PRIMARY MOUSE KIDNEY CULTURES

Materials and Methods

Media and solutions

Growth medium. All cultures are grown in Eagle's medium (EM) with fourfold concentration of amino acids and vitamins, and 5% or 10% horse serum.

Tris-buffered saline (TBS). NaCl 0.8%, KCl 0.038%, Na_2HPO_4 0.01%, $CaCl_2$ 0.01%, $MgCl_2 \cdot 6\ H_2O$ 0.01%, dextrose 0.1%, Tris (Sigma 7-9) 0.3%. Adjusted to pH 7.4 with 1 N HCl.

TD. As for TBS, but minus $CaCl_2$, $MgCl_2$, and dextrose.

Standard buffer. 0.01 M Tris (pH 7.9 at room temperature), 0.06 M KCl.

2 x Trypsin

Trypsin 1-300 (Nutritional Biochemicals Corp.), 5 grams
Mix trypsin in a small amount of water (100-150 ml), add:

Penicillin solution	10 ml
Streptomycin solution	1 ml
Phenol red solution	3 ml

Add distilled water to bring volume to 1 liter.
Let stand in refrigerator overnight, Seitz filter. Store in stoppered containers in the refrigerator.

When used, mix with an equal volume of 2 x TD.

Method

Amounts are given for 100 mice.

1) Kill mice (12-15 days old) by pressing at base of skull with blunt object (pencil or forcep) and by pulling tail.

2) Wash mice with duponol solution and 75% alcohol; with mice face down, pull back skin with a set of sterile scissors and forceps. With another set of scissors and forceps pierce peritoneum on both sides of spine in lumbar region. Apply slight pressure at side of incision, kidneys will be exposed for removal.

3)Kidneys are washed in TBS to remove blood and hair, and, after removal of the outer membrane, washed 3 times with TBS before being minced into small pieces (1 mm) with curved scissors.

4) Resuspend mince in 100 ml of 0.05% trypsin in TD. Incubate at 37° for one hour.

5) After incubation, pour total suspension into trypsinization flask. Stir 10 min. on magnetic stirrer.

6) Allow large clumps to settle. Pour off cell suspension into a second trypsinization flask containing 20 ml 0.25% trypsin. Stir 10 min. After 10 min. check length of tubules under microscope. If 50% of tubules are short (approximately the length of the smallest square on a hemocytometer slide--0.004 mm), pour off suspension into a centrifuge bottle and stop digestion with 20% horse serum; otherwise continue stirring until the desired length is reached.

7) Add 100 ml 0.05% trypsin in TD to first flask containing remaining mince and stir 10 min. before pouring into second flask and repeating Step 6. Four to five fractions are usually taken in this manner.

8) Centrifuge 10 min at 1100 rpm in International Centrifuge No. PR-2 with 250 ml bottles. Resuspend in EM with 10% horse serum. Wash pellet with EM with 10% horse serum in 40 ml centrifuge tubes three times or until free of mucous and debris; centrifuge after each wash at 1100 rpm for three minutes. Pool all fractions. Seed 0.04 to 0.05 ml pellet in 60 mm Falcon petri dish; 2 to 2-1/2 times pellet size for 100 mm dish.

Procedure takes four to five hours to complete and is done at room temperature - 65°-75° F.

Modified two fold Eagle's 5 liter

1, Thaw frosen sol. of amino acids and vitamins for 10 L Eagle
2, Dissolve L-Tyrosine 0.72 g in about 800 ml dist. water by heating up to boiling.
3, Dissolve Magnesium sulfate 7 H_2O 2 g in the tyrosine sol. (2)
4, Dissolve L-Cystine 0.48 g in about 250 ml 60°C dist water by adding 4-4.5 ml N.NaOH and swirl.
Weigh into a 6 L. flask:
5, Sodium chloride.....64.00 g
6, Potassium chloride..... 4.00 g
7, Calcium chloride anhydrous..... 2.00 g
8, Add dist water about 2 liter to dissolve the chlorides.
9, Add the Tyrosin and Magnesium sulfate sol. (2+3) to the sulution of the chlorides (8).
10, Add the amino acids
11, Phenol red sol 1 %.....15.00 ml
12, Sodium phosphate monobasic..... 1.25 g
13, Dextrose anhydrous..... 45.00 g
14, Inositol..... 0.07 g
15, Ferric nitrate sol. 0.01%.....10.00 ml
Cool in refrigerator.
16, Add: Streptomycin sulfate sol. 5 ml. (1g)
17, Penicillin G Potassium 5,000.000 units
18, Antimycotic sol. 10 ml. (n-butyl-p-hydroxybenzoate 0.02%)
19, Cystine sol. (4)
20, Sodium bicarbonate 37.00 g dissolved in 1 L.dist.water
21, Vitamine sol.
22, L-Glutamine.....5.84 g
23, Sodium pyruvate.....1.10 g
24, Make up the volume to 5 L. with dist.water.
25, Flush with CO_2 to almost neutral and filter.

Amino acids for 100 l Eagle

L Arginine HCl.....	8.40 g
L histidine HCl H_2O.....	4.20 "
L Isoleucine.....	10.48 "
L Leucine.....	10.48 "
L Lysine HCl.....	14.62 "
L Methionine.....	3.00
L Phenylalanine.....	6.60 "
L Threonine.....	9.52 "
L Tryptophane.....	1.60 "
L Valine.....	9.36 "
L Serine.....	4.20 "
Glycine	3.00 "

Dissolve the amino acids in about 1500 ml 60° C dist.water, make up to 2 liter. Divide in 300 ml flasks, 200 ml in each for 10 l E.
Freeze.

Vitamins for 100 l Eagle

Choline chloride	0.4 g
Nicotinamide	0.4 "
Sodium pantothenat	0.43
Pyrodoxal HCl	0.4 "
Thiamine HCl	0.4 "
Riboflavin	0.04

Dissolve in about 500 ml dist. water.
Dissolve Folic acid 0.4 g in 2-300 ml dist.water by adding 1 N NaOH dropwise until sol. is clear.(takes 1.8-2.0 ml)
Add Folic acid sol to the other vitamins. If necessary clear the sol. by addind more 1 N NaOH.
Make up the volume to 1 liter.
Divide to 100 ml portions, for 10 liter E.
Freeze.

Buffer solutions

Phosphate buffer sol.PBS.

Chemicals	gm/l	mM
Sodium chloride	8.0	136.8
Potassium chloride	0.2	2.5
Sodium phosphate dibasic	0.115	0.8
Potassium phosphate monobasic	0.2	1.47
Calcium chloride anhydrous	0.1	0.9
Magnesium chloride 6 H_2O	0.1	0.5

Tris buffer

Sodium chloride	8.0	40	20 ✓	136.8
Potassium chloride	0.38	1.9	9.7 ✓	5.0
Calcium chloride anhydrous	0.1	.5	1.5 ✓	0.9
Magnesium chloride 6 H_2O	0.1	.5	1.5 ✓	0.5
[illegible] phosphate dibasic	0.1	—		0.7
Sig[illegible] 7-9	3.0	15	45 ✓	25.0

Adjust To pH 7.4 ± HCl 12 N

TD

TD= Tris buffer without calcium and magnesium.

Dulbecco's Modified Eagle Medium

Chemicals	mg/L	mM
Sodium chloride	6400.0	109.4
Potassium chloride	400.0	5.4
Calcium chloride anhydrous	200.0	1.8
Magnesium sulfate 7 H_2O	200.0	0.8
Sodium phosphate monobasic	125.0	0.9
Dextrose anhydrous	4500.0	25.0
Inositol	7.0	0.04
Ferric nitrate 9 H_2O	0.1	0.00025
Sodium bicarbonate	3700.0	44.0
Sodium pyruvate	110.0	1.0
Phenol red	15.0	0.04
Amino acids		
L-Arginine HCl	84.0	0.4
L-Cystine	48.0	0.2
L-Glutamine	584.0	4.0
Glycine	30.0	0.4
L-Histidine HCl H_2O	42.0	0.2
L-Isoleucine	104.8	0.8
L-Leucine	104.8	0.8
L-Lysine HCl	146.2	0.8
L- Methionine	30.0	0.2
L-Phenylalanine	66.0	0.4
L-Serine	42.0	0.4
L-Threonine	95.2	0.8
L-Tryptophane	16.0	0.08
L-Tyrosine	72.0	0.4
L-valine	93.6	0.8
Vitamins		
Choline chloride	4.0	0.024
Folic acid	4.0	0.009
Nicotinamide	4.0	0.033
Calcium pantothenate	4.0	0.008
Pyrodoxal HCl	4.0	0.02
Riboflavin	0.4	0.001
Thiamine HCl	4.0	0.012
Antibiotics		
Penicillin G Potassium	500.000 units	
Streptomycin sulfate, equiv. base	100.0	0.17
Antimycotic		
N-butyl-p-hydroxybenzoate	0.2	0.001

Puck-Eagle Medium (PEM)

N16	40%
Eagle's	40%
NCTC	4%
[Serum	1%]

N16

SOLUTIONS FOR N16 STORED IN DEEP FREEZE

"N16 I" for 30 liters N16

L-Arginine HCl	1.125 g
L-Histidine HCl	1.125 "
L-Lysine HCl	2.40 "
L-Tryptophane	0.60 "
β-phenyl-L-Alanine	0.75 "
L-Methionine	0.75 "
L-Threonine	1.125 "
L-Leucine	0.75 "
DL-Isoleucine	0.75 "
DL-Valine	1.50 "
L-Glutamic	2.25 "
L-Aspartic	0.90 "
L-Proline	0.75 "
Glycine	3.0 "

Dissolve in 1.5 liters of H_2O (60°C) 150 ml
in 300 ml flask pro 3 liters N16.

"N16 II" for 30 liters N16

Hypoxanthine	0.75 g
L-Cystine	0.225 "
Thiamine HCl	0.15 "
Riboflavin	0.015 "
Pyridoxin HCl	0.015 "
Folic acid	0.003 "
Biotin	0.003 "
Choline	0.090 "
Ca Pantothenate	0.090 "
Niacinamide	0.090 "
i-Inositol	0.030 "

Dissolve in 100 ml of H_2O by adding 1N NaOH to it.
Make up to 150 ml.
5 ml in 15 ml tube pro 1 liter N16.

N16 (continued)

Steps:

No. 1

NaCl	6.4 g
KCl	0.4 "
$CaCl_2$ anhydr.	0.2 "
Dissolve in ≈	250 ml of H_2O.
Phenol red sol. 1%	1.5 ml
$MgSO_4 \cdot 7\ H_2O$	0.2 g

No. 2

L-Tyrosine	0.04 g
Dissolve in ≈	100 ml of H_2O by heating almost to boiling point.

No. 3 Add No. 2 to No. 1.

No. 4 Add solution of amino acids, labeled "N16 I" and stored in deep freeze.

No. 5 Neutralize by adding 1 N NaOH.

No. 6

Add $NaHPO_4 \cdot H_2O$	0.125 g
Dextrose	4.5 "
$Fe(NO_3)_3 \cdot 9\ H_2O$ sol.	1.0 ml

Cool in refrigerator.

No. 7

$NaHCO_3$	3.7 g
Dissolve in ≈	100 ml of H_2O.

Cool in refrigerator.

No. 8

Penicillin sol.	5.0 ml
Streptomycin sol.	0.5 "
Antimycotic sol.	1.0 "
L-Glutamine	0.2 g

No. 9 Add vitamins, etc., sol., labeled "N16 II" and stored in deep freeze.

No. 10 Add No. 7 ($NaHCO_3$ sol.) to it.

Make up to 1000 ml $\bar{c}$ cold H_2O.

Eagle's Medium (E)

Eagle's is usually made in batches of 10 liters, although for special purposes smaller quantities can be made.

VITAMINS: Amounts of vitamins sufficient for 100 liters are weighed, dissolved, and divided among 10 flasks to be stored in deep freeze.

	1 liter	100 liters
Choline	4 mg	0.4 grams
Nicotinamide	4 "	0.4 "
Pantothenic acid	4 "	0.4 "
Pyridoxal HCl	4 "	0.4 "
Thiamine	4 "	0.4 "
Riboflavin	0.4	0.04 "

Dissolve in about 500 ml distilled water.

Folic acid	4 mg	0.4 grams

Dissolve in 200-300 ml distilled water by adding 1 N NaOH dropwise until solution is clear (takes 1.5-1.6 ml total for 0.4 grams).

Add folic acid mixture to other vitamins; add more 1 N NaOH if necessary to clear the solution. Make up to 1 liter with distilled water in the volumetric flask and distribute in flasks and tubes (100 ml for 10 liters of Eagle's; 10 ml for 1 liter). Store in deep freeze.

Ferric nitrate stock solution for Eagle's:

$Fe(NO_3)_3 \cdot 9\ H_2O$	10 mg
Distilled water	100 ml

Eagle's Medium (continued)

AMINO ACIDS: Amounts of amino acids sufficient for 100 liters are weighed, dissolved, divided into 10 flasks, and frozen; one flask is used to make 10 liters of Eagle's.

	conc. in Eagle's 1 liter	100 liters
L-Arginine HCl	0.0840 gr	8.40 g
L-Histidine HCl-H_2O	0.0420 "	4.20 "
I-Isoleucine	0.1048 "	10.48 "
L-Leucine	0.1048 "	10.48 "
L-Lysine HCl	0.1462 "	14.62 "
L-Methionine	0.03 "	3.0 "
L-Phenylalanine	0.066 "	6.6 "
L-Threonine	0.0952 "	9.52 "
L-Tryptophane	0.016 "	1.6 "
L-Valine	0.0936 "	9.36 "
Glycine	0.03 "	3.0 "
L-Serine	0.042 "	4.20 "

Dissolve in a 2 l. volumetric flask, using about 1200-1400 ml of 60°C H_2O.

When amino acids are dissolved make up the volume to 2 l.

Divide in 300 ml flasks, 200 ml in each for 10 l.E, and in 50 ml flasks, 20 ml in each for 1 l.E.

Freeze.

Store in deep freeze.

Eagle's Medium (continued)

MIXING: Eagle's is made up as two-fold Eagle's (2xE): it is then either used as 2xE or is mixed equally with water to make regular Eagle's (E).

1. Thaw frozen solution of amino acids for 10 l E/or weigh and dissolve in 60°C distilled H_2O.

2. Thaw frozen solution of vitamins for 10 l E/or weigh and dissolve as indicated on page 1.

3. Dissolve L-Cystine 0.48 g in 200-300 ml 60°C distilled water by adding 3-4 ml of 1 N NaOH and shaking.

4. Dissolve L-Tyrosine 0.72 g in 500-600 ml distilled H_2O by heating up boiling.

5. Dissolve $NaHCO_3$ 37 g in 1 liter of distilled H_2O.

6. Weigh into a 6 l. flask:

NaCl	64 g
KCl	4 "
$CaCl_2$ anhydrous	2 "
Add distilled water	2 liters, dissolve chlorides.
$MgSO_4 \cdot 7\ H_2O$	2 g
Add No. 4, the still hot or warm L-Tyrosine solution.	
After $MgSO_4$ has been dissolved:	
$NaH_2PO_4 \cdot H_2O$	1.25 g
Inositol	0.07 "
Dextrose	45. "
$Fe(NO_3)_3$ sol.	10 ml (or $Fe(NO_3)_3$ 0.001 g)
Add No. 5, the $NaHCO_3$ solution.	
Cool in refrigerator.	
Add: Streptomycin solution	5 ml
Penicillin "	50 "
Antimycotic "	10 "

7. Pour No. 1 (amino acids) and No. 2 (vitamins) in a larger flask.
   Add 1% phenol red solution 15 ml.
   Add No. 3 (L-Cystine solution).
   Flush with CO_2 to make solution almost neutral.

8. Add No. 7 to No. 6.
   Make up the volume to 5 l. with distilled H_2O.

9. Dissolve L-Glutamine 5.84 g and sodium pyruvate 1.1 g in the cold medium.
   To make regular Eagle's dilute 1 part of 2xE with 1 part distilled H_2O.

10. Flush with CO_2 to almost neutral.

    Filter.

NCTC 109

VITAMINS FOR 1 LITER NCTC 109

I.	Choline chloride	1.25 mg
	Vitamin B_{12}	1.00 "
	Ascorbic acid	49.90 "
II.	Vitamin A (cryst. alc.)	2.5 "
	Calciferol (Vit. D)	2.5 "

Dissolve in 10 ml of 95% alcohol.
Use 1.0 ml pro 1 liter NCTC.

III.	Menadione (Vit. K)	2.5 mg

Dissolve in 10 ml of 95% alcohol.
Use 0.1 ml pro 1 liter NCTC.

IV.	α Tocopherol phosphate disodium salt	2.5 mg

Dissolve in 10 ml of 95% alcohol.
Use 0.1 ml pro 1 liter NCTC.

V.	Thiamine HCl	2.5 mg
	Riboflavin	2.5 "
	Pyridoxin HCl	6.25 "
	Pyridoxal HCl	6.25 "
	Niacin	6.25 "
	Niacinamide	6.25 "
	Pantothenate calc.	2.50 "
	Biotin	2.50 "
	Folic acid	2.50 "
	i-Inositol	12.50 "
	p-Aminobenzoic acid	12.50 "

Dissolve in ≈ 80 ml of H_2O by adding 2-3 drops of 1N NaOH.
Make up to 100.0 ml in volumetric flask.
Use 1 ml pro 1 liter NCTC.

COENZYMES FOR 1 LITER NCTC 109

DPN	Diphosphopyridine nucleotide (Cozymase, Coenzyme 1)	~~1.0 mg~~ 7.0 mg
CoA	Coenzyme A	2.5 "
TPN	Triphopyridine nucleotide monosodium	1.0 "
TPP	Thiamine Pyrophosphate Chloride = Cocarboxylase	1.0 "
FAD	Flavin Adenine dinucleotide	1.0 "
UTP	Uridine-5'triphosphate sodium salt	1.0 "

NCTC 109 (continued)

NCTC 109 - 1 liter

L-Alanine	31.48 mg
L-Alpha amino butyric acid	5.51 "
L-Arginine	25.76 "
L-Asparagine	8.09 "
L-Aspartic acid	9.91 "
L-Cysteine	26.00 "
D-Glucosamine	3.20 "
L-Glutamic acid	8.26 "
Glycine	13.51 "
L-Histidine	19.73 "
Hydroxy-L-Proline	4.09 "
L-Isoleucine	18.04 "
L-Leucine	20.44 "
L-Lysine	30.75 "
L-Methionine	4.44 "
L-Ornithine	7.38 "
L-Phenylalanine	16.53 "
L-Proline	6.13 "
L-Serine	10.75 "
L-Taurine	4.18 "
L-Threonine	18.93 "
L-Tryptophan	17.50 "
L-Valine	25.00 "

Dissolve in ≈ 100 ml of H_2O 60°C in a 1000 ml volumetric flask.

Add ≈ 200 ml of H_2O and:

NaCl	6.8 g
KCl	0.4 "
$CaCl_2$	0.2 "
$MgSO_4 \cdot 7\ H_2O$	0.2 "
Phenol red sol. 1%	2.0 ml
$NaH_2PO_4 \cdot H_2O$	0.14 g
Glucose	1.0 "
L-Tyrosine	16.44 mg

Dissolve in ≈ 100 ml of H_2O heating it almost up to boiling point.

Cool to room temperature.

Add to the amino acids-salts solution.

Cool in refrigerator then add:

NCTC 109 (continued)

Sodium acetate	50.0 mg
Sodium glucuronate	1.8 "
Glucuronolactone	1.8 "
Glutathione monosodium salt	10.10 "
L-Glutamine	135.73 "
Deoxyadenosine	10.0 "
Deoxycytidine HCl	10.0 "
Deoxyguanosine	10.0 "
Thymidine	10.0 "
5-Methyl-cytosine	0.1 "
Tween 80	1.25 "
Cysteine HCl	259.90 "

Neutralize by 1N NaOH.

Dissolve L-cystine 10.49 mg
in ≈ 50 ml of H_2O 60°C by adding 1-2 drops of 1N NaOH.

Cool to room temperature. Add to the solution above.

Add vitamins.

Add $NaHCO_3$ 2.2 g
dissolved in ≈ 200 ml of H_2O.

Add coenzymes.

Make up to 1 l c cold H_2O.

Flush c CO_2. Filter. Keep in refrigerator.