The Effect of Horse Serum Residue and Chemically Defined Supplements on Proliferation of Strain L Clone 929 Cells from the Mouse*

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In long-term tissue culture work a medium of embryo extract, horse serum, and saline is routinely used for the maintenance of strain L cells of single cell origin. Sanford *et al.* (6) tested the effects of the ultrafiltrates and residues of both the horse serum and chick embryo extract on the proliferation of clone 929 strain L cells. These workers determined that unfractionated horse serum in saline was capable of supporting proliferation to a limited extent, whereas the horse serum residue[1] fraction and saline were incapable of maintaining inoculum size (5). In this present study an effort has been made to ascertain whether the horse serum residue fraction, when supplemented with the addition of the amino acids found by Westfall *et al.* (8) to be present in the serum ultrafiltrate, is capable of supporting proliferation of strain L cells equivalent to that obtained in a medium containing whole horse serum; or whether additional supplementation with known growth-promoting agents is required.

MATERIALS AND METHODS

The method of obtaining and preparing the horse serum has been described (6). All the serum used in this study was obtained from one lot and was processed at the same time. A portion of it was ultrafiltered to provide the residue fraction, and the remainder was kept for use as whole serum.

Ultrafiltration of the pooled horse serum was carried out in a modified Simms' ultrafiltration apparatus (6, 7). Absence of protein in samples of all the ultrafiltrate was demonstrated by negative tests with the use of sulfosalicylic acid. The serum residue was reconstituted to original volume with Earle's balanced saline (1).

The commercially obtainable amino acids, amine, and amides were used in quantities to correspond to those found, by Westfall (8), free in horse serum ultrafiltrate. These, with the exception of L-cystine and L-tyrosine were put into solution in Earle's balanced saline containing double the concentration of salts. These composed part "A" of the amino acid mix (Table 1). L-cystine and L-tyrosine, part "B" amino acid mix (Table 1), were dissolved in 0.075 N HCl.

The supplements used in addition to the amino acids and amine and amides were those of Morgan, Morton, and Parker's (3) mixture 199 with the ommission of desoxyribose and sodium acetate, and were prepared according to their procedure.

All solutions were sterilized by passage through a #03-porosity Selas filter under pressure of a gas mixture of 5 per cent CO_2 in air. The horse serum and serum residue were first clarified through a Selas XFF porosity filter.

The percentage of components used in the test media are shown in Table 2.

The method for the preparation and handling of replicate cultures of Strain L cells of single cell origin has been described (2). This procedure was slightly modified in the present experiments. The cells were washed and suspended in a mixture of 0.1 per cent methocel of 4,000 cps, HG viscosity[2] dissolved in Earle's balanced saline. Each T-15 flask received 2.5 ml. of the culture medium to be tested and 0.5 ml. of the methylcellulose cell suspension. The cell suspension was stirred 20 min-

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[1] Washed serum residue refers to the large molecular portion of horse serum cleared of its freely diffusible small molecular weight substances by ultrafiltration through a collodion membrane.

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[2] Dow Chemical Co., Midlands, Michigan; Methocel, HG viscosity, 4,000 cps (centipoises).

utes prior to the dispensing of any cells and continuously during the planting.

Immediately prior to fluid renewals, 3 times a week, the cultures were centrifuged at 33 *g* (350 r.p.m.) for 20 minutes, and 2 ml. of the culture fluid was renewed. This speed of centrifugation has been found to be satisfactory in clearing the supernatant of cells and did not cause appreciable cell damage.

At intervals after planting, cultures from each test group were selected, and the number of nuclei in each culture was ascertained by the method for the measurement of proliferation in tissue culture (4). In those cases in which all cultures were not enumerated at one time, a table of random order was used to determine which cultures in each set would be enumerated.

TABLE 1

CONCENTRATION OF AMINO ACIDS IN TEST MEDIUM

Part "A" amino acids†	Source*	Amount (mg.)
Alanine	NBC	15.00
Alpha amino-butyric acid		2.00
Arginine·HCl	Merck	10.00
Asparagine	NBC	1.50
Aspartic acid	NBC	2.50
Glutamic acid	NBC	2.50
Glutamine	GB	20.00
Glycine	NBC	4.00
Histidine·HCl	Merck	7.00
Hydroxyproline	NBC	1.00
Isoleucine	NBC	8.50
Leucine	Merck	10.00
Lysine	Merck	15.00
Methionine	NBC	2.00
Ornithine·HCl	Schwarz	3.50
Phenylalanine	NBC	7.00
Proline	Merck	2.50
Serine	NBC	2.50
Taurine	NBC	1.50
Threonine	NBC	10.50
Tryptophane	NBC	5.50
Valine	NBC	12.50
Part "B" amino acids‡		
Cystine	Merck	50.00
Tyrosine	Merck	75.00

* NBC: Nutritional Biochemicals Corporation, Cleveland, Ohio; Merck: Merck and Company, Inc., Rahway, New Jersey; GB: General Biochemicals, Inc., Chagrin Falls, Ohio; Schwarz: Schwarz Laboratories, Inc., Mt. Vernon, N.Y.

† In 250 ml. balanced saline containing 2 × concentration of salts, at 2× concentration found by Westfall (8). All L forms were used.

‡ In 0.075 N HCl to 150 ml. with 3 × distilled water, with gentle heating. At 2 × the concentration found by Westfall (8). All L forms were used.

The present study consisted of three experiments. The intervals at which cultures were removed in the experiments differed. In no case was the proliferation of cultures determined earlier than 7 days, and the longest growth period studied was 21 days.

Each of the cultures was periodically examined microscopically; the last examination was just preceding treatment with citric acid. The visual observations were also used as a guide in determining the dilution to be used in the enumeration procedure.

RESULTS

Experiment 1 was carried for a total of 13 days (Chart 1). Neither the cultures on medium A nor those on medium E had maintained the original inoculum; each had a population decrease to well below the inoculum level. The difference noted between the cultures on these two media is not statistically significant.[3] The cultures tested in the remaining three media demonstrated in excess of a tenfold population increase over the inoculum. The effects produced by these three media are statistically significantly different, with the major difference between media B and D.

In Experiment 2 (Chart 1) the inoculum size was approximately one-half that of experiment 1, and the extent of proliferation in the cultures was determined at 10 days. At this time cultures exposed to medium E (horse serum residue and saline) did not show the drop in population that those on medium A (horse serum residue, saline and part "A" and part "B" amino acids) exhibited. The rates of proliferation of cultures on the three remaining media were similar to those seen in Experiment 1; the cultures on medium D showed less proliferation than did those on media C and B.

TABLE 2

PERCENTAGE OF COMPONENTS IN FINAL MEDIA TESTED

	A	B	C	D	E
Parts "A" and "B" amino acids of horse serum ultrafiltrate	40				
Parts "A" and "B" amino acids of horse serum ultrafiltrate plus ATP and vitamins of mixture 199		40			
Parts "A" and "B" amino acids of horse serum ultrafiltrate plus all supplements of mixture 199			40		
Whole horse serum				40	
Horse serum residue	40	40	40		40
Saline	20	20	20	60	60

In Experiment 3 (Chart 1) cultures were carried on media A and E for 1 week to confirm the results of the two previous experiments, and, since medi-

[3] The authors with to express their appreciation to Mr. Marvin Schneiderman, Biometry and Epidemiology Section, National Cancer Institute, for statistical advice and consultation.

um E also served as a control, the effect of aging on the residue fraction of the horse serum was tested. The three remaining media were tested for a total of 3 weeks, with culture removals at 7-day intervals. In this manner, the effects of all the media were compared at 7 days, and, in addition, the effects of media B, C, and D were compared at 14 and 21 days. Growth of cultures in media B, C, and D paralleled that evidenced in the previous indicating progressive deterioration.

Addition of amino acids, amides, and the amine of horse serum to this solution of residue (medium A) gave no significant improvement in the population level in any experiment; in the two shorter experiments this addition was definitely injurious.

When, however, the medium was further supplemented by vitamins and ATP (medium B), the population levels of the cultures all showed a sharp

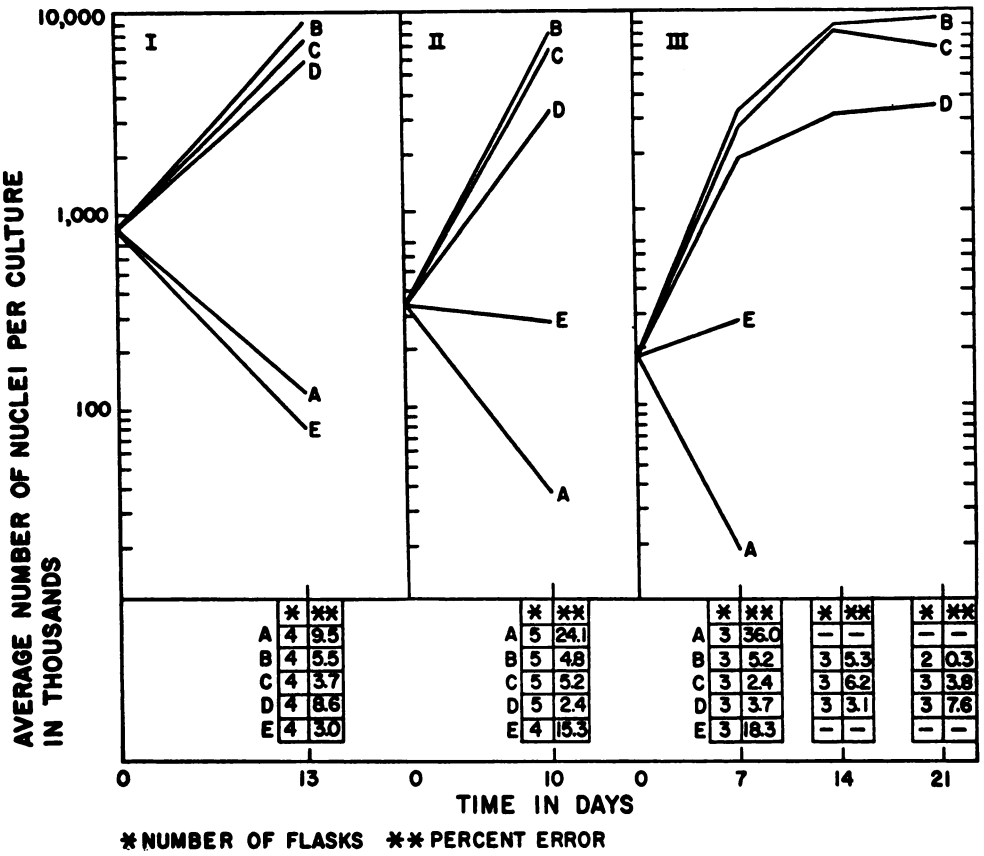

CHART 1.—Components of media tested in each experiment. *A:* Horse serum residue, amino acids, amides and amine of horse serum, and saline. *B:* Horse serum residue, amino acids, amides, and amine of horse serum, vitamins and ATP of mixture 199, and saline. *C:* Horse serum residue, amino acids, amides and amine of horse serum, all other supplements of mixture 199, and saline. *D:* Whole horse serum and saline. *E:* Horse serum residue and saline.

two experiments up to the 14th day. By the 21st day a minor decline in the number of nuclei of cultures on medium C was seen.

DISCUSSION

In the foregoing quantitative studies, under the experimental conditions used, cultures in horse serum residue and balanced saline (medium E) showed a very slight rise in population, compared with the inoculum level at 7 days (Exp. 3); at 10 days (Exp. 2) the population level had decreased, while at 13 days the population was much reduced, and continued superiority (statistically significant gains) over those of the whole horse serum medium (D).

Further addition to medium B of all other components of mixture 199 (medium C) gave population levels consistently and statistically significantly lower than for medium B; at 21 days this effect was even further increased.

It is apparent that some or all of these factors, not just the amino acids, amides, and amine, are essential to supplement washed serum residue for growth and proliferation.

From these experiments the medium which consists of the serum residue, amino acids, amides and amine of horse serum, and the vitamins and ATP, as prepared in mixture 199, was found to be superior to the naturally occurring medium of unfractionated horse serum; statistically significant gains in proliferation were obtained.

SUMMARY

Quantitative experiments were used to test the effect of supplements to a basic medium of the residue fraction remaining after ultrafiltration of horse serum. This study was carried out on washed cell suspensions of clone 929 strain L cells, originally obtained from a strain C3H mouse. These cultures were planted in T-15 flasks on glass substrate, and changes in population levels were determined by enumeration of the nuclei at 7-, 10-, 13-, 14-, and 21-day intervals.

The unsupplemented fraction of the horse serum was incapable of maintaining the inoculum level beyond 7 days.

This residue medium, supplemented with the amino acids, amides, and amine of horse serum was less effective in maintaining population levels.

The further addition of niacin, *p*-aminobenzoic acid, niacinamide, pyridoxine·HCl, thiamin·HCl, D-Ca pantothenate, *i*-inositol, choline chloride, riboflavin, ascorbic acid, glutathione, cysteine·HCl, biotin, folic acid, vitamin A, vitamin D (calciferol), Tween 80, menadione, vitamin E, and ATP, as contained in mixture 199 of Morgan, Morton, and Parker, gave population levels superior to those obtained by use of unfractionated horse serum. Under the experimental conditions used, addition of the other components of mixture 199 to this medium gave no added increase in proliferation and, in fact, indicated a possible inhibitory action.

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REFERENCES

1. Earle, W. R. Production of Malignancy *in Vitro*. IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells. J. Nat. Cancer Inst., **4**:165–212, 1943.
2. Evans, V. J.; Earle, W. R.; Sanford, K. K.; Shannon, J. E., Jr.; and Waltz, H. K. The Preparation and Handling of Replicate Tissue Cultures for Quantitative Studies. J. Nat. Cancer Inst., **11**:907–27, 1951.
3. Morgan, J. F.; Morton, H. J.; and Parker, R. C. Nutrition of Animal Cells in Tissue Culture. I. Initial Studies on a Synthetic Medium. Proc. Soc. Exper. Biol. & Med., **73**:1–8, 1950.
4. Sanford, K. K.; Earle, W. R.; Evans, V. J.; Waltz, H. K.; and Shannon, J. E., Jr. The Measurement of Proliferation of Tissue Cultures by Enumeration of Cell Nuclei. J. Nat. Cancer Inst., **11**:773–95, 1951.
5. Sanford, K. K.; Leadbetter, P. A.; Bryant, J. C.; Braker, W. P.; Evans, V. J.; and Earle, W. R. The Effects of Amino Acids as Supplements to Fractions of Serum and Embryo Extract on the Proliferation of Cells *in Vitro*. J. Nat. Cancer Inst., **14**:513–18, 1953.
6. Sanford, K. K.; Waltz, H. K.; Shannon, J. E., Jr.; Earle, W. R.; and Evans, V. J. The Effects of Ultrafiltrates and Residues of Horse Serum and Chick-Embryo Extract on Proliferation of Cells *in Vitro*. J. Nat. Cancer Inst., **13**:121–37, 1952.
7. Simms, H. S., and Sanders, M. Use of Serum Ultrafiltrate in Tissue Cultures for Studying Deposition of Fat and for Propagation of Viruses. Arch. Path., **33**:619–35, 1942.
8. Westfall, B.; Peppers, E. V.; Sanford, K. K.; and Earle, W. R. The Amino Acid Content of the Ultrafiltrate from Horse Serum. J. Nat. Cancer Inst., **15**:27–35, 1954.