPTION OF THE DRAWINGS
FIG. 1 illustrates graphically the growth rate of established mammalian cell lines at various glucose concentrations.
FIG. 2 illustrates graphically the efficiency of cell utilization of glucose at different concentrations.
FIG. 3 illustrates graphically the release of glucose from the secondary source starch using fetal calf serum which contains amylase and maltase;
FIG. 4 illustrates graphically the inactivation of serum enzymes with heat;
FIG. 5 illustrates graphically certain conditions resulting from the growth of V79 cells in various culture media containing glucose, starch, plus amylase or maltose plus maltase;
FIG. 6 illustrates graphically the growth of V79 cells on maltose in a culture medium containing serum with maltase inactivated by heat.
DESCRIPTION OF THE PREFERRED EMBODIMENTS

The provision of secondary glucose sources in culture media is useful in most cell cultures and is particularly useful in mammalian cell cultures. Such mammalian cells as 3T3 and 3T6 (mouse embryonic fibroblast lines), SV40 and SV40 (papovaviruses), and V79 (a line derived from Chinese hamster embryonic lung fibroblasts) as well as other mammalian cell lines can be grown in media containing secondary glucose sources.

Secondary glucose sources can be added to a wide variety of cell culture media. Some of the better known and widely used media include Eagle's medium, the Dulbecco-Vogt modification of Eagle's medium, and Ham's medium. It should be clear, however, that other media known to those skilled in the art can be provided with secondary glucose sources as described herein.

Suitable secondary sources of glucose can be a glucose-containing oligosaccharide or polysaccharide together with appropriate glucosidase activity. The release of glucose can be illustrated for the oligosaccharide maltose used in combination with the enzyme maltase as follows:

\[ \text{Maltose} \rightarrow \text{Glucose} \]

Similarly, the release of glucose from the polysaccharide starch by the enzymes amylase and maltase can be illustrated as follows:

\[ \text{Starch} \rightarrow \text{Glucose} + \text{Maltose} \]

Amylase hydrolyzes starch to glucose and maltose, and the maltose is hydrolyzed into glucose by the enzyme maltase. It is preferable, therefore, to include both amylase and maltase in a culture medium if starch is the secondary source of glucose.

All glucose-containing oligosaccharides or polysaccharides which can be broken down to release glucose by an appropriate glucosidase are suitable secondary sources. Examples of oligosaccharides containing glucose include, but are not limited to, maltose, sucrose, lactose, and raffinose. Suitable examples of glucose-containing polysaccharides include, but are not limited to, glycogen and starch. Other compounds containing glucose moiety which can be released may also be useful. These include, for example, synthetically prepared glucosides.

Secondary sources of glucose are particularly useful in cultures where it is desired to maintain a relatively constant but low concentration of glucose. Whereas most previous systems used glucose at the physiological range (1 mg/ml) or higher, it has been demonstrated that much lower concentrations can be used to achieve beneficial results. FIG. 1 illustrates how the doubling time of a number of established mammalian cell lines in the Dulbecco-Vogt modification of Eagle's medium supplemented with 10% dialyzed calf serum or fetal calf serum depends on the glucose concentration of the medium. These determinations were carried out by
inoculating 32 mm petri dishes with $10^9$ or $10^4$ cells in 2.0 ml of medium and counting the number of cells per culture at daily intervals. The doubling times taken from the exponential portion of the growth curves are independent of glucose concentration at the physiological range (1 mg/ml) or higher. Below 300 μg/ml the growth rates decline; but even at 1 μg/ml, a glucose concentration 1,000 fold lower than physiological, two of the lines grow with a doubling time of less than 50 hours without any initial delay attributable to adaptation. At still lower glucose concentrations, cell number does not increase and there is noticeable cell death and detachment. From these experiments, it can be seen that the preferred glucose concentration in a mammalian cell medium is from about one microgram to about 100 micrograms per milliliter of medium. Mass cultures can be grown indefinitely (>20 generations) at these glucose concentrations if the cell number is kept low and the cultures transferred before the glucose is consumed.

The efficiency of glucose utilization is also improved by using lower glucose concentrations. This is illustrated in Fig. 2 which shows the number of cells grown per unit of glucose consumed at varying glucose concentrations. Cultures with different initial glucose concentrations were formed by inoculating petri dishes containing 2.0 ml of Dulbecco-Vogt modification of Eagle’s medium and 10% dialyzed calf serum with $10^4$ cells which were allowed to grow to maximal cell density. This density was attained when all the glucose was consumed. These cultures were not refed. Ordinate values were derived from the ratio,

\[
\text{Final Cell Number - Initial Cell Number} / \text{Glucose Consumed}
\]

At 1–3 mg/ml, the number of cells produced is 5–20 fold greater than at the physiological concentration of about 1 mg/ml. At all glucose concentrations, the maximum cell number in fetal calf serum is 20–40% higher than in calf serum.

Doubling times and maximal cell density in low glucose concentrations were estimated for Figs. 1 and 2 as follows. Subconfluent, growing cultures in medium containing 4.5 mg/ml glucose and 10% calf serum were removed with isotypic EDTA and trypsin, diluted into glucose-free medium for counting, then centrifuged and resuspended in fresh glucose-free medium. $10^9$ or $10^4$ cells were plated in duplicate 32 mm dishes containing 2.1 ml of medium with 10% dialyzed calf serum and different glucose concentrations. Beginning two to four days later, cultures were trypsinized and the cells were counted daily until the cultures failed to increase in cell number and began to degenerate due to glucose starvation.

One method of conveniently adding the appropriate glucosidase to a culture medium is to select a serum supplement which contains the desired hydrolytic enzyme activity. As is known, different types of serum contain differing types and amounts of enzyme activity. For example, calf serum has been found to have threefold greater activity of α-amylase than fetal calf serum, whereas horse serum has less than 10% of the activity of fetal calf serum. Because of these differences, a convenient method of controlling the amount of any particular glucosidase activity in the culture is to select a serum containing the particular enzyme activity desired and to add the serum in an amount sufficient to provide the desired level of activity. Alternatively, another convenient method for reducing the rate of hydrolysis is to use heat to inactivate part of the enzymatic activity. A serum having too high an activity, for example, can be heated to an elevated temperature and cooled prior to its addition to the culture medium to provide the desired activity level.

The invention is further illustrated by the following examples:

**EXAMPLE 1**

**Digestion of Starch and Liberation of Glucose by Enzymes of Fetal Calf Serum under Cell Culture Conditions**

A 10% stock solution of starch was prepared by dissolving 5 grams of soluble starch, B grade (Calbichem) in 50 ml of glass distilled water by heating for about 30 minutes in a boiling water bath, and then autoclaving for 15 minutes at 25 psi.

One part of the stock starch solution was introduced into 20 parts of serum-free, Dulbecco-Vogt modification of Eagle’s medium. The medium was maintained at 37°C. for over 20 days, after which time there was no detectable decline in the amount of starch and no liberation of glucose.

The same procedure was followed except substituting medium containing 10% fetal calf serum. This time, starch disappeared with a half-life of about 35 minutes and was near the limit of detection 4 hours later. Glucose appeared in the medium, but less rapidly, since molecules containing fewer than 8 saccharide units give no color in the starch-iodine reaction, but must be further hydrolyzed to yield glucose. The glucose concentration in the medium reached 25 μg/ml within an hour, a concentration fully able to support cell growth. The concentration rose to 100 μg/ml by four hours, and continued to rise until, by about 10 days, a maximum of about 50% of the starch was converted to glucose.

Glucose and starch determinations were done as follows. The glucose oxidase reaction, coupled with the oxidation of a chromogen (“Glucostat special”, Worthington Biochemical Corp.) gives a yellow-brown color with maximum absorption at 400 nm. 0.6 ml of diluted unknowns, standards and a glucose-free blank were deproteinized by the successive additions of 0.3 ml of 2% ZnSO₄·7H₂O and 0.3 ml 0.46% NaOH and centrifuged at 4,000 g for 10 min. Equal volumes of the supernatant and glucostat reagent were mixed and incubated at 37°C.; the reaction was stopped by the addition of two drops of 4.0 N HCl. The length of incubation with the glucostat reagent was adjusted to make the absorption of the standard solutions proportional to their glucose concentration in the range of interest. The assay was accurate over a range of 1 μg/ml to 5 mg/ml.

A modification of the iodine method of Smith and Roe (1949) was used. Iodine reagent was prepared by adding 18 mg iodine dissolved in 1 ml ethanol, to 320 ml of 3.5 mM KI. Concentrated HCl was added to a concentration of 0.01 N. The reagent was stored in a brown bottle and was prepared fresh every other day. Starch solutions were diluted from 1:1 to 1:40 in iodine reagent so as to give absorption proportional to starch concentration over a range of 20 μg–5 mg per ml. The
color is stable for several hours at dilutions of 1:20 or 1:40 of sample to reagent, but fades rapidly at 1:1 dilution. The results of this Example are shown graphically in FIG. 3.

EXAMPLE 2
Inactivation of Serum Enzymes Liberating Glucose from Starch and from Maltose

Glucose-free, fetal calf serum, which contains amylase and maltase, was heated in a water bath under various conditions and then tested for its ability to release glucose from starch. The serum was dialyzed at 4°C for 24 hours against three changes of 20 volumes of isotonic phosphate buffered salt solution (pH 7.3) and finally for 10–18 hrs. against 5 volumes of glucose-free F12 medium (Ham, 1965). This serum supported growth as well as undialyzed serum, but glucose-free Dulbecco-Vogt medium could not substitute for Ham’s medium in the final dialysis without reduction in growth rate and cloning efficiency. The level of glucose in the dialyzed serum was below the limit of detection by the glucose oxidase assay (1 μg/ml). The serum was sterilized by filtration before use. FIG. 4 shows that the ability to release glucose from starch was virtually completely lost after heating at 65°C for 90 min. The maltase activity was more resistant; its inactivation required 30 min. at 70°C. In the absence of serum, the glucose oxidase assay of a 5 mg/ml maltose solution gave a value of 4 μg/ml of glucose equivalent; this may represent contaminating glucose.

EXAMPLE 3
Growth of V79 Cells on Starch or Maltose as Secondary Glucose Sources

V79 cells (5×10⁶) were inoculated into 32 mm petri dishes containing 2.0 ml of glucose-free Dulbecco-Vogt modification of Eagle’s medium. 4.5 mg/ml of either starch, maltose or glucose were added. The V79 cells grew on glucose liberated from starch or maltose by the enzymes of fetal calf serum with virtually no initial period of glucose starvation as shown in the upper panel of FIG. 5. The growth curves of V79 cells in starch, maltose or glucose were virtually identical, the doubling time being about 12 hours. The middle panel of FIG. 5 shows that cells grown in free glucose consumed nearly 90% of the added glucose in ten days. Glucose liberated from maltose or starch by the serum was not appreciably consumed by the cells for the first three days, but thereafter the increased cell number reduced the glucose concentration to very low levels, production and consumption presumably continuing concurrently.

The bottom panel of FIG. 5 shows how the pH of the medium was affected by the source of glucose in the culture. When the high concentration of free glucose was present from the outset, the pH eventually dropped below 6.6. In cultures containing secondary glucose sources, the pH fell very little; the final values were 7.0 for maltose and 7.1 for starch, scarcely lower than the starting value. From this data, it is clear that at the lower glucose levels maintained by sustained release from starch or maltose, more efficient glucose utilization results in much less acid production.

EXAMPLE 5
Growth of V79 Cells on Reduced Initial Concentrations of Glucose and Maltose

The procedure of Example 4 was followed except that an initial concentration of glucose of 500 μg/ml was used, and a low initial concentration of maltase activity was established by using a fetal calf serum supplement consisting of 9 parts heat inactivated serum (70°, 30 minutes) and one part unheated serum. Under these conditions, the growth rate in added glucose was a little more rapid than in maltose as shown in the upper panel of FIG. 6. By the fourth day, however, the glucose was no longer detectable in the medium; cell number did not increase further, and two days later began to decline, due to cell death and detachment. The maltose-grown cells reached a higher saturation density and remained healthy in appearance; free glucose could be detected in the medium throughout the experiment as shown in the bottom panel of FIG. 6.

EXAMPLE 6
Growth of V79 Cells on Glucose Released From Glycogen by Amylase in Fetal Calf Serum

V79 cells (10⁴) were inoculated into culture media prepared as in Example 3 but substituting glycogen for starch. Each day the cultures were examined by microscope and it was observed that the cells grew at the same rate and to the same density as a culture containing glucose, but the medium did not become acidic.

What is claimed is:

1. In the method of culturing mammalian cells in a culture medium which supplies nutrients including glucose to said mammalian cells:
   The improvement of maintaining the glucose concentration in said culture medium at a desired concentration by providing sustained release of glucose at a controlled rate by including in the culture medium a compound containing an enzymatically releasable glucosyl moiety and an enzyme activity capable of releasing glucose from said glucosyl-containing compound, said glucosyl-containing compound being present in an amount sufficient to provide sustained release of glucose and said enzyme activity being present at a level sufficient to maintain the glucose at the desired concentration.

2. The improvement of claim 1 wherein said enzyme activity is supplied by the presence in said culture medium of at least one hydrolytic enzyme.

3. The improvement of claim 2 wherein said hydrolytic enzyme comprises a glucosidase.

4. The improvement of claim 3 wherein said glucosyl-containing compound comprises an oligosaccharide.

5. The improvement of claim 4 wherein said oligosaccharide comprises maltose and said glucosidase comprises maltase.

6. The improvement of claim 3 wherein said glucosyl-containing compound comprises a polysaccharide.

7. The improvement of claim 6 wherein said polysaccharide comprises starch and said glucosidase comprises amylase and maltase.

8. The improvement of claim 6 wherein said polysaccharide comprises glycogen and said glucosidase comprises amylase and maltase.

9. The improvement of claim 1 wherein said enzyme activity is supplied to said culture medium by adding serum thereto.

10. The improvement of claim 9 wherein said serum comprises fetal calf serum.

11. The improvement of claim 5 wherein maltase is supplied to said culture medium by adding serum thereto.

12. The improvement of claim 11 wherein said serum
comprises fetal calf serum.

13. The improvement of claim 7 wherein amylase and maltase are supplied to said culture medium by adding fetal calf serum thereto.

14. The improvement of claim 1 wherein said enzyme activity is included in an amount sufficient to maintain a glucose concentration in said culture medium of from about 1 to about 100 micrograms per milliliter of medium.

15. The improvement of claim 4 wherein said glucosidase is included in an amount sufficient to maintain a glucose concentration in said culture medium of from about 1 to about 100 micrograms per milliliter of medium.

16. The improvement of claim 6 wherein said glucosidase is included in an amount sufficient to maintain a glucose concentration in said culture medium of from about 1 to about 100 micrograms per milliliter of medium.

17. The improvement of claim 9 wherein sufficient serum is added to maintain a glucose concentration in said culture medium of from about 1 to about 100 micrograms per milliliter.

18. The improvement of claim 9 wherein the enzyme activity in the serum added to said culture medium is reduced by heating the serum to an elevated temperature prior to its addition to the culture medium.

19. The improvement of claim 18 wherein said enzyme activity is supplied by the presence in said serum of at least one hydrolytic enzyme.

20. The improvement of claim 19 wherein said hydrolytic enzyme comprises a glucosidase.

21. The improvement of claim 20 wherein said serum comprises fetal calf serum.

22. In the method of growing mammalian cell cultures wherein serum containing glucosidase activity is added to a mammalian cell culture medium to release glucose from glucosyl-containing compound to maintain a desired glucose concentration:

the improvement of regulating the glucose concentration in said culture medium by regulating the glucosidase activity of the serum prior to its addition to the culture medium.

23. The improvement of claim 22 wherein the glucosidase activity in said serum is reduced by heating said serum.