[18] According to studies with purified cytochrome b-c preparations, by Stotz, E. H., M. Morrison, and G. Marinetti, in *Enzymes: Units of Biological Structure and Function*, ed. O. H. Gaebler (New York: Academic Press, Inc., 1956), pp. 409–413, the E'_0, at pH 7.4 of $-0.04v$ calculated by Ball[16] for cytochrome *b* would appear to be too low by $0.1v$.

[19] Weissman, S. I., *J. Chem. Phys.*, **29**, 1189 (1958).

[20] Singer, T. P., E. B. Kearney, and V. Massey, in *Enzymes: Units of Biological Structure and Function*, ed. O. H. Gaebler (New York: Academic Press, Inc., 1956), pp. 417–432.

[21] Michaelis, L., *J. Biol. Chem.*, **96**, 703 (1932).

[22] Commoner, B., B. B. Lippincott, and J. V. Passonneau, these PROCEEDINGS, **44**, 1099 (1958).

[23] Lippincott, B. B., Ph.D. thesis, Washington University (1959).

[24] Commoner, B., J. J. Heise, B. B. Lippincott, R. E. Norberg, J. V. Passonneau, and J. Townsend, *Science*, **126**, 57 (1957).

[25] Ehrenberg, A., and G. D. Ludwig, *Science*, **127**, 1177 (1958).

THE SUSTAINED GROWTH OF HUMAN AND ANIMAL CELLS IN A PROTEIN-FREE ENVIRONMENT

By HARRY EAGLE

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

Communicated by Herman M. Kalckar, February 8, 1960

Although a few strains of mammalian cells have been grown in a protein-free medium,[1–7] most of the cultures now available require protein, usually added as whole or dialyzed serum. The role of that protein is not clear. It has been found to promote the adhesion of cells to a glass surface, and their subsequent flattening;[8, 9] but this is not its only or perhaps even primary function, for it is also required by cells growing in suspension. Since the protein amino acids are utilized to only a minor degree for the synthesis of cell protein,[10] it seemed possible that it might be providing one or more essential compounds of small molecular weight, either initially bound to the protein, or formed by its degradation. This was borne out by the present experiments, which show that protein as such is not required for the growth of the human or animal cells so far studied. Its primary function is to provide as yet unidentified compound(s), sufficiently small to pass through a cellophane membrane, and the provision of which is promoted by the addition of a pancreatic extract.

Methods.—The dual culture apparatus used in most of these experiments was a modification of that used by Nurmikko[11] and by McLimans *et al.*,[12] and consisted of two cylindrical culture vessels, each with a horizontal side tubulation terminating in a ground glass flange. Sterile silicone grease was spread on each flange, a cellophane membrane made from $^{24}/_{32}''$ Visking clear cellophane tubing was interposed, and the two vessels were then joined. The cell culture in each cylinder was kept dispersed by a suspended and free-spinning magnetic bar. The courtesy of Dr. William F. McLimans in providing a prototype model in advance of publication is greatly appreciated. The cell lines used were human strains KB and HeLa, a cloned subculture of HeLa (S3), and a mouse fibroblast, strain L 929. The suspension cultures used as inoculum were grown in a minimal basal medium containing the 28 demonstrably essential growth factors[13] supplemented with 5 per cent dialyzed human or horse serum.

In setting up the experiments, a culture in the logarithmic phase of growth was centrifuged, and the cells re-suspended in 100 ml of the protein-free basal medium to a count of 200,000 cells/ml. This was then placed in one arm of the culture vessel. The other arm contained 100 ml of uninoculated medium supplemented with 1–5 per cent dialyzed serum or a mixture of dialyzed serum and pancreatic extract. The latter was added as "Viokase," a powdered pancreatic extract prepared by the Viobin Corporation (Monticello, Illinois), used at a final concentration of 0.1–0.25 per cent powder. The insoluble portion was removed from the stock "2.5%" solution in 0.85 per cent NaCl by centrifugation prior to dialysis and sterilization through a sintered glass filter.

The cultures were counted daily, and replaced with enough fresh medium to keep the counts in the range 180,000–250,000 cells/ml. The fluid in the "feeder" compartment was replaced to an equal degree, but with a minimum 20 per cent replacement even when the cell culture required no dilution.

TABLE 1

THE GROWTH OF CELLS IN A PROTEIN-FREE MEDIUM EQUILIBRATING ACROSS A CELLOPHANE MEMBRANE WITH A "FEEDER" COMPARTMENT CONTAINING SERUM PROTEIN

	Composition of "Feeder" Compartment			
Dial. serum (5–20%)	+	+	+	+
"Non-essential" amino acids*	0	+	0	+
Pancreatic extract* (0.25%)	0	0	+	+
Cell line studied ↓	Growth in Protein-free Compartment			
HeLa	0	0	+	+
HeLa-S3	0,0	0,0,0	+	+
929	0,0,0	0,0,0,0,0	+,+	+
KB	0,0,0,0,+	+,+	+	+,+

* Alanine, aspartic, asparagine, glutamic, glycine, proline, and serine, each at 0.1 mM.
† "Viokase," made by Viobin Corporation, Monticello, Illinois. A large part of the powder fails to dissolve, and was removed by centrifugation prior to dialysis and glass filter sterilization.

Results.—1. *The growth of human and animal cells in a protein-free environment, separated by a cellophane membrane from a "feeder" compartment containing serum protein:* When cells were grown in suspension in a basal medium containing only the 28 demonstrably essential and chemically defined factors (amino acids, vitamins, salts, and glucose),[13] separated by a cellophane membrane from the same medium supplemented with serum protein, growth was occasionally obtained with one of the 4 cell lines tested (cf. first 2 columns in Table 1, and middle section of Fig. 1). In the successful cultures, the essential growth factor(s) deriving from the protein, and diffusing through the cellophane membrane, were apparently being provided in growth-limiting concentration. Thus, in one experiment, the generation time decreased progressively from 3.6 to 2.7 to 2.0 days as the concentration of dialyzed serum in the "feeder" compartment was increased from 5 to 10 to 20 per cent. Although the addition of the 7 nutritionally non-essential amino acids (alanine, aspartic acid, asparagine, glutamic acid, glycine, proline, and serine) each at 0.1 mM, often promoted growth, this was neither regular nor reproducible (Table 1). Preliminary equilibration of the cell-free fluids in the 2 compartments for 24 hr at 37° prior to inoculation of the protein-free compartment also had no regular effect.

In the meantime, it had been found[14] that when sterile dialyzed serum was stored

at icebox temperatures, free amino acids gradually appeared, most of which reached concentrations of 0.03–0.08 mM after 6 weeks. The rate of that proteolysis was materially increased at 37°. These results suggested that the growth-promoting effect of serum in the foregoing experiments might have been due to a similar slow breakdown of protein, and might be materially increased by adding appropriate enzymes to the serum-containing "feeder" compartment. The latter proved to be the case.

2. *The growth-promoting effect of pancreatic extract added to the "feeder" serum-containing compartment:* All four cell lines tested could regularly be grown in a protein-free medium, apparently indefinitely, if the culture was equilibrated across a dialysis membrane with the same medium supplemented with as little as 1 per cent dialyzed serum and 0.1–0.25 per cent of a pancreatic extract ("Viokase"). The growth-promoting activity of the enzyme preparation was not affected by its preliminary dialysis. Illustrative experiments with the HeLa-S3, KB, and L-929 strains are summarized in Figure 1. In other experiments, cells have been cultured for periods of up to 48 days, during which time they increased 15 millionfold.

TABLE 2

THE EFFECT OF THE DIALYZED SERUM CONCENTRATION IN THE "FEEDER" COMPARTMENT ON THE GENERATION TIME OF CELLS IN THE PROTEIN-FREE COMPARTMENT

	Pancreatic Extract* in "Feeder" Compartment	"Non-essential" Amino Acids	Concentration of Dialyzed Serum in "Feeder" Compartment: 20	10	5	2	1.5	1	0.8
			Generation Time, Days						
HeLa-S3	+	+	...	...	1.5	1.4	1.6	1.7	...
929	+	0	...	2.1	1.9	2.5	...	...	...
	0	+	...	...	1.9	...	...	...	...
KB	0	0	2.0	2.7	3.6	...	...	...	...
	+	+	2.6	...	4.2	...	...	...	...
	+	0	...	...	1.9	...	2.9	3.7	∞
	+	+	...	...	2.3	1.8	1.3	1.7	∞

The same minimal and chemically defined growth medium,[13] containing the 28 essential growth factors, was present in both compartments.
* Dialyzed Viokase at 0.25%.

When the enzyme preparation was removed from the "feeder" compartment, the growth rate slowed within a few days and the cells usually died.

The generation time of cells growing in the protein-free compartment, expressed as a function of the serum concentration in the "feeder" compartment, is shown in Table 2. As there shown, the provision of 1 per cent dialyzed serum (approximately 0.07 per cent protein) in the "feeder" compartment, supplemented with 0.1 per cent pancreatic extract, sufficed for sustained and rapid growth. Lower concentrations of serum were ineffective.

Discussion.—It was clear from the rapid proliferation of the cells in the protein-free compartment that this growth was not due to the selective multiplication of a small fraction of the population, but involved most of the original inoculum. Further, when cells which had been growing in the protein-free environment for 4 weeks were then placed in ordinary monolayer or suspension cultures, they died in the absence of added serum protein, and grew only at the normally effective concentrations.

As indicated by the growth-promoting and usually essential role of the pancreatic enzymes, the essential growth factor(s) which were diffusing into the cell culture

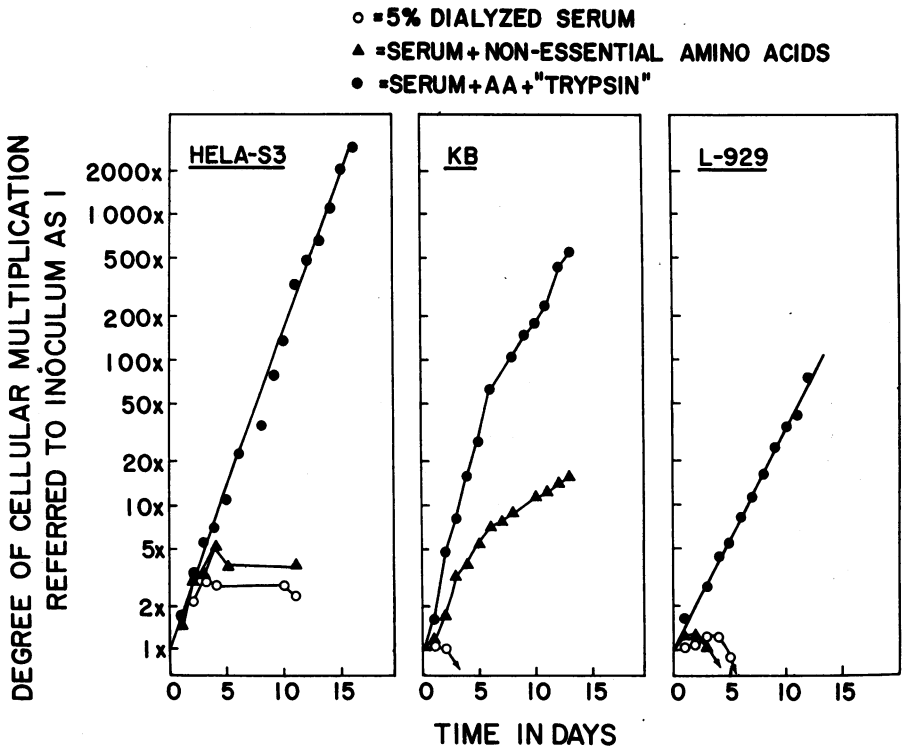

FIG. 1.—The rapid and sustained growth of mammalian cells in a protein-free medium, continuously equilibrated across a cellophane membrane with the same medium supplemented with serum protein and a pancreatic extract. The basal serum-free medium is one arm of the dialysis apparatus was equilibrated for 24 hr at 37° against either (*a*) the same growth medium supplemented with 5 per cent dialyzed horse serum along, (*b*) the dialyzed serum medium plus the 7 nutritionally nonessential amino acids (alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, and serine) each at 0.1 mM, and (*c*) the dialyzed amino acid serum medium plus "trypsin" (0.25% Viokase). After 24 hr, identical serum-free inocula, prepared by the centrifugation of suspension cultures in the logarithmic phase of growth, were added to the 3 serum-free culture arms. Thereafter, the serum-free culture fluid was removed and replaced as necessary at 1- to 2-day intervals to keep the cell count in the range 180,000–250,000 per ml. Simultaneously, the fluid in the "feeder" compartment was replaced, and to the same degree, with the appropriate serum-containing medium.

from the serum- and enzyme-containing "feeder" compartment were clearly not unchanged proteins. When cell-free medium was equilibrated for 24 hr across a cellophane membrane with medium supplemented with 20 per cent serum, with or without added pancreatic extract, the protein-free compartment still failed to give a visible turbidity either on heating, or on the addition of trichloracetic acid or sulfosalicylic acid. Further, complement fixation tests on the fluids on the two compartments, using rabbit antisera versus horse or human serum, showed no antigenically active protein on the one side of the membrane, while the fluid in the serum-containing compartment gave a positive reaction at dilutions of 1:1000–1:8000.

There was thus no evidence for the diffusion across the cellophane membrane of compounds behaving chemically or immunologically like serum protein. The essential growth factor(s) dialyzing across the membrane may have been originally bound to the serum protein, or may have been formed from it on incubation. The

growth-promoting activity of streptogenin for certain strains of lactobacilli and streptococci illustrates the latter possibility; while there are at least two analogies for the possibility that preformed growth-active substances were initially bound to the serum protein. Saprophytic treponemeta can be grown in a chemically-defined medium supplemented only with serum albumen; and, in that case, the albumen has been shown to serve solely as the carrier for an essential but toxic lipid.[15] More recently, it has been found that serum protein binds half-cystine residues which permit the growth of human and animal cells in an otherwise cystine-free medium.[16]

Whether the compounds necessary for cell growth were initially bound to protein, or were formed by its degradation, their provision to the cells was greatly accelerated by the addition of a dialyzed pancreatic extract to the "feeder" compartment, presumably because of the resulting proteolysis. Since dialyzed serum contains proteolytic enzymes,[14] it is possible that a similar degradation of serum protein may be directly responsible for its growth-promoting effect in ordinary cell cultures.

The regularity with which the suspension cultures so far tested can be grown in a protein-free environment suggests that, if intact protein is indeed necessary for cell growth in a monolayer, its primary role may be to permit the initial adhesion and flattening of the cells[8, 9] rather than their subsequent growth.

Studies are continuing with respect to the growth-promoting activity of proteins other than those in serum, the specific enzyme(s) in the pancreatic extract which are active, and the chemical nature of the postulated growth-active substance(s) formed. A number of crystalline proteolytic enzymes have had qualitatively the same effects as the crude pancreatic extract; and a number of serum protein fractions have been as effective as whole serum.

Summary.—1. Human (HeLa, HeLa-S3, KB) and mouse (L-929) cells could be regularly grown in suspension culture in a protein-free and chemically defined basal growth medium, if that culture was equilibrated across a cellophane membrane with medium containing 1–5 per cent dialyzed serum and a dialyzed pancreatic extract ("Viokase"). Only occasional and relatively slow growth was obtained when the enzyme preparation was omitted from the "feeder" compartment containing protein.

2. It is suggested that the primary role of serum protein in suspension cultures of mammalian cells is to provide essential growth factor(s) of small molecular weight, either initially bound to the serum protein, or formed from it on proteolysis.

The expert assistance of Miss Mina Levy and Mr. Aaron E. Freeman in the conduct of these experiments is gratefully acknowledged.

[1] Healy, G. M., D. C. Fisher, and R. C. Parker, *Canad. J. Biochem. Physiol.*, **32**, 323 (1954).

[2] Evans, V. J., J. C. Bryant, M. C. Fioramonti, W. T. McQuilkin, K. K. Sanford, and W. R. Earle, *Cancer Research*, **16**, 77, 87 (1956); McQuilkin, W. T., V. J. Evans, and W. R. Earle, *J. Natl. Cancer Inst.*, **19**, 885 (1957); Evans, V. J., H. A. Kerr, W. T. McQuilkin, W. R. Earle, and R. N. Hull, *Am. J. Hyg.*, **70**, 297 (1959).

[3] Waymouth, C., *J. Natl. Cancer Inst.*, **17**, 315 (1956); *ibid.*, **22**, 1003 (1959).

[4] Rabson, A. S., and F. Y. Legallais, *Nature*, **181**, 1343 (1958).

[5] Pumper, R. W., *Science*, **128**, 363 (1958).

[6] Katsuta, H., T. Takoaka, K. Mitamura, I. Kawada, H. Kuwabara, and S. Kuwabara, *J. Exptl. Med.* (Japan), **28**, 191 (1959).

[7] Holmes, R., *J. Biophys. Biochem. Cytology*, **6**, 535 (1959).
[8] Fisher, H. W., T. T. Puck, and G. Sato, these PROCEEDINGS, **44**, 4 (1958); *J. Exptl. Med.*, **109**, 649 (1959).
[9] Lieberman, I., and P. Ove, *Biochem. & Biophys. Acta*, **25**, 449 (1957); *J. Biol. Chem.*, **223**, 637 (1958); Lieberman, I., F. Lamy, and P. Ove, *Science*, **129**, 43 (1959).
[10] Eagle, H., and K. A. Piez, *J. Biol. Chem.* (in press).
[11] Nurmikko, V., *Experientia*, **XII**, 245 (1956); *App. Microbiol.*, **5**, 160 (1957).
[12] McLimans, W. F., F. L. Glover, and E. V. Davis (in press).
[13] Eagle, H., *Science*, **130**, 432 (1959).
[14] Piez, K. A., H. Eagle, L. Levintow, and V. I. Oyama (in preparation).
[15] Oyama, V. I., H. G. Steinman, and H. Eagle, *J. Bact.*, **65**, 609 (1953).
[16] Eagle, H., V. I. Oyama, and K. A. Piez, *J. Biol. Chem.* (in press).

*METABOLISM AND MORPHOLOGY OF RIBONUCLEOPROTEIN PARTICLES FROM THE CELL NUCLEUS OF LYMPHOCYTES**

BY JOHN H. FRENSTER,† VINCENT G. ALLFREY, AND ALFRED E. MIRSKY

THE ROCKEFELLER INSTITUTE

Communicated February 8, 1960

Incubation of isolated nuclei of calf thymus lymphocytes with C^{14}-labelled precursor compounds results in rapid labelling of the proteins and ribonucleic acids (RNA) of the nuclei.[1] Some of the proteins and RNA can then be extracted from the nuclei by neutral buffer solutions.[1] This paper describes the differential ultracentrifugation of such extracts which results in the isolation of classes of ribonucleoprotein particles, each of which can be characterized by its ease of extraction, its sedimentability, its composition of protein and RNA, and its rate of intranuclear protein and RNA metabolism. Unlike cytoplasmic ribonucleoprotein particles which are potassium-dependent,[2] these nuclear particles require a sodium environment for their metabolic activity in the nucleus; furthermore, unlike cytoplasmic particles,[3, 4] the intranuclear metabolism of these nuclear particles is resistant to preincubation of the nuclei with ribonuclease (RNAase) but sensitive to pre-incubation of the nuclei with deoxyribonuclease (DNAase).

It is also shown here that after extraction from the cell nucleus, the isolated nuclear ribonucleoprotein particles remain capable of active protein and RNA metabolism in a nucleus-free system.

Previous studies of the cell nucleus by electron microscopy have demonstrated the occurrence of discrete particles of 100–300 Å diameter in the nucleolus,[5–7] on the lateral loops of lampbrush chromosomes,[8] on the chromosomal rings of Balbiani,[9] in the blebs[10] and pore annuli[11, 12] of the nuclear membrane, and throughout the nuclear sap.[13] Particles of similar size are shown here to be extracted from the nuclei of calf thymus lymphocytes, and to be composed of sub-units of 15–25 Å diameter.

The heterogeneity of the RNA of the cell nucleus has been shown previously by radioautography of labelled nuclei,[14] by isolation of labelled nuclear RNA species,[15, 16] by determination of the base composition of isolated nuclear RNA species,[17] and by histochemical staining of nuclear ribonucleoprotein species throughout the mitotic cycle.[18] Composition and metabolic heterogeneity is also