identical in the 2 specimens indicating the absence of a selective proliferation of non-labeled cells.

In conclusion it appears that liver regeneration in contradistinction to red cell regeneration is the result of multiplication of the general liver cell population rather than multiple divisions of a small segment of that population.

Summary. A study of hepatic regeneration in rats was carried out to see whether the stimulus of partial hepatectomy promotes proliferation of parenchymal liver cells or, in analogy with red cell regeneration, promotes the differentiation of stem cells to new hepatic parenchymal cells. By labeling a large proportion of hepatic cells with tritiated thymidine it was shown that the labeled cells rather than the presumably unlabeled stem cells were responsible for hepatic regeneration following partial hepatectomy.

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A Chemically Defined Medium for Growth of Animal Cells in Suspension. (28038)

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The growth of tissue cells (L cells) in suspension in a chemically defined medium, NCTC 109 with methylcellulose, has been reported by Bryant *et al.*(1). Bakken *et al.* (2) reported that this medium also supported the growth of suspension cultures of a line of human skin cells. Merchant and Hellman (3) have recently described the growth of L-M strain mouse cells in suspension culture using chemically defined 2X Eagle's medium. A serum-free medium containing lactalbumin hydrolyzate described by Higuchi(4) that supports the growth of several cell lines in monolayer cultures was modified for the growth of tissue cells in suspension culture by Tribble and Higuchi(5).

This report presents a chemically defined medium formulated as a result of experiments with a cat kidney cell line, and its successful application for the growth of 5 other cell lines.

Materials and methods. Preparation of the medium. Salts (except sodium bicarbonate), glucose, amino acids, and glutamine were dissolved together in hot distilled water. Vitamins, stored at –20°C as a 100X stock solution, were added to the cooled mixture. Sodium bicarbonate and phenol red dissolved together in a small amount of water were added and the resulting solution was sterilized by filtration through a 0.2 μ membrane filter and stored at 5°C as a 5X solution. Prior to inoculation, insulin, methylcellulose, streptomycin and penicillin were added aseptically and the final volume adjusted by addition of sterile distilled water. Methylcellulose was stored as a sterile 2% solution; antibiotics were stored at –20°C at 100X concentrations. The medium used for determining amino acid requirements was prepared by adding aseptically to the filtered salts-vitamin solution individual amino acids (100X

concentration) sterilized by autoclaving at 121°C for 15 minutes. Similarly, the medium used for vitamin requirements was prepared by adding aseptically individual vitamins sterilized by filtration. Sodium pyruvate, KCl, NaH_2PO_4, $CaCl_2$, and $MgCl_2$ were omitted from the basal medium preparation when it was tested for growth-supporting activity. These compounds were sterilized by autoclaving as 100X solutions and added aseptically.

Cell lines. Mouse fibroblast (L) cells and HeLa cells were obtained commercially from Microbiological Associates, Bethesda, Md. Rat spleen, monkey kidney, guinea pig kidney, and cat kidney cells were established in our laboratories. Growth of these cell lines in the serum-free lactalbumin hydrolyzate medium has been described by Tribble and Higuchi(5). All cells were free of PPLO.

Incubation and cell enumeration. Growth of suspension cultures in the defined medium was initiated from cell monolayers grown in defined medium (less methylcellulose) or from other suspension cultures grown in the serum-free lactalbumin medium. Most initial cell concentrations were 1×10^5 to 2×10^5 per ml. Glass beads were used in place of trypsin for suspending cells from monolayer cultures according to Nagle(6). Suspension cultures were incubated at 34 to 36°C in rubber-stoppered 100-ml serum bottles containing 25 ml of medium in a New Brunswick Gyrotory incubator shaker operating at 124 to 130 rev/min. At appropriate intervals, numbers of viable cells were determined in the hemocytometer by the trypan blue procedure of McLimans *et al.*(7). Media were changed by centrifuging the serum bottle cultures at 1000 rev/min for 10 minutes, decanting the supernatant, and replacing it with fresh medium.

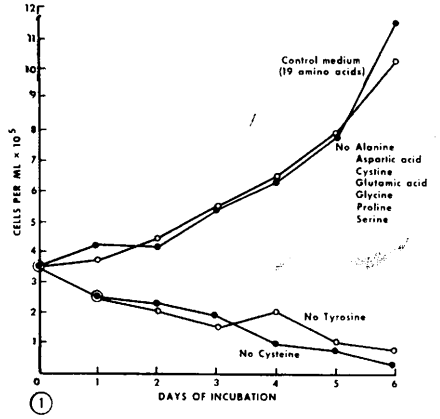
Results. Amino acid requirements. The serum-free lactalbumin hydrolyzate medium reported by Higuchi(4) for the growth of tissue cell monolayers served as a basis for the present study. Replacement of the hydrolyzate portion of this medium with known amino acids provided a relatively simple chemically defined medium (Table I). The

TABLE I. Chemically Defined Medium for Growth of Animal Cells in Suspension.

Component	Conc, mg/l	Component	Conc, mg/l
Amino acids:		*Salts:*	
L-Arginine · HCl	100	NaCl	7400
L-Cysteine · HCl	75	KCl	400
L-Histidine · HCl	60	$NaH_2PO_4 \cdot H_2O$	100
L-Isoleucine	150	$NaHCO_3$	500
L-Leucine	300	$CaCl_2 \cdot 2H_2O$	265
L-Lysine · HCl	300	$MgCl_2 \cdot 6H_2O$	275
L-Methionine	60	*Carbon sources:*	
L-Phenylalanine	120	Glucose	1000
L-Threonine	135	Na pyruvate	110
L-Tryptophan	60	*Vitamins:*	
L-Tyrosine	120	D-Biotin	1.0
L-Valine	150	Choline Cl	1.0
L-Glutamine	450	Folic acid	1.0
Antibiotics, etc.:		Niacinamide	1.0
Methocel, 15 cps	1000	Ca pantothenate	2.0
N.P.H. insulin	200 u/l	Pyridoxal · HCl	1.0
Streptomycin	100 mg/l	Thiamine · HCl	1.0
Penicillin	100,000 u/l	i-Inositol	1.0
Phenol red	10 mg/l	Riboflavin	.1
		B_{12}	.002

18 amino acids reported to be present in lactalbumin hydrolyzate at the relative concentrations supplied by the manufacturer plus cysteine were used in formulation of the defined medium. Preliminary studies with cat kidney cells indicated that a concentration of amino acids approximately equivalent to 0.37% lactalbumin hydrolyzate supported growth of monolayer cultures.

The individual amino acids and concentrations required for growth of cat kidney cells in suspension cultures were determined. Basal media similar to that reported in Table I containing the 10 essential amino acids for the rat: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine were prepared, omitting individually the remaining 9 amino acids. Results (Fig. 1) with these media demonstrated that both tyrosine and cysteine also were required. Omission of any one of the other 7 amino acids resulted in growth nearly equivalent to that obtained with the control medium containing all 19 amino acids. This curve is a composite representing growth obtained in media from which alanine, aspartic acid, cystine, glutamic acid, glycine, proline, or serine were individually omitted. The re-



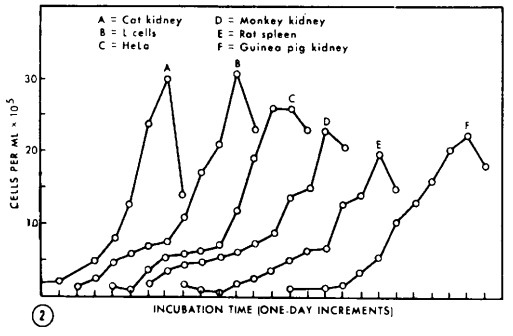


FIG. 1. Amino acid requirements for growth of cat kidney cells in suspension culture.

FIG. 2. Growth of tissue cell suspension cultures in a chemically defined medium.

quirement for the 12 amino acids for growth of cat kidney cells was reexamined, omitting these amino acids from the medium one at a time. All 12 amino acids were required for growth. Similarly, no growth occurred in the absence of glutamine.

The effect of amino acid concentration was determined in experiments in which growth was measured at 6 days in media containing individual amino acids at one-half or 2 times the concentration given in Table I. All media were changed at 4 days; pH was not adjusted. Results indicated that growth was not significantly enhanced by altering concentrations of most of the amino acids. However, reduction in cysteine or isoleucine concentration reduced growth (to about 12% and 25% respectively of the growth of the control culture). Growth was also reduced (to about 30% and 8% respectively) by increasing the concentrations of cysteine or tyrosine. Concentrations of the 12 amino acids and glutamine shown in Table I were employed in the remaining experiments.

Vitamin requirements. The effect of vitamins on growth of cat kidney cells was determined by comparing growth in media (Table I) from which vitamins had been omitted singly. A 0-hour population of 3.3×10^5 washed cells per ml was employed. After 4 days of incubation all cultures yielded cell counts approaching 1×10^6 per ml except the culture without choline ($<1 \times 10^5$ per ml). At this time, the medium was replaced with fresh medium to give a cell population of about 2.0×10^5 per ml and the cultures reincubated. Failure of growth in all cultures (except control) indicated a requirement for each vitamin that was evident only after the cellular "vitamin pool" had been apparently depleted. The marked response to omission of choline in the first growth cycle was confirmed in several separate experiments.

Requirements for sodium pyruvate, potassium chloride, sodium phosphate, calcium chloride, and magnesium chloride. These salts were tested individually at 0.5X, 1X, and 2X, the concentrations contained in the lactalbumin hydrolyzate medium(4). Growth from an initial population of washed cells of 2.2×10^5 cells per ml was measured after 6 days of incubation. Death occurred upon omission of any single compound from the medium. One-half X concentrations supported limited growth and 2X concentrations were inhibitory. Growth with 2X pyruvate was equivalent to the control.

Requirement for insulin. Growth of cat kidney cells in a basal medium (Table I) containing varying concentrations of protamine zinc insulin (NPH Iletin, Lilly) or zinc insulin (Iletin, Lilly) was determined in a similar manner. No growth occurred in absence of insulin. Two-tenths unit per ml of protamine zinc insulin appeared to be optimal; 0.4 unit per ml of zinc insulin supported growth approaching that obtained with the protamine zinc preparation.

Requirement for methylcellulose. The effect of methylcellulose (Methocel, 15 cps, Dow Chemical Co.) on growth was determined as above at concentrations of 0, 0.05,

0.1 and 0.2%. Optimum concentration was 0.1%; reduced growth (40% of control culture) accompanied by clumping of cells was obtained in absence of methylcellulose.

Growth of other cell lines in the chemically defined medium. The growth of cat kidney, L, HeLa, monkey kidney, guinea pig kidney, and rat spleen cells in suspension cultures is compared in Fig. 2. All cell lines had been established and maintained for varying periods of time in the defined medium in suspension culture prior to this experiment. Media were changed daily after the first 2 days of incubation. Maximum growth obtained varied from 2.0×10^6 cells per ml in 11 days (rat spleen cells) to 3.1×10^6 cells per ml in 9 days (L cells).

Discussion. The serum-free, lactalbumin hydrolyzate medium of Higuchi(4) that is supplemented with vitamins, pyruvate, methylcellulose, catalase, insulin, and monoolein supported growth of several cell lines in monolayer cultures. Tribble and Higuchi(5) demonstrated that the lactalbumin hydrolyzate medium also supported growth of 18 cell lines in suspension culture. The suspended cell culture system used by these workers permitted an accurate and rapid determination of growth by direct counting of viable cells in trypan blue and eliminated problems that occur in monolayer cultures, *e.g.*, failure of cells to adhere to glass surfaces and sensitivity of cells upon removal from the glass for transfer to another culture.

The chemically defined medium of this report may be compared with 2 chemically defined media [medium NCTC 109 of McQuilkin *et al.*(8) and minimum essential medium of Eagle(9)] that also have been used for growth of cells in suspension culture. Concentrations of most amino acids of medium NCTC 109 vary from one-fifteenth to about one-third the levels of those in the medium described here. In addition NCTC 109 contains 11 amino acids that are not present in ours. Cysteine · HCl concentration (75 mg per liter) in our defined medium was critical; little or no growth occurred below 37.5 mg per liter or above 150 mg per liter. Cysteine · HCl concentration of medium NCTC 109 is 259.9 mg per liter. This is the only amino acid in our medium that is used at a higher concentration in NCTC 109. Medium NCTC 109 contains additional vitamins and cofactors (a total of 69 components with methylcellulose) not contained in the presently described medium.

Our medium is basically similar to Eagle's minimum essential medium, differing in the following respects: individual amino acid concentrations of Eagle are from one-sixth to approximately equal the levels of the present medium. Biotin and Vit. B_{12} are added to our medium in addition to the 8 vitamins present in Eagle's medium.

The chemically defined medium as described contains insulin and thus differs further from the other two media. Insulin was used in the medium of Higuchi(4) who found that under conditions of insulin deficiency in HeLa cultures, a marked excess of lactic acid accumulated in relation to amount of glucose utilized. Lieberman and Ove(10) had previously described the stimulatory activity of insulin for animal cells in tissue culture and presented evidence that the hormonal activity was identifiable with the growth stimulatory activity. Recent studies in our laboratory have indicated the possibility that a mixture of several amino acids may satisfy the insulin requirement for strains of cat kidney, L and HeLa cells. However, a positive assignment for insulin function in the defined medium must await further study.

Several factors are critical for the culture system described. The medium should be prepared carefully in the sequence given to eliminate precipitate formation in preparation or subsequent storage. The optimal incubation temperature is 34 to 36°C; temperatures approaching 37°C and higher are increasingly inhibitory. The speed of the rotary shaker cannot be altered greatly; at speeds below 120 rev/min some cells did not remain in suspension, and at speeds exceeding 130 rev/min satisfactory growth was not consistently obtained. Finally, the medium was developed by submerged culture technics and as a consequence does not support growth of all the cell lines described when employed in monolayer cultures. Modification of the medium may yield a chemically defined me-

dium permitting satisfactory growth in monolayer cultures.

The medium as described for suspension culture appears to be nutritionally complete for the 6 cell lines tested. Growth was maintained continuously as cell populations of 5 $\times$ 10^5 to 1 $\times$ 10^6 cells per ml for several months by changing the medium on alternate days and reducing cell populations at the time of medium change. Maximal cell populations attained in the defined medium have approached those of the lactalbumin medium (5). Populations higher than those shown in the growth curves were obtained with L cells by increasing medium changes to twice daily, indicating a possible requirement for a better buffering system or addition of an adsorbant for toxic cellular products.

The defined medium is relatively simple in chemical composition and, combined with the suspension culture method, should be of value in studying aspects of cell physiology such as response to hormones, drugs, enzymes, etc., as well as in obtaining a basic knowledge of nutritional similarities and differences for various cell lines.

Summary. A chemically defined medium is described that supports the growth of L, HeLa, monkey kidney, rat spleen, guinea pig kidney, and cat kidney cells in continuous suspension culture. Cell populations of 2.0 $\times$ 10^6 per ml to 3.1 $\times$ 10^6 per ml were obtained. The complete medium contains 33 components plus antibiotics and phenol red. The requirement for each component was determined for growth of a cat kidney cell line.

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A Disposable, Constricted Tissue Culture Tube. (28039)

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The incubation of tissue culture tubes at a slight angle, whether in rolled or stationary tubes, has presented problems. Inclining culture tubes at an angle of about 5° was designed to localize cellular monolayers at the distal end of the tube. If held horizontal, the monolayers would be spread over a large surface, requiring correspondingly larger volumes of medium and extensive microscopic scanning for detection of cell morphology. Furthermore, the proximity of cells to the opening of the conventional tube, if unslanted, makes the culture more subject to contamination. Because of the slanted position, we have found it necessary to seed primary monkey kidney (MK) cells in tubes with 300,000 cells per tube, and other cells, such as the stable HeLa line, with 50,000 cells, to assure an adequate attachment of cell population. Only a small percentage of cells attach at the upper area of the distal third of the tube. The majority of the cells slide towards the butt of the tube, with increased attachment rates near the base. The largest portion of the inoculated cells attach at the butt and proliferate from this area towards the upper portion of the tube, eventually meeting the centers of cell proliferation in the upper areas. This produces a cellular monolayer but of varying texture. With growth, such cultures develop a tendency to slough from the glass at the base of the tube