MOLECULAR GROWTH REQUIREMENTS OF SINGLE MAMMALIAN CELLS

III. Quantitative Colonial Growth of Single S3 Cells in a Medium Containing Synthetic Small Molecular Constituents and Two Purified Protein Fractions*

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Plate 73

(Received for publication, February 9, 1959)

Previous studies on the molecular growth requirements for colony formation by single mammalian cells *in vitro* (1–3) have demonstrated that these requirements are strongly influenced by the genetic constitution and degree of homogeneity of the cell strain employed; by the medium of previous cultivation of the cells and their treatment before inoculation; and by the treatment to which the macromolecular components of the medium had been subjected. When serum proteins were systematically fractionated, it was found that growth of single S3 HeLa cells required two separate protein fractions, albumin and an α-globulin (1). The present paper describes a medium containing synthetic micromolecular constituents and two purified protein fractions, which regularly produces colonies from single S3 HeLa cells plated in Petri dishes, with an efficiency approaching 100 per cent.

Methods and Materials

The cell strains employed are designated S3-9 and S3-9-IV, both of which are subclones of the HeLa S3 cell (4) and which behaved similarly in the present experiments. Cell stocks were cultured as monolayers on neutraglas bottles in the following medium: N16—40 per cent, saline F—30 per cent (see reference 5), human serum—20 per cent, horse serum—10 per cent (see reference 4). In some experiments the cell stock was cultured exclusively in the medium of Table I. Monolayer dispersal was effected by removal of the medium; a rapid rinse of the

* Contribution 84 from the Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver. This work was supported by a grant from The National Foundation, Inc., and from The Damon Runyon Memorial Fund for Cancer Research.

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cell layer with 10 ml. of 0.05 per cent trypsin (Difco 1–300 or Worthington crystalline trypsin) in saline G (5); and an incubation at 37°C. for 5 minutes with a second addition of 3 to 5 ml. of the above trypsin solution. In the present serum-free experiments the enzyme action could not be stopped by serum, as in our earlier procedure (4). Instead, the cell suspension was rapidly diluted serially in N15 (40 per cent) + saline G (60 per cent) to a concentration such that an inoculum of 0.10 ml. furnished 100 or 200 cells. Similar results were obtained

TABLE I

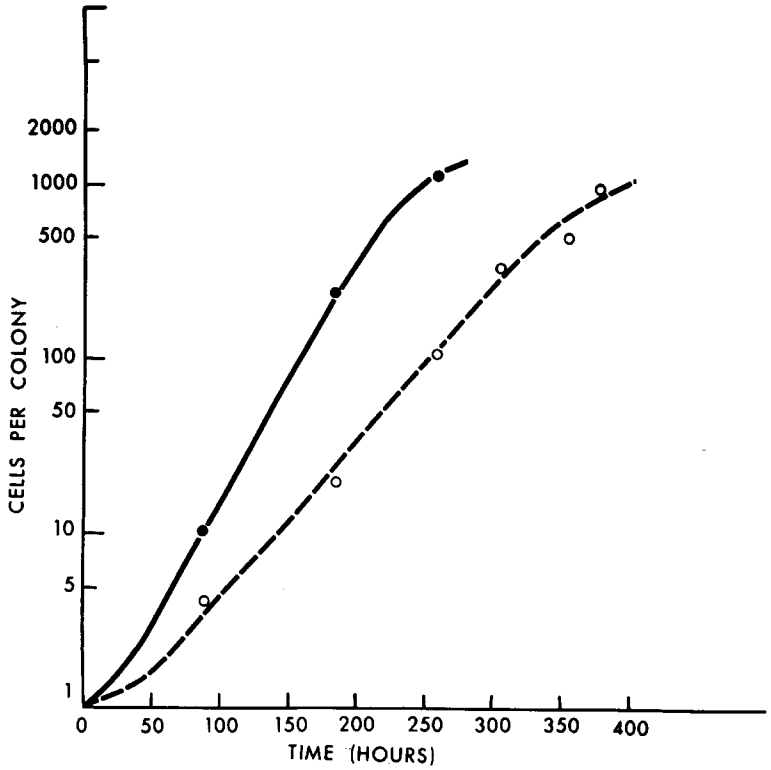
Definition of the Solutions Employed

A. Serum-free medium which supports quantitative growth of single S3 HeLa cells on glass:—

1. Amino Acids	*gm./liter*		*gm./liter*
L-Arginine HCl	0.015	L-Valine	0.010
L-Histidine HCl	0.015	L-Glutamic acid	0.030
L-Lysine HCl	0.032	L-Aspartic acid	0.012
L-Tryptophan	0.008	L-Proline	0.010
β-Phenyl-L-alanine	0.010	Glycine	0.040
L-Methionine	0.010	Glutamine	0.080
L-Threonine	0.015	L-Tyrosine	0.016
L-Leucine	0.010	L-Cystine	0.003
L-Isoleucine	0.005		
2. Vitamins and Growth Factors			
Hypoxanthine	0.010	Choline	0.0012
Thiamine HCl	0.0020	Ca pantothenate	0.0012
Riboflavin	0.00020	Niacinamide	0.0012
Pyridoxine HCl	0.00020	Inositol	0.0004
Folic acid	0.00004	Vitamin B-12	0.0010
Biotin	0.00004		
3. Salts and Other Small Molecules			
NaCl	7.40	$CaCl_2 \cdot 2H_2O$	0.016
KCl	0.285	$NaHCO_3$	1.20
$Na_2HPO_4 \cdot 7H_2O$	0.29	Glucose	1.10
KH_2PO_4	0.083	Phenol red	0.0012
$MgSO_4 \cdot 7H_2O$	0.154		
4. Proteins			
Normal serum albumin	2.0	Fetuin	2.0

B. Trypsinization and dilution solutions have been described elsewhere. (5)

when the medium of Table I was used as a diluting fluid. Inoculated Petri dishes were incubated without medium change, under conditions of careful temperature, CO_2 and relative humidity regulation, as previously emphasized (5). Petri dishes incubated 8 to 16 days were fixed and stained, as described (4), for evaluation of cell growth. The fetuin employed was prepared from fetal calf serum[1] according to the methods devised by Pedersen (6) and Deutsch



TEXT-FIG. 1. Growth curves of single S3 cells incubated at 37.5°C. in the medium of Table I (open circles) and in an optimal medium containing 30 per cent serum (solid circles) (4). The generation time in the logarithmic portion is 21 hours in the optimal serum-containing medium and 34 hours in the serum-free medium.

(7), slightly modified by ourselves (8), and albumin was obtained from several sources and by different preparative procedures, as explained in the following text. Growth curves were performed as described elsewhere (4).

RESULTS

Table I lists the composition of the medium which produces growth of single S3 cells, with an efficiency close to 100 per cent, when the cells are dispersed

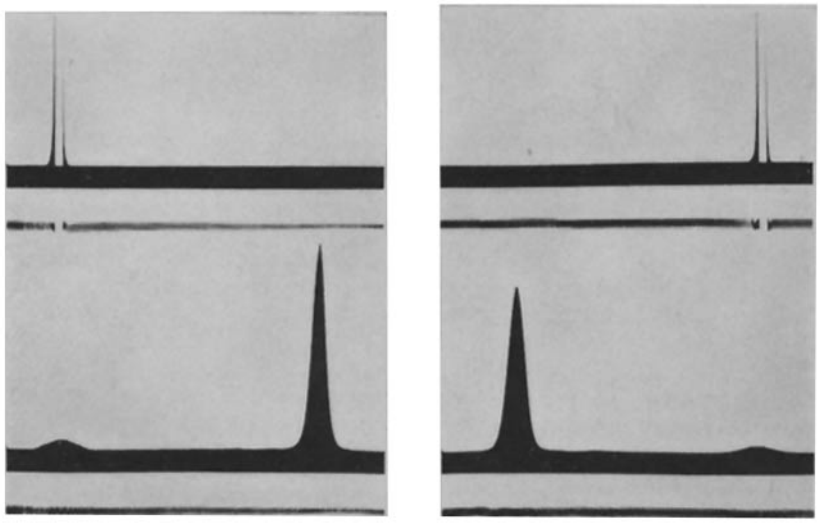
[1] Available from the Colorado Serum Company, Denver.

and plated by the prescribed technique. Fig. 1 *a*, *b* presents a typical Petri dish which was inoculated with 200 cells, in the medium of Table I, and incubated for 15 days with no change of medium. Text-Fig. 1 shows a typical growth curve in this medium, compared with that in a medium supplemented

TABLE II

Demonstration of the requirement for both fetuin and albumin for colony formation. Single cells were plated in Petri dishes in media containing the basal micromolecular medium of Table I, supplemented or not with the purified protein fractions as shown (*A*). In addition, in order to achieve equilibration with any micromolecular components of serum which might render fetuin and/or albumin unnecessary each of the media was previously dialyzed for 72 hours against 100 volumes of an optimal medium containing 30 per cent whole serum (*B*). The Petri dishes were then incubated for 14 days.

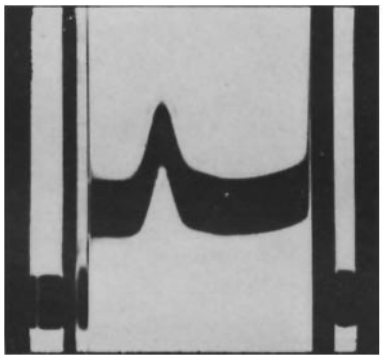
Supplement to basal micromolecular medium of Table I	Plating efficiency	
	(*A*) Without dialysis	(*B*) With dialysis
	per cent	*per cent*
None	0	0
Albumin	0	0
Fetuin	0	0
Albumin plus fetuin	77.5	94
Whole serum	108	—



TEXT-FIG. 2 A. Descending (right) and ascending (left) boundary patterns obtained in electrophoresis of a 1 per cent solution of a serum albumin preparation (Cutter) used in this study. This experiment was performed in barbital buffer of 0.1 ionic strength at pH 8.6. The mobility of the major component is -6.0×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, and of the trace (less than 1 per cent) component -4.2×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$.

with whole serum. The data demonstrate that the growth rate of the single cells in the medium of Table I is somewhat lower but comparable to that obtained in whole serum.

In a test of the long term growth of these cells in the serum-free medium, a colony was picked from a plate like that in Fig. 1, and cultivated continuously in the medium of Table I. Sustained growth has been maintained for more than 6 months and 120 generations, which have included two separate single cell isolations, demonstrating that the medium described is adequate for indefinite cell multiplication. Cells previously grown in the medium defined in Table I produce colonies when plated in this same solution with the same efficiency as



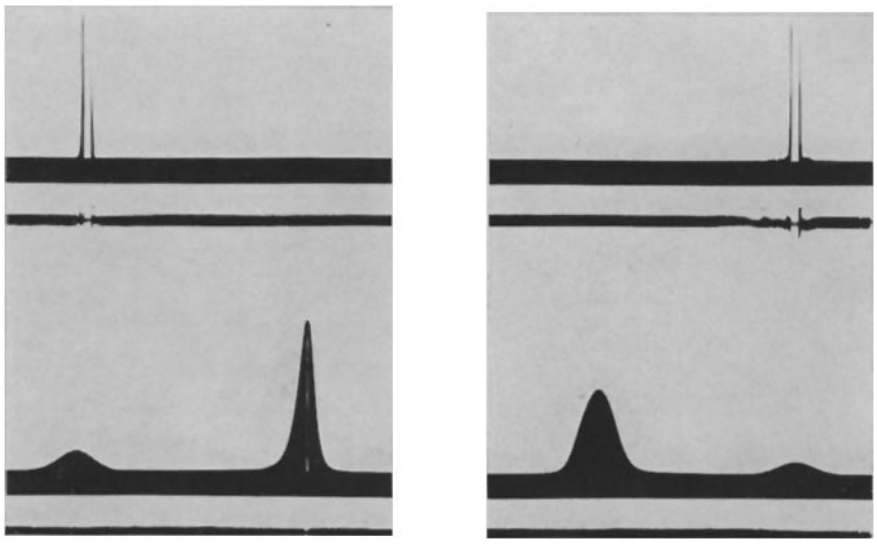
TEXT-FIG. 2 B. An ultracentrifugal boundary pattern of the active serum albumin preparation used in this study. The photograph is of a 1.2 per cent solution of the protein in a phosphate buffer of ionic strength 0.1 and pH 7.0 after 64 minutes at 59,780 R.P.M. (250,000 g).

do cells cultured in the serum-containing nutrient solution. It can be concluded that the medium of Table I is sufficient for long term growth; that no special adaptation to this medium is required; and that growth occurs from all or most of the cells of the original stock, rather than from rare mutants.

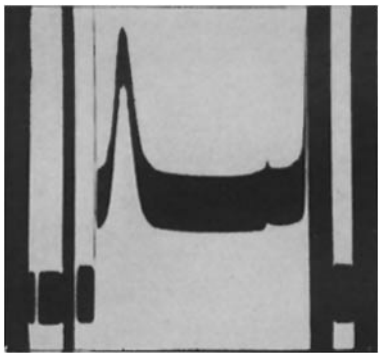
If either of the macromolecular components of this medium is omitted, no growth of cells under the specified conditions is observed even after 14 days of incubation (Table II). In preliminary experiments neither of the functions of these two proteins could be filled by substitution of dextran, gelatin, γ-globulin, or agar.

Albumin prepared by Cohn fractionation and sterilized by filtration and heating at 60°C. for 10 hours is satisfactory for growth of single cells and can be obtained commercially. Most of the experiments here described were carried out with such normal human serum albumin, available as a routine commercial preparation, supplied by Cutter Laboratories, Berkeley.[2] The Cutter albumins

[2] Grateful thanks are expressed to the Cutter Laboratories for gifts of a number of albumin preparations. A similar, commercially available preparation of Merck, Sharp and Dohme, Inc., Rahway, New Jersey, is also satisfactory. However, crystalline bovine albumin, as



TEXT-FIG. 3 A. Descending (right) and ascending (left) boundary patterns obtained in electrophoresis of a 1 per cent solution of the fetuin preparation used in this study. This experiment was performed in barbital buffer of 0.1 ionic strength at pH 8.6. Identical patterns were obtained with field strengths between 5 and 11 volts /cm.



TEXT-FIG. 3 B. An ultracentrifugal boundary pattern of the fetuin preparation used in this study. The photograph is of a 1.0 per cent solution of the protein in a phosphate buffer of ionic strength 0.1 and pH 7.0 after 40 minutes at 59,780 R.P.M. At 150 minutes the pattern consists of 1 symmetrical peak.

employed are at least 97 per cent homogeneous electrophoretically. Albumins prepared in our laboratories by isoelectric precipitation were equally active.

supplied by Pentex, Inc., Kankakee, Illinois, or Armour, Chicago, was found to be toxic in its original form. It acquired some growth competence after dialysis against saline, but the plating efficiency achieved with this material remained less than that of the other albumin preparations described.

In Text-fig. 2, electrophoretic and ultracentrifugal patterns are presented of an albumin preparation used in these experiments, which are shown to be approximately 99 per cent homogeneous with respect to both of these properties. If the concentration of albumin in the medium is reduced below 1 mg./cc. or increased above 5 mg./cc., both the growth rate and plating efficiency of the cells fall significantly.

The macromolecular component which satisfies the other growth need of single S3 cells was first identified as an α_1-globulin found in mammalian serum

TABLE III

Comparison of some further properties of these fetuin preparations with those previously described by other investigators. A correspondence in electrophoretic mobility, sedimentation constant, and trichloroacetic acid solubility was demonstrated earlier (8).

Property	These preparations	Fetuin of Pedersen (6) or Deutsch (7)
$(NH_4)_2SO_4$ concentrations in which precipitation occurs	38–45 per cent saturation	38–45 per cent saturation (6)
Molecular weight	44,600 ± 2000* (by light scattering method)	45,000 (7) (by sedimentation and diffusion)
Nitrogen	15 ± 2 per cent	13.39 per cent (7)
Hexose	7 ± 1 per cent	9.5 (7)
Hexosamine	5.5 ± 1 per cent	8 (7)
Sialic acid	4.1 ± 1.3 per cent‡	
Antitryptic activity	Positive	Positive (7)
Heat coagulation	Not coagulated	Not coagulated (7)
Minimum concentration needed for glass attachment and stretching of S3 cells (8)	1.0 μg./ml.	—

* Determined on this preparation by Dr. C. M. Kay (9).

‡ Determined by the method of Winzler (17) using a human serum pool as a standard. The relatively large uncertainty in our value is due to inconstancy of this standard.

(1, 16*a*). All of the growth functions of this material were found to be supplied by preparations of fetuin, the name given by Pedersen (6) to an α_1-globulin fraction with glycoprotein characteristics which occurs in high concentration in calf fetal serum. The electrophoretic and ultracentrifugal behavior of the fetuin preparations used in this study are shown in Text-fig. 3, and demonstrate the material to be more than 97 per cent homogeneous with respect to these two properties. All the fetuin preparations we have employed exhibited the same specific activity with respect to cell stretching and growth.

The correspondence between our preparation and the fetuin system with respect to electrophoretic mobility, sedimentation constant, and solubility in 5 per cent trichloroacetic acid have been demonstrated previously (8). Other properties of the preparation used in these experiments are listed in Table III.

The list of micromolecular components of Table I was developed by tests of combinations of media and specific compounds first proposed by other laboratories studying animal cell nutrition (10). Except for a few changes, this particular formulation has been employed as routine in this laboratory for several years as a basal medium for growth of various mammalian cells (4, 5). Not all of the molecules listed may be essential to the growth of single S3 cells. Thus, some like hypoxanthine, appear to be expendable in short term experiments, although long term tests necessary for certainity of such a conclusion are not yet completed. Substances like vitamin B-12 operate to increase the growth rate or render the plating efficiency somewhat more reliable, in contrast to nutrilites like inositol and glutamine without which no growth whatever occurs, as has previously been demonstrated by Eagle (10 *d*) in massive cultures.

The medium listed in Table I will not promote growth of single cells of the S1 HeLa clone which has an aneuploid constitution similar to that of the S3 cell (4, 11); nor of euploid human cells, nor of cells of the Chinese hamster (12).

DISCUSSION

In earlier phases of this study it was shown that single S3 cells could form colonies with maximal plating efficiency in a medium consisting of a synthetic micromolecular portion plus dialyzed serum. On continued dialysis, the serum protein lost its ability to promote cell growth, but recovered such competence if additional small molecular supplements, including ascorbic acid and cholesterol, were added (1). While this result suggested that bound micromolecules released from the serum proteins require replacement in the medium, the present experiments favor an alternative or additional interpretation: as a result of prolonged dialysis or other treatment, some serum proteins develop growth toxicity which can be offset by provision of specific small molecules. This may explain the ability of purified fetuin and albumin to support growth in a medium which is simpler than that required when highly dialyzed whole serum protein is employed. In experiments now in progress, cell toxic protein fractions have been recovered from serum dialysates.

The present work does not establish the exact molecular identities of the proteins required for growth of the single S3 cell under the specific conditions employed by us. The difficulty of precise identification of the specific molecular form or forms of a protein preparation which is homogeneous with respect to some criteria, is readily demonstrable in the case of serum albumin. This protein, which is one of the best characterized, has been crystallized and shown to migrate as a single boundary in an electric field over a wide pH range, and to sediment largely as a monomer with only a few per cent of a dimer. Nevertheless, advanced chromatographic, immunologic, and fractional precipitation procedures have shown it to contain a variety of different molecular species. At least six different components have already been resolved from albumin (13). Whether one, a few, or all of these components are uniquely or alter-

natively requisite for the albumin contribution to S3 growth here described remains to be determined. Nor is anything known of the nature of the albumin function—whether it be to serve as an essential nutrilite itself; to provide a dissociable component, or a frank impurity, undetectable by the methods so far employed, which constitutes the actual active substance; to assist in transport of some other molecule; to regulate metabolic balance; or to detoxify media (14) containing injurious levels of certain constituents.

The situation with the fetuin system is even more obscure than that of albumin because of the smaller amount of study which it has received. Fetuin is the name which has been given to the α_1-globulin of fetal calf serum. Like albumin, fetuin has been shown to be physically heterogeneous (7), despite the fact that it shows a single electrophoretic boundary at pH 8.6 in 0.1 ionic strength barbital buffer (see Text-fig. 3). Furthermore, it has also been shown to be heterogeneous by immunologic procedures (15). Hence, all of the unanswered questions listed in the foregoing paragraph with respect to albumin apply with even greater pertinence here. Moreover, as we earlier indicated, it is still unknown whether the activity in fetuin is molecularly identical with the α-globulin of adult serum which was shown by Lieberman and Ove (16 *a*) and independently by us (1, 8), to possess growth-promoting activity for mammalian cells. Fractionation studies begun earlier to resolve such questions are still in progress in this laboratory. In the meantime, Lieberman and Ove (16 *b*), while confirming our findings that the fetuin fraction contains the growth-promoting activity, have presented evidence suggesting that such preparations may be separated on a diethylaminoethylcellulose column into components with different physical properties and different biological activities, though these two sets of properties were not correlated. Our own experiments on the fractionation of fetuin will be described later.

Experiments on the kinetics of interaction of cells and glass surfaces are also in progress and these have demonstrated that the stretching and growth-promoting properties of fetuin can be strongly adsorbed on glass and remain fully active, but will not so adsorb to the cells themselves. Preliminary experiments indicate both fetuin and albumin to be needed in the macromolecular form. Attempts by means of microbiological assay to demonstrate presence of small molecular nutrilites bound to these molecules have been negative in the case of folic acid, thiamine, riboflavine, and niacin, although small amounts of biotin and vitamin B-12 activity have been demonstrated in the albumin, and B-12 alone in the fetuin preparations.

Indefinite growth of some cell strains in the absence of added protein has been described (10 (*b*)). This may reflect differences in different cell metabolic patterns, or may be yet another example of how massive cell populations may be more self-sufficient nutritionally than single cells. It is possible that when their functions are clarified, the proteins here found necessary for growth of single cells might conceivably become expendable.

While large areas of ignorance remain, the present experiments now make possible reliable and quantitative single cell plating in a medium more precisely defined than those utilizing either whole serum or its totality of macromolecular components. All constituents of the medium in Table I are available commercially, except fetuin, and it can be prepared readily and reproducibly in substantial quantities (8). The behavior of cells in such media has been much more reliable over a period of more than a year, than in mixtures relying on dialyzed serum protein. The medium here defined permits more careful control of experiments designed to search for individual cells with differing genetic constitution or adaptive state; and to quantitate the effects of physical, chemical, and biological agents on the growth of single mammalian cells.

SUMMARY

Two purified serum protein fractions, fetuin and serum albumin, will replace whole or dialyzed serum in supporting the growth of single S3 HeLa cells in an otherwise chemically defined nutrient solution.

In the serum-free medium, single S3 cells will form macroscopic colonies with essentially 100 per cent efficiency.

The generation time of S3 cells in the serum-free medium is approximately 50 per cent greater than that observed in an optimal, serum-containing medium.

All components of the serum-free medium are available commercially, except fetuin, which can easily be prepared in substantial quantities.

The problem of the purity of the protein preparations and of their possible roles in promoting cell growth is discussed.

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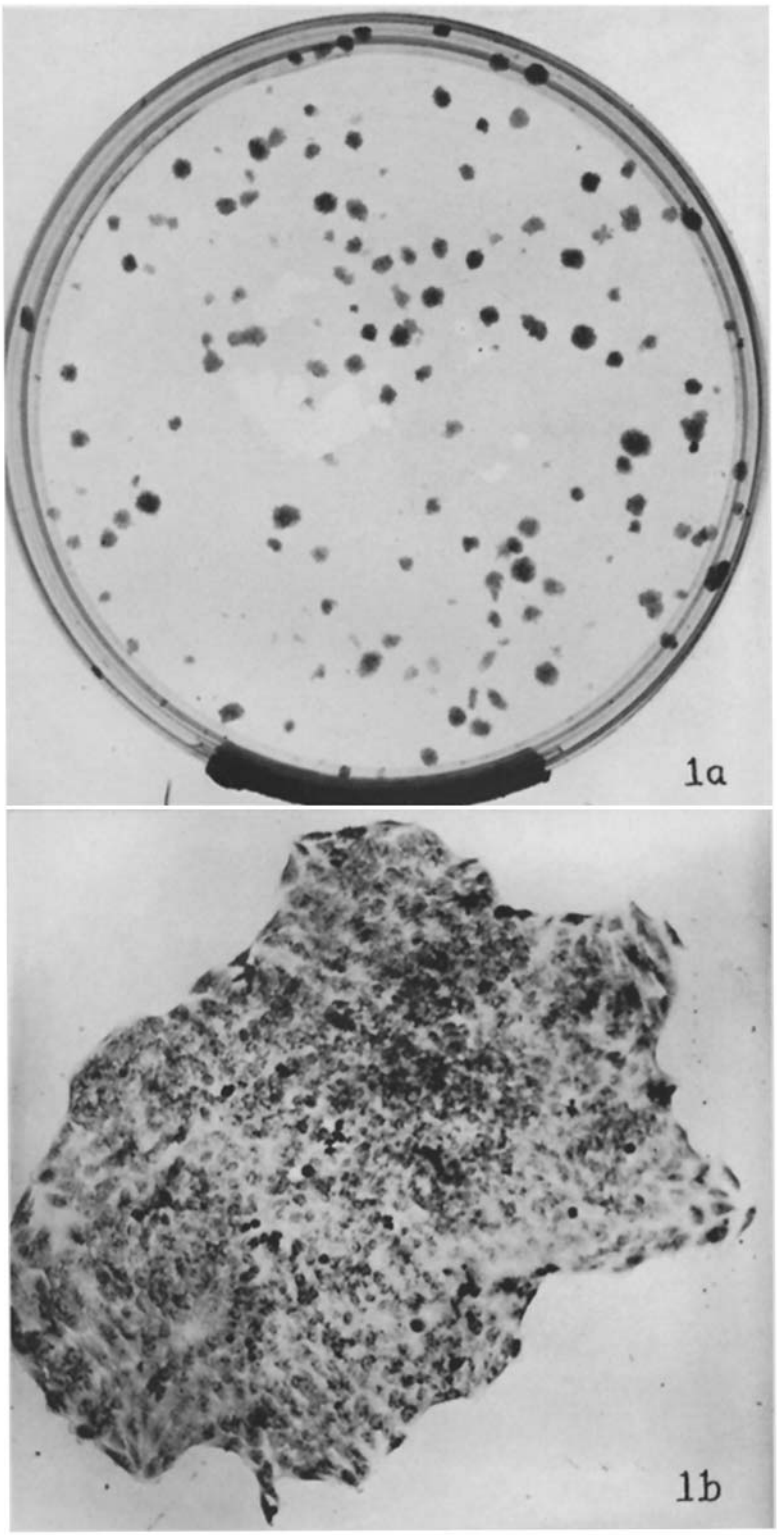
EXPLANATION OF PLATE 73

FIG. 1 *a*. A photograph of a Petri dish seeded with 200 cells and incubated for 14.7 days in the medium of Table I. 155 colonies developed, corresponding to a plating efficiency of 77.5 per cent. × 2.

FIG. 1 *b*. Enlargement of a typical colony on the Petri dish shown in Fig. 1 *a*. × 106. These colonies characteristically exhibit such a closely packed configuration when grown in the medium of Table I.



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