in microtome sections of biological material. These examples of the industrial and research uses of isotopes, taken almost at random from an enormous field, can do no more than illustrate their already great and growing importance.

Fundamental Research

We have been both entertained and instructed by our sessions on fundamental research. New giant accelerators have been described to us, and we have heard that the cosmic ray workers, flying large stacks of photographic emulsions in Comet proving trials, have been able to obtain an enormous amount of new data on the collision of protons ten thousand times more energetic than any which can be produced by the largest planned accelerators. We have also heard of the new discovery, by the orbiting satellites, of intense belts of 40-million-volt protons, 1000 kilometers or so above the earth in particular latitudes. The great question of why pions and nucleons exist, with their particular masses and particular interactions, remains totally unanswered, in spite of the wealth of new knowledge produced by the accelerators. Strange particles accumulate and now total 31. The theoreticians have a new occupation of inventing new rules and waiting to see whether the latest strange particle obeys them. Feynman has predicted that 20 years hence our successors may be convening a "Conference on the Peaceful Uses of Strange Particles."

New Tools

In the field of nuclear data we have heard that the present situation leaves no room for complacency, since present reactor technology requires much more precise information, which we shall have to work hard to obtain. To help in this, important new tools providing enormously powerful pulses of neutrons are becoming available.

In the chemical sessions we have heard of the isolation of weighable amounts of berkelium, and that the chemists look forward to going well beyond element 102, aided by expensive reactors with neutron fluxes up to 10^{16} per square centimeter per second, which they hope benevolent governments will supply in the future. The chemical effects of fission fragments appear to be much higher than anticipated, and this may have important technological consequences. There has been a rapid advance in solvent-extraction technology, and long-chain amines and long-chain derivatives of phosphoric acid have been synthesized, with highly specific activities. Such developments could have applications far outside the world of atomic energy.

We have had a rich feast—perhaps too rich—at this conference, not only from the lectures but from the exhibitions, which have enabled us to see in a few days, in an exciting visual way, work proceeding throughout the world. We have also held innumerable discussions in small groups to amplify the knowledge gained in our formal sessions. This is the classical method of cooperation in the scientific world. We will go away with a great deal to think about, and this conference, like the 1955 conference, is likely to have a profound effect on the future development of atomic energy.

Requirements for Growth of Single Human Cells

"Nonessential" amino acids, notably serine, are necessary and sufficient nutritional supplements.

Royce Z. Lockart, Jr., and Harry Eagle

A number of human cell strains serially propagated in monolayer culture have been shown to have the same nutritional requirements for growth (*1*). A minimal medium containing the essential 13 amino acids, eight vitamins, five ions, and glucose, supplemented with dialyzed serum, permitted the apparently indefinite propagation of all these cultures, with a generation time in the logarithmic phase of growth of approximately 20 to 24 hours. However, when cultures were initiated with a relatively small inoculum, and, in particular, when cloning was attempted with several cell lines by the method of Puck and Fisher (*2*), the cells failed to grow in the same minimal medium which permitted the growth of heavily seeded cultures. As is shown below, the additional factors required for the growth of these small inocula proved to be the "nonessential" amino acids, which, for the growth of heavily seeded cultures, need not be added to the medium; in many experiments serine alone sufficed.

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Methods

The present experiments (*3*) were carried out with four serially propagated human cell cultures: (i) the stock HeLa strain; (ii) the S3 HeLa clone isolated by Puck *et al.* (*4*); (iii) a human conjunctival culture (*5*); and (iv) the KB strain (*6*). The cultures were grown in suspension in "spinner" cultures, as described by McLimans *et al.* (*7*). With such suspension cultures, the only manipulation of the cells required in the preparation of the inoculum was that of dilution; this obviated the cellular damage incident to the dispersal of stationary monolayer cultures by Versene, trypsin, or mechanical means.

As suggested by McLimans *et al.* (*7*), the medium contained the essential amino acids and vitamins at twice, and phosphate at ten times, the usual concentrations, while calcium was omitted

in order to minimize cell clumping. The cultures were used in the logarithmic phase of growth, and cells were counted in a hemocytometer. In most experiments the number of cells per clump averaged between one and two. One hundred clumps, 50 to 83 percent of which were single cells, were plated in 5 milliliters of experimental medium in 60-millimeter Petri dishes, with three to five replicate cultures for each experimental mixture. The cultures were incubated at 37°C in a 5-percent CO_2 atmosphere, and the number of cell colonies was counted after 8 to 9 days' incubation. In some experiments, the rate of growth was determined by counting the number of cells per clone on days 2, 4, and 6.

Results

1) *The effect of dialyzed serum on plating efficiency and clonal growth rates of human cells.* The human cell lines tested in these experiments had a generation time of 20 to 24 hours in culture and could be kept in the log phase of growth indefinitely. The cells retained their capacity to adhere to glass surfaces and were able to initiate growth with no apparent lag. Similar results have been reported for the L strain and for monkey-kidney cells by Gwatkin *et al.* (*8*).

In heavily seeded cultures, human serum which had been dialyzed for 24 to 72 hours against running tap water, then dialyzed for 4 hours against distilled water, or against salt solution, was just as effective as whole serum. In cloning experiments, however, dialyzed serum was usually far less effective than whole serum in terms of both (i) the plating efficiency and (ii) the rate of clonal growth.

The plating efficiency with dialyzed serum was extremely variable. With some lots, 10-percent dialyzed serum was as effective as whole serum, but in other experiments it was wholly ineffective, in that only a few clones could be seen after 9 days (see Fig. 1). At serum concentrations of 1 to 5 percent, the plating efficiency with dialyzed serum was regularly lower than with whole serum tested at the same concentration, and in the same experiment (Table 1). This decreased plating efficiency in dialyzed serum was not due to the failure of the cells to adhere, but rather to the fact that many of the cells went through only a few generations and then stopped growing.

The clones which developed in dialyzed serum were consistently smaller than those observed in whole serum. As shown in Fig. 2, the smaller colony size in the dialyzed serum medium was usually referable to a decreased rate of growth and decreased with the concentration of dialyzed serum.

2) *The effect of nonessential amino acids on the growth of single cells in dialyzed serum media.* The addition of Tween-cholesterol (*9*) did not affect either the plating efficiency or the size of the clones obtained in dialyzed serum, and the addition of a number of cofactors (see *9*) was similarly ineffective. However, when the basal medium with dialyzed serum was supplemented with the seven nonessential amino acids (alanine, aspartic acid, asparagine, glycine, glutamic acid, proline, and serine), each at 0.1 to 0.2 m*M*, the plating efficiency and the clonal size (the latter reflecting the rate of growth) were equal to or in excess of those observed with medium containing the same amount of whole serum (see Table 1 and Fig. 1). Single HeLa, HeLa S3, conjunctiva, and KB cells could now regularly be cloned with a plating efficiency of 50 to 100 percent in 5 milliliters of medium supplemented with 2 to 5 percent dialyzed serum, and the rate of clonal growth was equal to that observed in a medium supplemented with 10 percent whole serum.

As shown in Table 1 and Fig. 1, serine was the most important of the seven "nonessential" amino acids which permit the growth of single cells in a minimal medium supplemented with dialyzed serum, and in most experiments serine alone was as effective as the complete mixture. The maximally effective concentration was on the order of 0.01m*M*. Glycine was regularly less active than serine. This probably reflects the relative inefficiency with which glycine is converted to serine in these cell cultures (*8a*).

Preliminary experiments indicate that the anomalous requirement by small inocula for ordinarily nonessential nutrilites is, at least in part, due to the loss of such nutrilites from the cell pool into the medium at a rate which may exceed the biosynthetic capacity of the cell. The possibility may also be considered that whole serum provides not only the preformed "nonessential" amino acids and compounds derived from them but growth factors which facilitate their biosynthesis or retention by the cell, and

Table 1. Data illustrating the reduced plating efficiency of human cells in a minimal growth medium supplemented with dialyzed serum and the effect of supplementation with "nonessential" amino acids.

Cell strain	Serum concentration in medium (%)	Plating efficiency* in basal medium supplemented with			
		Whole serum	Dialyzed serum		
			Alone	Plus 7 "nonessential" amino acids†	Plus serine
HeLa	5	16 ± 3‡	1.2	64 ± 7	48 ± 2
HeLa-S3	10	86 ± 7	0	96 ± 11	75 ± 10
HeLa-S3	5	83 ± 2	9 ± 1	79 ± 3	
HeLa-S3	2	34 ± 13	3 ± 2	65 ± 11	
HeLa-S3	1	0	0	23 ± 4	
HeLa-S3	0.5	0	0	0	
Conjunctiva	10	37 ± 4	8.7 ± 2	43 ± 4	35 ± 5
Conjunctiva	5	36 ± 2	2.0 ± 0.8	37 ± 5	26 ± 5
Conjunctiva	2	24 ± 2	0.8 ± 0.5	22 ± 0.2	20 ± 1
Conjunctiva	10	70 ± 22	89 ± 4		
Conjunctiva	5	88 ± 10	37 ± 6	84 ± 11	102 ± 5
Conjunctiva	2	38 ± 11	3 ± 3	38 ± 18	5 ± 2(!)
KB	10	45 ± 1	7 ± 2	40 ± 6	
KB	5	34 ± 5	5 ± 1	44 ± 6	43 ± 8
KB	2	20 ± 2	5 ± 1	32 ± 4	32 ± 2
KB	1	0	0.7	9 ± 5	19 ± 7
KB	0.5	0	0	4 ± 2	

* Percentage of cell clumps inoculated which grew out in 8 to 9 days to form visible clones containing more than 100 cells; smaller but viable clones were not counted. In most of the experiments the average number of cells per clump in the inoculum was less than 1.5, and 50 to 83 percent of the clumps inoculated were single cells.
† Alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, and serine, each at 0.1 to 0.2m*M*.
‡ Average of 3 to 5 plates, plus or minus standard error (range of variation/number of plates).

which are lost on dialysis. The fact that in the present experiments, and contrary to the findings of Sato, Fisher, and Puck (*9*), neither added cholesterol nor cofactors were necessary for the growth of single cells in a dialyzed serum medium may perhaps be referable to the varying methods used for the preparation of the cell inoculum. In the present experiments, the inoculum was prepared by the dilution of cells growing in suspension. The physical and chemical changes produced by the dispersion of monolayer cultures with trypsin or Versene are thereby avoided.

Serine has here been shown to be required for the regular growth of single human cells deriving from serially propagated cultures. Neuman and McCoy (*10*) have recently shown that isolated Walker carcinosarcoma 256 cells, unlike heavily inoculated cultures, require either pyruvate, oxalacetate, or α-ketoglutarate for growth; and yet other compounds may be required for the clonal growth of other cell lines. It is possible that in the cultivation of mammalian cells directly from the animal host, specific metabolites may similarly be required which are not necessary for the propagation of already established cell lines.

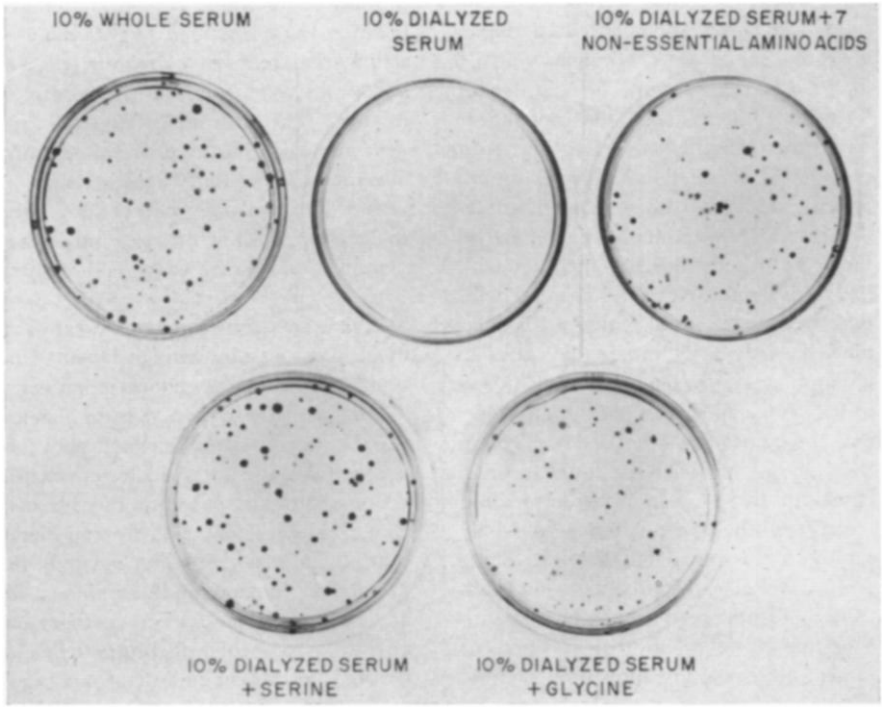

Fig. 1. The effect of "nonessential" amino acids on the plating efficiency of S3 HeLa cells in dialyzed serum. The seven nonessential acids included alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, and serine, each at 0.1 to 0.2m*M*.

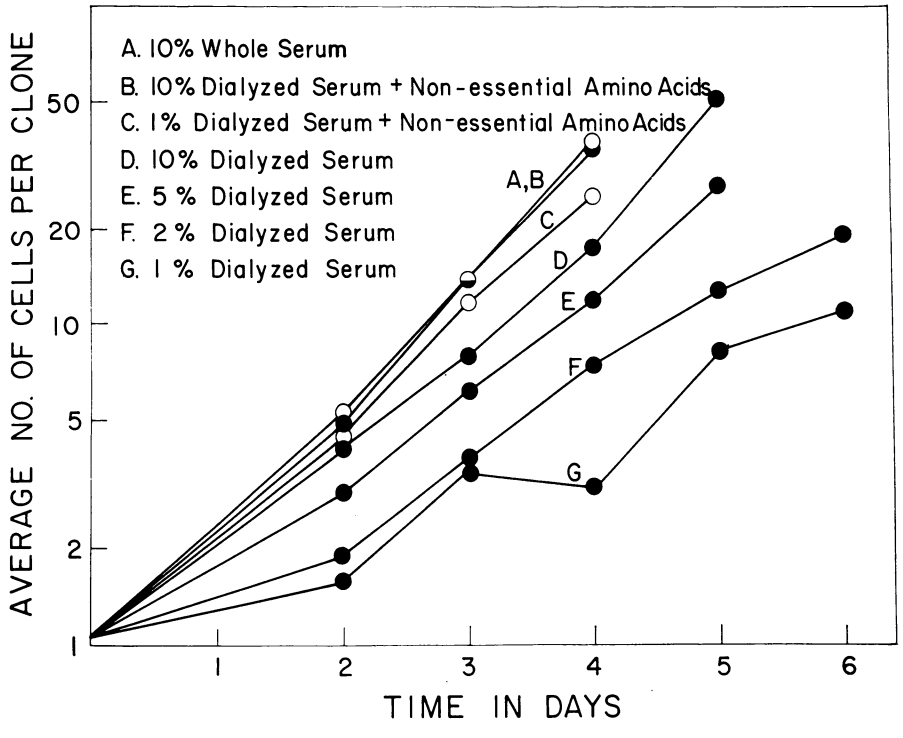

Fig. 2. The effect of "nonessential" amino acids on the clonal growth rate of HeLa S3. The seven nonessential acids included alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, and serine, each at 0.1 to 0.2m*M*.

Summary

A minimal growth medium supplemented with dialyzed serum, which sufficed for the propagation of a wide variety of human cell strains in heavily inoculated monolayer and suspension cultures, did not permit the regular or optimal growth of small numbers of HeLa, HeLa S3, conjunctiva, or KB cells deriving from suspension cultures. At threshold concentrations of serum, the plating efficiency of single cells was greatly reduced as compared with their plating efficiency in a medium containing dialyzed serum instead of whole serum, and the clones which did develop grew at a slower rate. The nutritional deficiency could be overcome by adding the seven amino acids which are ordinarily not nutritionally essential. In most of the experiments serine alone sufficed.

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