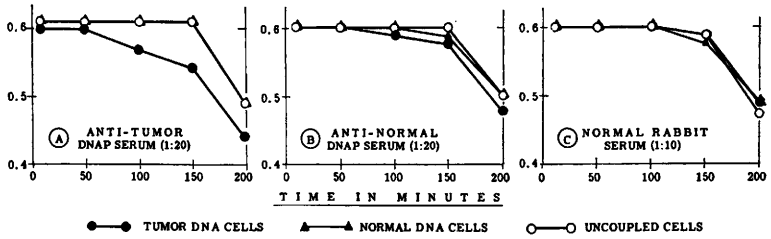

FIG. 2. Effect of DNA-ase upon settling rate of coupled and uncoupled cells in presence of rabbit antiserums and normal rabbit serum.

substance to stimulate antibody formation or to be detected by precipitin or agglutination reaction.

However, it must be admitted that it is difficult at present to understand why a hapten of the complexity of DNA would not precipitate with antiserum. This observation suggests that there might be an insufficient number of specific reactive sites per DNA molecule or that non-specific factors are involved, as observed by Ingraham(5) with respect to non-agglutination of formalin-treated red cells.

Summary. Formalinized erythrocytes exposed to purified DNA under the proper conditions can then be specifically agglutinated with antiserum, which has been prepared against the DNA-protein complex. Inhibition of the reaction by homologous DNA is strong evidence that agglutination is an antigen-antibody reaction and not merely a non-specific agglutination. The removal of this reactivity by desoxyribonuclease lends further support to the belief that DNA is functioning as the hapten. Trypsin does not alter the agglutination reaction.

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Growth-Promoting Properties of Pyruvate, Oxalacetate, and α-Ketoglutarate For Isolated Walker Carcinosarcoma 256 Cells. (24025)

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Puck and his associates devised procedures for growing isolated human cells in tissue culture(1,2). The medium consisted of whole serum, amino acids, vitamins, and balanced salt solution. Media containing dialyzed serum as replacement for whole serum did not sustain growth of single cells of the S3 colony strain of HeLa cells. Supplementation of the medium with cholesterol and a variety of coenzymes, however, enabled the cells to grow (3).

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The present communication concerns rapid growth of isolated Walker carcinosarcoma 256 cells induced by pyruvate, oxalacetate, and α-ketoglutarate in media containing dialyzed serum.

Methods. The basal medium (Table I) contained amino acids previously found essential or stimulatory for massive cultures of Walker tumor as well as 5 nonessential amino acids(4). Bovine serum was dialyzed against 2 changes of 100 volumes of Earle's solution at 6°C each for 12-24 hours on a shaker. Earle's solution used was modified to contain 3 g glucose/l, and an adjusted NaCl level to compensate for osmotic changes. Cells for inocula were grown from freshly excised tumor tissues in medium similar to the basal medium except that it contained 10% whole bovine serum. The inoculum was prepared by removing the used medium and separating cells by treatment with 0.05% trypsin (Bacto-Difco 1:250) 5 to 10 minutes at room temperature with occasional gentle agitation. Petri dishes were prepared in duplicate to contain 100 cells and graduated quantities of test compounds in 5 ml of basal medium. The dishes were incubated at 37°C for 7 days in air mixed with 7-9% CO_2 to maintain pH at approximately 7.2. For some of the work, cells were prepared by chopping fragments of excised tumor immersed in basal medium until a milky suspension of cells formed. This suspension was filtered through a pledget of glass wool. The inoculum obtained was utilized in the same manner as trypsinized cells.

Results. In early studies the cells produced colonies with 50-100% plating efficiency in basal medium containing whole bovine serum. Use of dialyzed serum for determination of amino acid requirements of isolated cells, however, resulted in formation of few or no colonies. Addition of coenzyme A, co-carboxylase, diphosphopyridine nucleotide, flavinadenine dinucleotide, and cholesterol as suggested by Sato *et al.*(3) did not improve the response of the cells. Intermediary metabolites, pyruvate,† oxalacetate,‡ or α-ketoglutarate,§ proved quite efficacious in promoting high plating efficiency and rapid cell division (Table II). The effects were detectable at

TABLE I. Basal Medium for Study of Growth-Promoting Effects of Intermediary Metabolites and Other Factors upon Isolated Walker Carcinosarcoma 256 Cells.

Constituent	Conc.*
Amino acids	mM/l
L-Arginine • HCl	.2
L-Histidine • HCl • H_2O	.1
L-Lysine • HCl	.2
Glycine	.1
DL-Alanine	.3
DL-Serine	.5
DL-Threonine	.3
DL-Valine	.3
L-Leucine	.3
L-Isoleucine	.3
L-Aspartic acid	.15
L-Glutamic acid	.15
L-Asparagine • H_2O	.3
L-Glutamine	1.5
DL-Phenylalanine	.2
L-Tyrosine	.1
DL-Tryptophan	.03
L-Proline	.15
Hydroxy-L-proline	.15
L-Cysteine • HCl • H_2O	.2
DL-Methionine	.2
Vitamins	μg/ml
Thiamine • HCl	.2
Riboflavin	.2
p-Aminobenzoic acid	1
Pyridoxine • HCl	.5
Pyridoxal • HCl	.5
Nicotinic acid	.5
Nicotinamide	.5
Calcium pantothenate	.2
Biotin	.2
Folic acid	.2
Vitamin B_{12}	.000075
Choline chloride	5.0
Inositol	9.0
Ascorbic acid	.5
Glutathione	.5
Salts	g/l
NaCl	6.46
KCl	.4
$MgSO_4 \cdot 7H_2O$	.2
$NaH_2PO_4 \cdot H_2O$	.14
$NaHCO_3$	2.20
$CaCl_2$	.2
Misc.	
D-Glucose	4.0 g/l
Phenol red	2.5 μg/ml
Potassium penicillin G	50 "
Streptomycin sulfate	50 "
Dialyzed bovine serum†	15% (vol)

* Concentration in final culture medium.
† Dialyzed 2 times for 12-24 hr against 100 vol of Earle's sol.

† Nutritional Biochemicals Corp., and Distillation Products Industries.

‡ Mann Research Laboratories, Inc.

§ H. M. Chemical Co.

TABLE II. Response of Isolated Walker Carcinosarcoma 256 Cells to Selected α-Keto Acids.*

Metabolite, mM/l	Pyruvate		Oxalacetate		α-Ketoglutarate	
	Colonies	Cells/colony†	Colonies	Cells/colony†	Colonies	Cells/colony†
.0	0	38– 60	4	11– 40	0	0
.01	11	43– 65	16	31– 119	17	14– 22
.02	30	98– 171	9	118– 221	36	67–304
.05	81	660– 728	90	804–1048	87	400–468
.1	85	660–1060	89	529–1140	79	572–772
.2	89	888–1148	94	1412–1484	97	616–748
.5	98	1243–1398	90	1192–1664	99	988–996

* Initial inoculum was 100 cells/petri dish; cultures were incubated 7 days at 37°C.
† Actual cell counts in 2 colonies representative of estimated 60% of colonies.

0.01 mM/l and almost optimal at 0.05 mM/l. In presence of these intermediates, plating efficiency approached 100%. The number of colonies in duplicate petri dishes usually agreed within less than 10%. The colony size easily attained 100 cells in 7-day incubation periods. Colony size did vary possibly because of the heterogeneous cell population, occasional colony formation from more than a single cell, or local variation of conditions in culture vessels. Small differences in generation times could result in appreciably different colony size after 10 generations which occurred in 7 days. A measure of cellular growth, however, was obtained by counting cells in colonies representative of 60% of the colonies.

Intermediary metabolites displayed similar activity, and this activity was the same when equivalent molar amounts of all combinations of these compounds were tested. Succinate, fumarate, citrate, iso-citrate, and *cis*-aconitate, and lactate were inactive. The related L-alanine, L-aspartic acid, L-glutamic acid, L-glutamine, and L-asparagine were already included in the basal medium which supported little or no growth; and nonessential amino acids—L-alanine, L-aspartic acid, L-glutamic acid, L-proline, and L-hydroxyproline—in quantities as high as 10 mM/l did not produce the stimulatory effects noted here. Furthermore, growth-promoting properties of active metabolites were not diminished by omission of the nonessential amino acids, ascorbic acid, and glutathione.

Cells prepared directly from freshly excised tumor responded to intermediary metabolites as did the trypsinized cells. These fresh cells were not used for routine studies because debris and proportion of damaged cells rendered initial enumeration less reliable.

One effect of dialysis of serum on clonal growth of isolated Walker cells was the removal of pyruvate, oxalacetate, and α-ketoglutarate from the serum. These compounds are reported present in blood at approximately the levels (0.1 to 0.2 mM/l) (5) which supported optimal growth of cells. Massive cultures of the Walker tumor did not require these compounds and media employed by Sato *et al.*(3), Eagle(6), McQuilkin *et al.*(7), and Healy *et al.*(8) were devoid of these intermediary metabolites. Fischer's Medium 605(9), however, did contain them. The role of these metabolites in survival of isolated Walker cells is, at present, unknown; but these results suggest many interesting facets of tumor cell metabolism. For example, α-keto acids are well known for their participation in transamination processes in addition to their role in energy production, and these reactions may assume a more important role under conditions of clonal growth. The inclusion of coenzymes in the present medium could not be used in lieu of pyruvate, α-ketoglutarate, and oxalacetate.

Although future studies of exhaustively dialyzed serum or serum fractions may reveal requirements for further small molecular compounds, the present media containing twice dialyzed bovine serum and pyruvate, oxalacetate, or α-ketoglutarate is practical and effective for investigation of nutritional requirements of isolated Walker tumor cells and its clonal strains. Our experiments utilizing such media invariably led to a high plating efficiency and rapid growth of isolated Walker carcinosarcoma 256 cells.

Summary. When Walker carcinosarcoma 256 cells were grown under clonal growth technics, 50-100% plating efficiency was noted in a medium containing whole bovine serum. When dialyzed serum was used in the medium, few or no colonies were formed. Addition of pyruvate, α-ketoglutarate, and/or oxalacetate to the substrate resulted in plating efficiencies of 90-100%. Activity of these 3 metabolites was the same irrespective of addition of individual compounds or combinations thereof. Other Kreb's acids and metabolites tested failed to promote survival and growth of isolated Walker cells.

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Observations on Arteriovenous Communications in Lungs of Dogs.* (24026)

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The presence of precapillary arteriovenous communications in mammalian lungs has been one of the interesting questions of pulmonary vascular physiology. The study of Prinzmetal *et al.*(1) is often cited as evidence for these vascular communications. However, other workers(2,3) have suggested that precapillary shunts in the lungs are either very small or are possibly not extant at all. Because of the importance of these vascular passages, if present, in the economy of the pulmonary circulation, the following investigation was carried out.

Method. Mongrel dogs (8-14 kg) were anesthetized with sodium pentobarbital, and conventional Starling heart-lung preparations were set up. The arterial cannula was provided with a large side arm so that left ventricular output could be diverted to collecting vessels. Blood was heparinized. Pulmonary arterial pressure was recorded with a mercury manometer connected into the right upper lobar arterial branch. Cardiac output was adjusted to 400-500 ml/minute. Peripheral resistance was 80-90 mm Hg. Mean arterial pressure was usually 10-15 mm Hg. The preparations functioned without visible dilatation of the hearts. Lucite microspheres‡ in 2 g quantities were suspended in dog plasma colored with Evans blue dye and were rapidly introduced into the right atrium through stoppered venous cannula. Spheres of 75 μ and 250-300 μ were assorted by screening and agitation and the 2 sizes of spheres were used in all experiments since this quantity produced moderate rises in pulmonary arterial pressure. Two grams of 75 μ spheres comprise 1 million particles by count and 2 g of 250-300 μ particles number 500,000. After introduction of the plasma-sphere suspension, as the dye appeared in the arterial cannula, the cardiac output was diverted for 45 seconds through a stainless steel screen (325 mesh) to sieve out spheres which passed through the

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