ence of such a reaction is, of course, most improbable. Interaction of the product, glutamate, at low concentration with the enzyme used had been shown by calorimetry to be absent, and full activity of the enzyme in such tests as Figure 1, *b*, was ascertained by addition of glutamine to the sample in a second calorimetric test after the equilibrium run.

In summary, it has been shown experimentally that the calorimetric determination of free-energy, heat, and entropy changes as outlined in these PROCEEDINGS[5] may be applied to a seemingly irreversible reaction. The standard free-energy change of glutamine hydrolysis observed is $\Delta F^\circ_{310} = -3{,}430$ cal/mole. The sum of this quantity and whatever value is accepted for the free energy of the glutamine synthetase reaction is the free energy of ATP hydrolysis. For one example, with the value of $-4{,}300$ cal/mole at pH 7.0 and 37° C.,[2] the free energy of ATP hydrolysis is $\Delta F' = -7{,}730$ cal/mole, the heat change $\Delta H = -4{,}800$ cal,[10] and the standard entropy change $\Delta S' = +9.45$ e.u.

It is our pleasant obligation to thank Professor H. A. Krebs, in whose department these experiments were carried out with the microcalorimeter of the Bio-Energetics Division, Naval Medical Research Institute, for continuous encouragement and advice. Thanks are also due to Drs. T. Spencer and K. Bascombe, of the Department of Physical Chemistry, Oxford, for their calculation of activity coefficients used in this paper and to Dr. K. Burton for valuable advice and criticism.

[1] By "practically irreversible" we mean that the reverse reaction, though it of course exists cannot be practically shown by any ordinary method.

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*ON THE FUNCTIONS OF X-IRRADIATED "FEEDER" CELLS IN SUPPORTING GROWTH OF SINGLE MAMMALIAN CELLS**

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In earlier publications we have described a method whereby suspensions of single mammalian cells can be plated in Petri dishes containing nutrient medium, so that each cell multiplies in isolation to form a macroscopic colony.[1, 2] While some cell

lines were found to give rapidly multiplying colonies with almost 100 per cent plating efficiency on plates containing only the standard nutrient solution, others grew poorly or not at all in the media available unless the plate was also inoculated with a "feeder" cell layer, consisting of about 10^5 cells previously exposed to a completely lethal dose of X-irradiation.[2, 3] In the presence of such a "feeder" layer of nonmultiplying cells, the test cells exhibited maximal plating efficiencies and growth rates. Two different mutants (S1 and S3) of the HeLa cell, of human carcinomatous origin, have been isolated and quantitatively characterized on the basis of the need of one of these (S1), but not the other (S3), for assistance by a feeder layer order for growth to occur in a specific medium.[3]

The experiments here to be described were undertaken to clarify the mechanisms by which a feeder layer of nonmultiplying cells can operate to support growth of single cells in an otherwise incompetent medium.

METHODS AND MATERIALS

Specific details of the preparation of monodisperse cell suspensions, their plating in Petri dishes and incubation in an atmosphere of 5 per cent CO_2, have been previously recounted.[2, 4] All plates, unless otherwise indicated, received 4.5 cc. of the Complete Growth Medium, as previously described (Table I of another paper[4]), except that the horse serum was omitted and the amount of human serum was often varied between the values of 8 and 20 per cent, without significant change in the results. Other specific changes in the medium, like the production of inositol deficiency or the addition of specific antisera, are described in connection with the appropriate experiments. No agar was employed in any of the present platings. The clonal HeLa strain, S3, which had been isolated from the parental HeLa cell population[3] was used for these experiments.

Where feeder cells were utilized, these were prepared by exposure to an X-ray dose of 2,000 r.[2] To each feeder plate, 10^5 of these cells, washed to eliminate carry-over of nutrients, and the new test medium were added before addition of the inoculum of test cells. The ability of this X-ray dose completely to prevent colony formation by the feeder cells themselves has been thoroughly demonstrated.[2, 5]

The cells were incubated for 9–12 days. Plates containing feeder cells sometimes were given a change of medium after 6 or 7 days of incubation if the pH fell sufficiently to indicate depletion of nutrients.

EXPERIMENTAL RESULTS

Two obvious mechanisms by which a feeder layer might operate to promote the growth of single test cells are (1) by direct supply of essential molecules which are absent from the medium and (2) by removal of inhibitory substances which might be present. Experiments were designed with model systems to determine whether a feeder layer can indeed act in either or both of these capacities.

1. *Test of the Ability of Feeder Cells To Relieve a Nutritional Deficiency of the Medium.*—Test of this first mode of action of a feeder cell layer was carried out by plating cells of the S3 HeLa strain with and without a feeder layer in a medium deficient in a known metabolite. The nutrilite chosen for this experiment was inositol, which had been demonstrated by Eagle to be required for the growth of several mammalian cells.[6]

Following Eagle,[6] dialyzed serum was substituted for whole serum in the nutrient solution, in order to eliminate inositol. Eagle had reported[6] that while other mammalian cells under these conditions showed no growth unless inositol was added to

such a medium, the behavior of his HeLa culture was variable. With the single-cell plating technique here employed, the results are always reproducible: No growth whatever of single cells is obtained with an inositol-free medium; addition of inositol (0.4 μg/cc) to such a medium restores the plating efficiency (i.e., the per cent of the single cells plated which grow into macroscopic colonies) close to the theoretical value.

Experiments were then carried out to determine whether addition of a feeder layer of washed, irradiated cells would permit growth of single S3 cells even in the absence of inositol. The results unequivocally demonstrated that the presence of the feeder layer removes the requirement for an external supply of inositol in the growth of single S3 cells. A typical experiment is illustrated in Table 1. In Figure 1 are shown photographs of plates from such an experiment. The conclusion may be drawn that one of the ways in which a feeder layer can function is by relieving a nutritional deficiency of the medium for the growth of single cells.

TABLE 1

REPRESENTATIVE EXPERIMENT DEMONSTRATING THAT A FEEDER LAYER OF X-IRRADIATED AND WASHED S3 CELLS WILL OVERCOME AN INOSITOL DEFICIENCY OF THE NUTRIENT MEDIUM AND SO SUPPORT COLONIAL MULTIPLICATION OF SINGLE, UNIRRADIATED S3 CELLS *

	PLATING EFFICIENCY (PER CENT)	
	No Inositol Added	0.4 μg/cc Inositol Added
"Feeders" absent	0	61
"Feeders" present	80	79

*100 washed S3 cells were plated in triplicate in the inositol-free growth medium (standard nutrient solution of Table I of another paper [Marcus, Cieciura, and Puck, *J. Exptl. Med.*, **104**, 615, 1956], 40 per cent; dialyzed human serum, 8 per cent; and Hanks saline, 52 per cent), with and without the addition of inositol and of washed feeder cells, respectively, as indicated. The averaged number of colonies per plate for each set of conditions is equal to the plating efficiency, as given.

2. *Test of the Ability of Feeder Cells To Neutralize a Toxic Medium.*—It has been not infrequently reported that mammalian cell strains are intoxicated by serum obtained from certain human subjects and not from others.[7] It has been presumed that the basis for such specific inhibitory action lies in the presence of antibodies or similar substances in certain sera, capable of preventing growth of some kinds of cells. Hence an experiment was designed to test whether a feeder layer could protect an inoculum of viable cells against the action of toxic antibodies.

Antibodies to the S3 cell were prepared by intramuscular inoculation of rabbits at weekly intervals with 10^7 washed HeLa cells suspended in 1.0 cc. of Hanks saline. At various time intervals, beginning one week after the third such injection, blood was drawn and serum prepared. Such sera were collected and frozen, and constituted a standard antibody preparation used for all the experiments here presented.

The toxic action of such antibodies for HeLa cells has been described by other investigators.[8] The use of the single-cell plating procedure makes possible precise titration of this toxic antibody action, as will be described elsewhere. An experiment was performed to determine whether the presence of a feeder-cell layer could enable single S3 cells to multiply in the presence of an otherwise inhibitory concentration of antibodies. An inoculum of 100 S3 cells was added (*a*) to a set of plates containing nutrient medium plus a completely toxic concentration of S3 antiserum and (*b*) to an identical set of plates containing, in addition, 10^5 X-irradiated S3 cells as a feeder system. The data of a representative experiment are presented

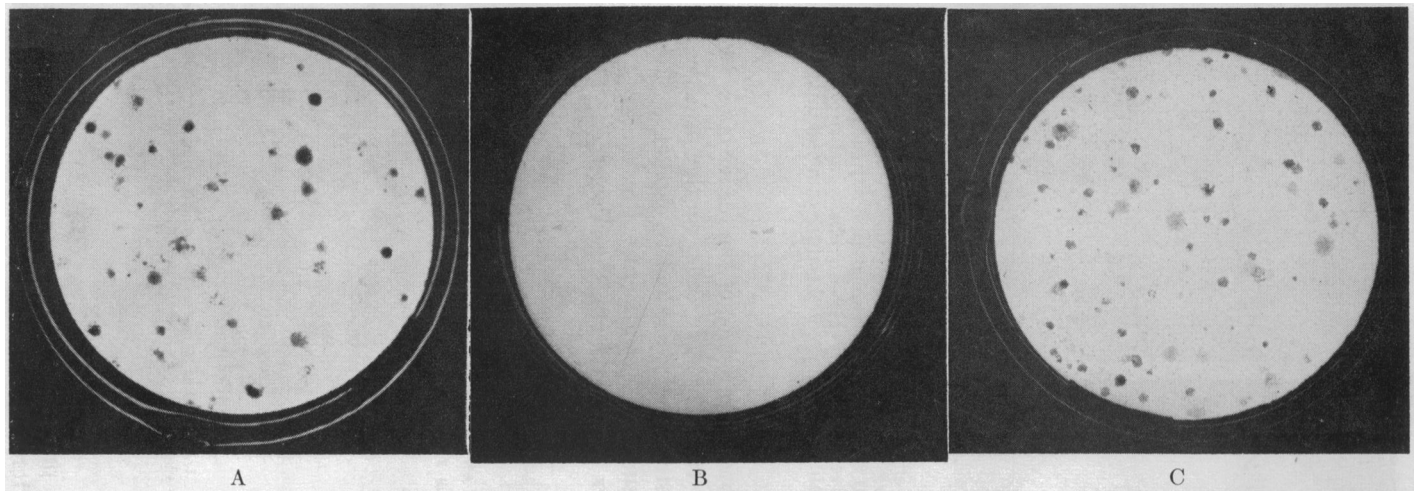

FIG. 1.—Typical plates demonstrating that in the presence of an irradiated feeder-cell layer single S3 cells can form colonies in a medium lacking inositol. (Actual size.) *A*, inositol added, no feeders; *B*, no inositol, no feeders; *C*, no inositol, but feeders present. All three plates received an identical inoculum of 100 washed S3 cells in inositol-deficient medium consisting of Standard Nutrient Solution, 40 per cent, dialyzed human serum, 10 per cent, and Hanks solution, 50 per cent. Plate *A* in addition received 0.4 μg./cc of inositol. Plate *B* received no further addition. Plate *C* received 10^5 washed S3 cells which had been irradiated with 2,000 r, to serve as a feeder system. The absence of any colonies on Plate *B* contrasts with the almost theoretical yields obtained on Plates *A* and *C*. The background of gray specks on Plate *C* represents feeder cells which have developed into giants (see Puck and Marcus, *J. Exptl. Med.*, **103**, 653, 1956).

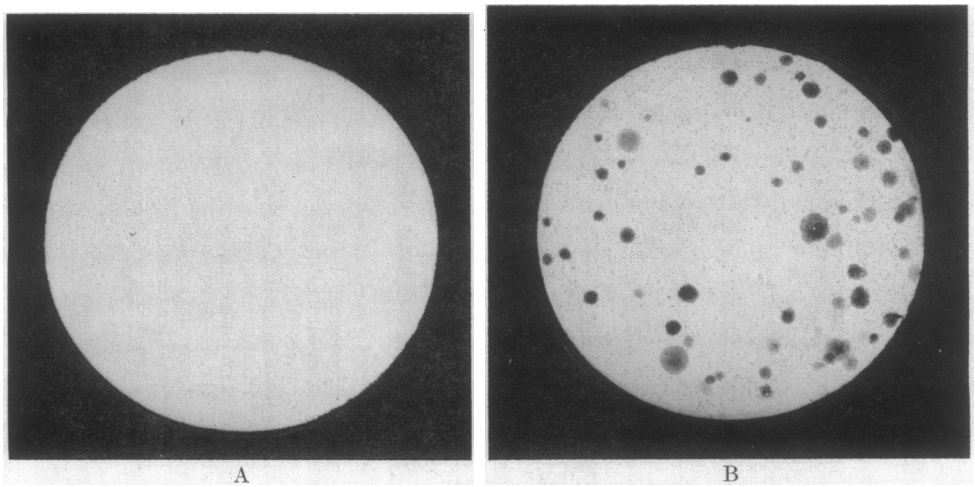

FIG. 2.—Plates demonstrating the effect of a feeder system in permitting cell growth in a medium containing an otherwise toxic amount of antiserum. Both plates received 100 S3 cells in 4.5 cc. of Complete Growth Medium plus 0.8 per cent of S3 antiserum. In Plate *A* the antiserum has completely destroyed the reproductive capacity of the test cells. In Plate *B* the presence of 10^5 irradiated S3 cells has largely neutralized the effect of the antiserum and produced excellent colonial growth.

in Table 2 and demonstrate how the presence of the feeder layer permits maximal plating efficiency and growth rate of single cells, in the presence of antiserum which otherwise completely destroys cell reproductive capacity. Figure 2 presents photographs of plates from such an experiment. Presumably, of course, this detoxifying action of the feeder layer is effected by adsorption of the antibody on the feeder cells.

TABLE 2

DEMONSTRATION OF THE ABILITY OF AN IRRADIATED LAYER OF S3 FEEDER CELLS TO PROTECT AN INOCULUM OF LIVE, SINGLE CELLS FROM AN OTHERWISE TOXIC CONCENTRATION OF S3 ANTISERUM *

Concentration of Added Antiserum (Per Cent)	Feeder Cells (10^5 S3 Cells Irradiated with 2,000 r)	Plating Efficiency (Per Cent)
0	Absent	85
0	Present	80
0.4	Absent	0
0.4	Present	72
0.8	Absent	0
0.8	Present	54

* All plates received Complete Growth Medium plus 10 per cent human serum. In addition, antiserum and feeder cells were added as indicated. Each plate received a live-cell inoculum of 100 S3 cells, which constituted the last addition before incubation.

DISCUSSION

Any of three factors might prevent multiplication of single mammalian cells plated in vitro: (*a*) trauma suffered by the cells in the course of the initial separation from the whole animal or in the dispersal of a cell mass into its individual constituents, (*b*) lack of some essential metabolites, or (*c*) the presence of inhibitory agents in the medium provided. We have elsewhere[2] demonstrated the need for

gentleness in the disaggregation of cells and have shown that quantitative and reproducible growth of each cell plated can be achieved only if conditions are not so drastic as to cause extensive leakage of cell substance into the extracellular fluid. A chemically defined, synthetic medium which will promote unlimited mammalian cell multiplication does not yet exist except for the case of one particular cell.[9] Thus it is necessary to rely on natural sources like serum or embryo extract to supply the as yet unidentified molecular requirements in cultivating most animal cells. However, these complex mixtures usually contain inhibitory substances as well as essential metabolites, and undoubtedly it is the failure to obtain a suitable balance between these which has made it difficult in the past to achieve single-cell growth in high efficiency from a variety of different tissues. We have previously shown that a feeder layer can make such single-cell growth feasible with the same simplicity and high efficiency which is characteristic of microbial systems.[3] The present experiments offer a basis for understanding how such a feeder layer can operate and, indeed, why, as is often the case, single mammalian cells fail to grow under conditions where massive inocula reproduce readily and continuously.[10] The use of a feeder system may make it possible automatically to correct nutritional deficiencies or toxic actions of the biological fluids needed to supplement media in current use. Theoretically, at least, the use of such a feeder system should permit successful single-cell plating of any strain which can at present be grown in massive inocula.

While the use of a feeder system will never replace the need for identification of the complete chemical requirements for growth of each cell strain, it furnishes a powerful tool permitting many kinds of investigation which otherwise could not be carried out at the present time. By making possible single-cell plating, it permits recognition and isolation of mutant markers which can be used as a basis for studies of mammalian cell genetics;[3] more quantitative means of study of cell growth and the effect thereon of various agents (e.g., X-rays)[5] than has before been possible;[11] and more rapid and convenient preparation of clonal cell stocks.

When a single cell has multiplied sufficiently, the resulting colony obviously can perform for itself the functions required initially by the feeder cells. Consequently, the need for the feeders should decrease as growth proceeds.

In this connection, the advantage of using single-cell platings rather than massive inocula for determination of absolute nutritional requirements of mammalian cells becomes obvious. The ability of cells to "feed" each other may mask the presence of certain nutritional requirements. If a mixture of different kinds of nutritional mutants is present in the stock, growth may occur on the addition of only those molecules which are synthesized by none of the cells present. Hence the apparent nutritional requirements may not be complete for any single cell type present in the mixture and may vary significantly for mixtures with different compositions, which may be present in the stocks of different laboratories. Our recent demonstration of the existence of different nutritional mutants in the parental HeLa cell population illustrates the nutritional heterogeneity of such common cell strains.[3]

We have used the feeder technique with human cells originating from a wide variety of noncancerous tissues, including conjunctiva, appendix, liver, kidney, bone marrow, spleen, and skin, as well as several cancerous tissues.[4, 12] In no case have we failed to obtain single-cell multiplication with high plating efficiencies. In many cases it has become possible eventually to dispense with the feeder system,

either because a better medium was ultimately devised or possibly because, in some cases, the cells eventually became sufficiently adapted to the given conditions in vitro to the point where the feeder system was no longer required. It is possible that the feeder system may also be an effective tool for study of cell-cell interaction for specialized cell functions other than reproduction.

Experiments are in progress to determine how wide a variety of human tissues can be successfully plated as single cells, with and without feeder systems, and what kinds of specificities may be involved in the ability of cells of one kind to act as feeders for another in a given medium.

SUMMARY

1. Model studies designed to elucate how a feeder layer of X-irradiated, non-reproducing cells can promote colonial growth of single mammalian cells in an otherwise incompetent medium reveal two ways in which feeder cells can function: They can relieve a specific nutritional deficiency of the medium, and they can neutralize the toxic action of specific cell antibodies.

2. The use of feeder systems as tools for the study of various aspects of mammalian cell behavior is discussed.

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*THE PRODUCTION OF TUMOROUS ABNORMALITIES IN FERN PROTHALLI BY IONIZING RADIATIONS**

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The appearance and behavior of tumorous growths which occur occasionally in fern prothallial cultures have been discussed both morphologically[1] and cytologically[2] in previous papers. These tumors were considered "spontaneous," in that they appeared relatively rarely and their occurrence could be ascribed to no