The constancy of concentrations of hexoses, hexosamine and sialic acid of stroma obtained from red cells stored over a 45-day period indicates that these sugars are not involved in the metabolic processes of erythrocytes.

Qualitative analysis of the acid hydrolysates of stroma by paper chromatography showed the presence of large amounts of galactose, smaller amounts of glucose and mannose and a trace of fucose. Excellent resolution without trailing was observed for each of the sugars. A number of investigators(9,10) found galactose and glucose present in stroma. In addition, McCrae(11) found fucose present in his mucoprotein preparation. This appears to be the first study in which mannose is reported to be present in stroma.

Summary. Human red cell stroma has been found to contain 2% hexoses, 1.2% hexosamine and 1.2% sialic acid. These stroma values remain constant during storage of whole blood in the cold for 45 days. Monosaccharides found in stroma were galactose, glucose, mannose and fucose.

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Serumless Medium for Cultivation of Cells of Normal and Malignant Origin.* (25797)

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Development of serum-free media for growth of cells in culture generally has culminated in a medium containing a protein fraction from serum or one suitable for "adapted" cell strains. Waymouth grew strain "L" mouse fibroblasts in media in which Armour fraction V and peptone were substituted for serum(1). Fisher *et al.* successfully grew colonies of human cells in media containing serum albumin and fetuin(2). Lieberman and Ove demonstrated the growth promotive properties of insulin, serum "sticking" factor and catalase in media containing serum albumin(3). Certain lines of strain "L" mouse fibroblasts have been cultivated in completely defined media(4,5,6). Recently, monkey kidney(7), Chang liver cells(8) and mouse lung(9) have been adapted to similar media sometimes supplemented with peptone. The present report concerns growth of a variety of cell cultures in media devoid of serum proteins and without a preliminary period of adaptation.

Methods. One ml of whole medium containing 10,000-15,000 Walker carcinosarcoma 256, KB, human heart, sarcoma 180 cells or 100,000 primary green monkey kidney cells was pipetted into 16 x 125 mm tubes placed in a slanted position in racks. The cultures were established (plated) by incubation for 24 hours at 37°C in an atmosphere of 8%

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TABLE I. Serumless Medium for Growth of Cell Cultures.

Constituent	Quantity	Constituent	Quantity	Constituent	Quantity
Amino acids	(mM/l)	Vitamins	(μg/ml)	Minerals	(g/l)
L-alanine	.1	Ascorbic acid	.5	NaCl	6.46
-arginine • HCl	.2	Biotin	.2	$NaHCO_3$	2.20
-asparagine • H_2O	.3	B_{12}	.00075	Na_2HPO_4	.14
-aspartic acid	.1	Calcium pantothenate	.2	KCl	.40
-cysteine	.2	Choline • HCl	5.0	$CaCl_2$	.20
-glutamic acid	.1	Folic acid	2.0	$MgSO_4 \cdot 7H_2O$	.20
-glutamine	1.5	Glutathione	.5	Glass distilled water	to 1000 ml
glycine	.1	Inositol	9.0		
-histidine • H_2O • HCl	.1	Nicotinamide	.5	Special additives	(mM/l)
hydroxy-L-proline	.1	Pyridoxine • HCl	.5	Pyruvate	1.0
-isoleucine	.3	Pyridoxal • HCl	.5	Mucate	.1
-leucine	.3	Riboflavin	.2		(μg/ml)
-lysine • HCl	.2	Antibiotics		Salmine sulfate	5.0
-methionine	.1	Penicillin G, sodium	50	Insulin*	1.0
-phenylalanine	.1	Streptomycin sulfate	50	Methyl oleate†	15.0
-proline	.1	Indicator		Folinic acid	.1
-serine	.25	Phenol red	2.5		(mg/ml)
-threonine	.1	Carbohydrates	(g/l)	Lactalysate‡	2.0
-tryptophan	0.03	Glucose	3.0		
-tyrosine	.1				
-valine	.1				

All constituents except salmine, insulin and methyl oleate are dissolved together in balanced salt solution (Earle's solution). Salmine sulfate, methyl oleate and insulin are added separately.

* Crystalline zinc insulinate, 23.6 U.S.P. units/ml.

† Tween 80 (15.0 μg/ml) and ETOH (600 μg/ml) used to dispense oleate.

‡ EDAMIN DR, (pancreatic digest of lactalbumin), Sheffield Chemical, Norwich, N. Y.

carbon dioxide-92% air. Medium was removed and tubes were rinsed with Earle's solution. The cells then were overlaid with 1 ml of test medium containing graded amounts of the test compounds. Incubation was continued for 3-4 days until cells attained maximal growth in presence of optimal amounts of test compounds. The medium was not replenished. Growth was measured by the method of Oyama and Eagle(10). The whole medium in which the cells were established was similar to that previously described(11). It contained L-isomers of essential and non-essential amino acids as well as 0.2 mM pyruvate and 5% bovine serum. Serum was omitted from test media. Fatty acid esters were added to media as 2.0% alcoholic solutions containing 2.0% Tween 80 (or Tween 20). For continued passage, cells were grown in T-15 flasks. To transfer to fresh containers, the cells were released from glass surfaces with 0.2 ml of 0.05% crystalline trypsin added directly to medium. An equal quantity of 0.1% trypsin inhibitor (ovomucoid) then was added to stop tryptic activity. The cells were collected by centrifugation and 1/10 to 1/20 of the cells were replanted in 3 ml of serumless medium in T-15 flasks.

Results. Attempts were made initially to grow Walker carcinosarcoma 256 cells in serumless media by appropriately supplementing chemically defined and required amino acids, vitamins, minerals, and glucose. After a series of studies, the medium shown in Table I was derived. In addition to amino acids, vitamins, minerals and glucose, it contains insulin, salmine sulfate, methyl oleate, pyruvate, mucate, folinic acid and lactalysate. The growth promoting effects of the critical constituents (except mucate) were demonstrated as shown in Table II. Each material stimulated growth when added to the otherwise complete medium. In absence of lactalysate, the cells responded favorably to D-galacturonate. However, with inclusion of lactalysate the beneficial effects of D-galacturonate were not observed consistently and apparently were masked or effectively replaced by ingredients of lactalysate. In later studies mucate, available in samples of greater purity than the galacturonate, exhibited similar borderline activity. This compound was retained as

a constituent of the medium in view of possible long term effects and to serve in conjunction with purification studies of lactalysate factor(s). D-glucuronate, which has been reported to benefit cells in culture(4), appeared inactive.

Lieberman and Ove found insulin to stimulate growth of HeLa and appendix A1 cells (3) and showed that salmine enabled cells to adhere to the glass vessel(12). In the present study both of these substances behaved as growth factors. Zinc tested as a trace metal was inactive.

Pyruvate which promoted growth of isolated Walker carcinosarcoma 256 cells(11) also stimulated growth of cells in the serumless medium.

The requirement for methyl oleate or related compounds is particularly notable. Table III illustrates growth response of Walker carcinosarcoma 256 cells to methyl esters of arachidonic, linolenic, linoleic and oleic acids. Methyl oleate and linoleate were the most effective. Optimal concentration of these unsaturated fatty acids extended over a narrow range, and slightly greater concentrations produced marked inhibitory effects. Tween 80 (polyoxyethylene sorbitan monooleate) possessed a degree of activity but not to an extent which obscured the activity of the fatty acids. Methyl cellulose or Tween 20 (polyoxyethylene sorbitan mono-laurate) when used as suspending agents yielded similar results. Methyl stearate was somewhat active. The methyl esters of caproic, capric, lauric, myristic and palmitic acids did not noticeably stimulate. Esters were employed in these studies instead of the free acids to avoid precipitation of insoluble calcium and

TABLE II. Growth Response of Walker Carcinosarcoma 256 to Supplements in Serumless Medium.

Insulin		Salmine sulfate		Methyl oleate		Pyruvate		Lactalysate		Folinic acid	
μg/ml	Cell protein/tube	μg/ml	Cell protein/tube	μg/ml	Cell protein/tube	mM/l	Cell protein/tube	mg/ml	Cell protein/tube	μg/ml	Cell protein/tube
.0	96	.0	100	0	12	.0	78	.0	24	0	86
.001	106	.1	86	1	44	.01	124	.1	26	10^{-4}	86
.05	138	.5	128	3	94	.1	150	.5	102	10^{-3}	114
.1	140	1	124	5	130	.5	162	1	122	10^{-2}	106
.5	172	3	162	8	150	1.0	184	1.5	128	10^{-1}	136
1	192	5	166	10	166	1.5	166	2	136	1	132
2	174	8	192	15	182	2.0	158	3	150	3	102
5	158	10	152	20	156	2.5	132	4	86		
				30	104						
				40	82						

Basal medium in Table I used in these studies.

TABLE III. Effect of Unsaturated Fatty Acid Methyl Esters on Growth of Walker Carcinosarcoma Cells.

Conc. (μg/ml)	Arachidonate	Linolenate	Linoleate	Oleate
	(μg cell protein/tube)			
.0	12	12	12	12
.1	26			
.5	50	40		
1	72	76	46	44
2	68			
3	54	98	136	94
5	10	40	140	130
8				150
10		6	92	166
15			54	182
20			8	156
30				104
40				82

Basal medium in Table I used in these studies. Esters were diluted from alcoholic solutions containing .2% ester and .2% Tween 80.

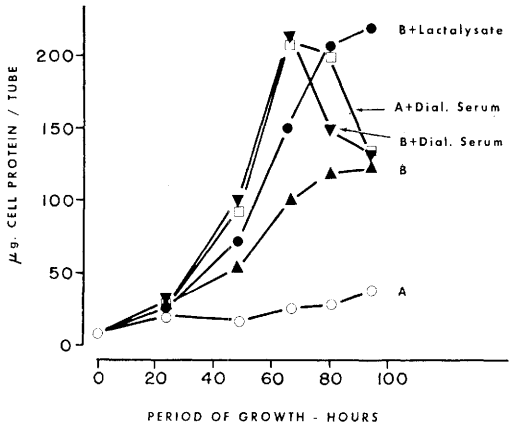

FIG. 1. Growth rates of Walker carcinosarcoma 256 in media with and without additives and serum. A. Medium, Table I, containing 0.2 mM pyruvate, but no other special additives. B. Medium, Table I, containing additives except lactalysate. 5% dialyzed bovine serum included as indicated.

magnesium salts. Ethanol was non-toxic at levels as high as 4 mg/ml.

Lactalysate is the only chemically undefined constituent of the serumless medium. Isolation of the active factor(s) is now in progress in this laboratory. Yeast extract also stimulated though less effectively. Bactopeptone and Bacto-tryptone were inactive.

Fischer reported a sparing effect of folinic acid for a peptone requirement of leukemic cells growing in a medium also containing serum(13). Folinic acid indeed stimulated Walker tumor cells growing in the serumless medium containing lactalysate despite the presence of 2 μg of folic acid per ml. Folinic acid, however, did not replace the requirement for lactalysate.

Variation of amino acids and vitamins, each as a group, and L-glutamine through a concentration range of several-fold showed optimal levels of these groups of nutrients to be that given in Table I.

Catalase, 3, 3′, 5-triiodothyronine, and versene employed by Lieberman and Ove(3) were not included in the present medium because activity for these compounds could not be demonstrated.

The stimulatory effects of insulin, salmine, pyruvate, oleate, folinic acid, and lactalysate recorded for Walker carcinosarcoma 256 cells, were observed to be similarly active for KB, human heart, sarcoma 180, and primary green monkey kidney cells. Evidently these factors have a broad applicability for cell cultures.

Relative growth rates of Walker carcinosarcoma 256 on different media with and without serum are shown in Fig. 1. The simple medium containing only amino acids, vitamins, minerals, glucose, and pyruvate supported little growth. Stimulation resulted from supplementation with chemically defined compounds but lactalysate was required to achieve a growth rate approximating that obtained with serum.

An important criterion of adequacy of a medium is its capacity to support long term growth and repeated subculture. At present the Walker carcinosarcoma 256, KB, human heart, and sarcoma 180 have been carried for 16-20 transfers in serumless medium with no apparent deterioration. A special regimen was not adopted for maintenance of the cultures at most rapid states of proliferation; nevertheless, growth has been such that cultures were routinely subdivided 10-fold every 5-6 days. Evidently, prolonged culture of the cells did not depend upon residual serum protein. Protective or other contributory factors of dialyzed serum were not completely supplied by the present medium for, in addition to a somewhat slower growth rate, inocula as small as 10,000 cells per T-15 flask exhibited poor viability.

As a further criterion for capacity of the serumless medium to support growth, Walker tumor cells were found to grow with a generation time of 25 hours in this medium in cell suspension systems. These suspension cultures were maintained continuously by transfer of 1/8 of the suspension to fresh media at 3-4 day intervals.

Summary. A serumless medium suitable for prolonged growth and repeated subculture of Walker carcinosarcoma 256, sarcoma 180, KB and human heart cells *in vitro* was described. In addition to amino acids, vitamins, minerals and glucose, it contained insulin, salmine sulfate, pyruvate, mucate, methyl oleate, folinic acid and lactalysate. The latter group of materials with exception of mucate was required for maximal growth rates,

comparable to those on media containing dialyzed serum. Suspension cultures of Walker carcinosarcoma 256 were grown with continuous passage in the serumless medium.

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Essential Duration of Parabiosis and Development of Tolerance to Skin Homografts in Mice.* (25798)

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Immunological tolerance of F_1 hybrid skin homografts can be induced in mice of their parent strains after establishing a "F_1 hybrid to parent" parabiosis(1,2). Similarly, female mice of either the A or C57Bl (Subline 1) strains were made tolerant of male skin isografts by placing adult females in parabiosis with the corresponding males(3). Finally, establishment of parabiosis between animals of the Z strain made tolerant of Ce strain tissue by intravenous injection of Ce cells at birth and previously non-conditioned isologous Z mice led to transfer of tolerance. Thus the Z strain animals conditioned only by having been placed in parabiosis with Z animals tolerant of Ce tissue lost their ability to reject homologous skin transplanted from Ce individuals(4). On the basis of these results we postulated that induction of tolerance by the technic of parabiosis might be due to a continuous exchange of all the transplantation antigens, perhaps in the form of intact cells between the 2 parabionts(2,3,4). However, recent experiments performed in this laboratory indicate that length of time during which parabiosis between homologous individuals must be maintained to achieve suppression of the homotransplantation reaction varied with strain of mice used, suggesting that time of foreign antigen exposure and exchange necessary to induce immunological tolerance in a given individual by this method, might perhaps be dependent on degree of histocompatibility difference between the individual and its parabiotic partner, *i.e.*, the more similar the 2 partners, the shorter the length of time in parabiosis required to achieve immunological tolerance.

Our purpose is to document observations bearing on this point and to demonstrate that duration of parabiosis required to produce tolerance is indeed a function of degree of histocompatibility difference between individuals and is also a characteristic of the strains of animals involved regardless of histocompatibility difference.

Method. In one group of experiments mice of the Z† strain and F_1 hybrids resulting from

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† Z is used to designate mice of C_3H strain originally obtained from Dr. J. J. Bittner's mouse colony.