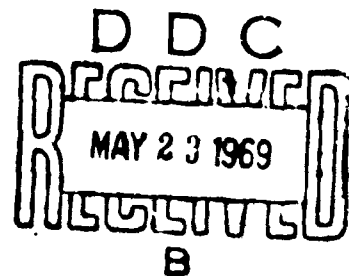


AD 687218

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A SIMPLIFIED METHOD OF TRYPSINATING AND GROWING
OF RABBIT KIDNEY CELL MONOLAYER CULTURES
COUNTRY: USSR

TECHNICAL TRANSLATION

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FSTC-HT-23-728-68

A SIMPLIFIED METHOD OF TRYPSINATION AND GROWING
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I.S. Kuchmasov and M.S. Yudina

SOURCE: VETERINARIYA
(Veterinary)
No. 1, pp. 22-24, 1966
USSR

Translated for FSTC by Techtran Corporation

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UNCLASSIFIED
Security Classification

DOCUMENT CONTROL DATA - R & D		
(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)		
1. ORIGINATING ACTIVITY (Corporate author) Foreign Science and Technology Center US Army Materiel Command Department of the Army		2a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED
3. REPORT TITLE A SIMPLIFIED METHOD OF TRYPSINATION AND GROWING OF RABBIT KIDNEY CELL MONOLAYER CULTURES		2b. GROUP
4. DESCRIPTIVE NOTES (Type of report and inclusive dates) Translation		
5. AUTHOR(S) (First name, middle initial, last name) I. S. Kuchmasov and M. S. Yudina		
6. REPORT DATE APR 9 1969	7a. TOTAL NO. OF PAGES 7	7b. NO. OF REFS N/A
11. CONTRACT OR GRANT NO. a. PROJECT NO. 980360102 c. 9223628 2301 d.		8a. ORIGINATOR'S REPORT NUMBER(S) FSTC-HT-23- 728-68 8b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report) ACSI Control Number (None)
10. DISTRIBUTION STATEMENT Distribution of this document is unlimited.		
11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY US Army Foreign Science and Technology Center
13. ABSTRACT The authors recommend the hot (37°) single-step method for trypsinization of kidneys of baby and adult rabbits. This method was found to be superior to others tried in yield of cells per gram of tissue, trypsin consumption, growth achieved, and economy. The use of Hottinger muscle digest as the nutritive medium for cultivation insures great availability of rabbit cultures for institutes and practical laboratories. The use of monolayer rabbit kidney cultures in veterinary virology is promising because of the possibility of creating standard conditions for obtaining tissue.		

DD FORM 1473 1 NOV 66
1 NOV 66 1473 1 JAN 68, UNCLASSIFIED
REPLACES FORM 1473 1 JAN 68, UNCLASSIFIED

UNCLASSIFIED
Security Classification

UNCLASSIFIED
Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Trypsinization Tissue culture Rabbit kidney cells Monolayer culture Virology						

UNCLASSIFIED
Security Classification

A SIMPLIFIED METHOD OF TRYPSINATION AND GROWING OF RABBIT KIDNEY CELL MONOLAYER CULTURES

The method of trypsination of monkey liver proposed by Dulbecco and Fogt [8], subsequently improved by Jongner [11], Rappaport [10], Dofer, *et al.* [2, 3], is widely accepted in virology as a method of hot fractional trypsination, which allows one to obtain steady growth of monolayer cultures with the optimal yield of cells in 1 g of tissue.

However, this method of trypsination has two essential disadvantages which reduce its value -- the great expense of trypsin and labor. Therefore, many investigators are undertaking measures toward simplification of the trypsination process.

Thus, Bodien [7] proposed a cold, 2-stage method of trypsination, A. A. Syelivanov [5], I. N. Dobrova [1] and others -- hot, 1-stage and continuous methods of trypsination of kidney of monkey and of other animals, and also amniotic membranes.

In the foreign literature there are reports of development of a method of hot fractional trypsination of rabbit kidneys, in the domestic -- A. A. Sviridov [4] with a hot, 1-stage trypsination of kidneys of 1 to 2 day old rabbits.

We have not found comparative studies of the various methods of trypsination of rabbit kidneys in the literature, despite the fact that these cultures possess a broad spectrum of sensitivity to many viruses (hoof and mouth disease, Aveski's¹ disease, etc.).

In the present work we set the goals:

- a) comparative study of hot fractional, hot single-step (37°) and cold (+4°) methods of trypsination of rabbit kidneys from 2 days to 2 months in age;

1 Transliterated from the Russian. Exact spelling uncertain. - Tr.

b) to find the most economical, simplest, most accessible method of trypsinization under practical conditions;

c) to explore the possibility of culturing monolayer cultures without changing the nutritive medium;

d) maximally simplify the entire process of obtaining tissue cultures, beginning with treatment of the glass and ending with trypsinization.

Methods

Treatment of dishes, stoppers, and rubber tubes. For a period of two years, bacteriological test tubes and flasks from the usual domestically produced glass were used for tissue culture.

Dust was washed from a new dish with tap water and it was left for one day in 1% solution of sulfuric acid. Then it was washed with the help of a brush in a hot solution of trisodiumphosphate (150 g trisodiumphosphate in 10 l of water) and was rinsed 5-6 times with cold running tap water. In order to neutralize the alkali, the dish was washed in 2% solution of hydrochloric acid for 5-10 minutes, then again rinsed with tap water, and then 3 times with distilled.

The plate was dried in a desiccator, arranged and sterilized 2-3 times with dry heat at 160-180°. A controlled sterility of the plates is the blackening of sucrose, which is placed in the desiccator in test tubes on each shelf.

Dishes formerly in use were treated by the same method, but beginning with washing in trisodiumphosphate solution.

New rubber tubes and stoppers were first washed in tap water, boiled for one hour in 5% solution of sodium bicarbonate, and carefully rinsed with tap water, boiled 2-3 times in distilled water, washed 3 times in the same water, air dried, mounted and autoclaved for a period of one hour at 2 atm.

Stoppers on tubes formerly in use were washed and boiled 30 minutes in distilled water, dried and autoclaved. Tappettes were washed with potassium dichromate according to the generally accepted method. They were sterilized with dry heat for two or three hours at 160-180°.

Solutions and Nutritive Media

a) Hanks' solution was prepared according to the generally accepted method. Water was first distilled in a D-1 still, and then deionized

in a "Elgastat". In order to prepare a solution called "chemically pure" or "analytical grade", salts were used.

The solution was autoclaved in two stages for ten minutes at 0.7 atm. on two successive days. After being autoclaved the first time, the solution was kept for one day in a thermostat of 37°, and after the second day it was kept at room temperature for a month and more under cotton plugs. Before use, a sterile 7.5% solution of sodium bicarbonate at pH 7.2 was added to it.

b) 7.5% solution of sodium bicarbonate was prepared in deionized water, sterilized by filtration through a Zeiss filter, stored in flasks or test tubes with rubber stoppers in the cold at +4° for several months.

c) 0.25-0.3% solutions of "Londonskiy" or "Disco" trypsin were prepared in Hanks' solution, pH 7.4-7.5, activity according to Casein of 125-250 units. It was sterilized by filtration through a Zeiss or Sal'nikov filter, dependent on the volume of the prepared solution. It was kept at a temperature of 4-6° for 2 months, and the activity was insignificantly reduced. Before use, about 100 units of penicillin and streptomycin per ml were added to the trypsin.

d) 0.5% solution of lactalbumin hydrolysate was prepared by the generally accepted method in Hanks' solution, the pH brought to 7.4 with sodium bicarbonate, then reduced to 6.7-6.8 by bubbling CO₂ through the solution, filtered through a Sal'nikov filter. Before use, 10% native bovine serum and about 100 units of penicillin and streptomycin per ml were added.

e) 0.5% solution of Hottinger muscle hydrolysate in Hanks' solution with 10% native bovine serum were prepared according to the protocol of G. Ye. Pankova and D. A. Tsuvyerkalov [6].

Trypsinization. a) Preparation of tissue. Kidneys from rabbits bled immediately before trypsinization were extracted with observance of the rules of Asepsis. First the capsule was removed, then the cortical and cerebral substance of the kidneys were minced into small pieces (2-3 mm) with scissors, rinsed with Hanks' solution, 0.25 or 0.3% trypsin solution was poured in and it underwent trypsinization.

b) Hot fractional method of trypsinization. Small pieces of kidney tissue in a trypsin solution was dispersed by means of mixing in a magnetic stirrer in a flask of 0.25 liter volume and 37°. After 30 minutes the suspension of digested tissue fragments was decanted, and to the small pieces of tissue were added a new portion of the

trypsin solution and it was mixed for 10 minutes.

The cell suspension in the trypsin solution was decanted into centrifuge bottles and placed in a refrigerator (+4°), and a new portion of trypsin added to the small pieces of tissue. The process of trypsinization was continued until complete separation of all the tissue was achieved (mixing time -- 10 minutes).

The collected cell suspensions were centrifuged at 1,000 rpm for 10 minutes, the cell precipitate was resuspended in nutritive medium. The concentration of cells is brought up to the 300-400 thousand per ml and about 1 ml inoculated into test tubes and about 150 ml into 1.5 liter flasks. The medium was changed after the second day of cultivation under stationary conditions of 37°.

c) Hot single-step method of trypsinization. Small pieces of minced tissue were dispersed in the trypsin solution in a magnetic stirrer at 37° in a single process until complete separation of all cells was achieved. The time of trypsinization was 2.5-3 hours for kidneys of 1.5-2 month old rabbits and 1-1.5 hours for kidneys of 2 day old rabbits.

d) Cold single-step of trypsinization. Small pieces of kidney tissue in trypsin solution were separated in our refrigerator at +4-10° in a magnetic stirrer for a period of 17 hours. Further treatment of the cells and culturing was the same as in preceding methods.

e) Method of calculating cells. A sample of the cell suspension in nutritive medium was diluted 5-fold with physiologic solution of NaCl, then 1 ml of the diluted suspension was taken with 1 ml of 0.1% solution of crystal violet in a 0.1 M solution of citric acid. The 10-fold diluted cell suspension was loaded into a Goryaev chamber. All cells were counted which had complete cytoplasm, membrane and nucleus. Cell conglomerates, in which it was not possible to count nuclei, were counted as one cell. The cell concentration in 1 ml of suspension was determined by the formula:

$$X = \frac{a \cdot A \cdot 4000}{b \cdot 270} \cdot 1000,$$

where: X = the quantity of cells in 1 ml of suspension,
a = the quantity of cells in the Goryaev chamber,
b = the dilution of cells.

Results

The average yield of viable cells in 1 g of tissue was 34.8 million with the hot fractional method, 37.3 with the hot single-step, and 21.6 million with the cold (Table).

The great variation in yield in viable cells depended upon the individual characteristics of the rabbits (age, fatness, etc.), and also on the use of fresh or old trypsin solutions. The younger the rabbits, the greater the yield of cells; the fresher the trypsin solution, the more rapid and more complete the breakdown of the small pieces of tissue.

In the test tubes and flasks upon seeding 300-400 thousand cells in ml of nutritive medium, stable growth of monolayer cultures was obtained in all experiments.

Yield of Viable Cells, Outlay of Trypsin, and Time of Breakdown of Tissue with Various Methods of Trypsinization of Rabbit Kidney.

		a) Способы трипсинизации											
e)	f)	b) горячий дробный				c) горячий одношаговый				d) холодный одношаговый			
		g)	h)	i)	j)	g)	h)	i)	j)	g)	h)	i)	j)
число опытов	возраст кролика (мес.)	время трипсинизации (час.)	расход трипсина (мл)	количество ткани (г)	время выдержки на 1 г ткани (мин.)	время трипсинизации (час.)	расход трипсина (мл)	количество ткани (г)	время выдержки на 1 г ткани (мин.)	время трипсинизации (час.)	расход трипсина (мл)	количество ткани (г)	время выдержки на 1 г ткани (мин.)
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Key: a, Method of trypsinization; b, Hot fractional; c, hot single-step; d, cold single-step; e, number of experiment; f, age of rabbits (months); g, days; h, time of trypsinization (hours); i, outlay of trypsin (ml); j, quantity of tissue (g); k, yield of cells in 1 g of tissue (millions)

With the hot fractional and the hot single-step method the trypsinization of kidneys of 1-2 day old rabbits a monolayer was obtained after 2 days without change of medium, from adult rabbits, on the fourth to fifth day with a change in medium after two days.

With the cold method of trypsinization, monolayer cultures of kidneys of adult rabbits were obtained on the sixth to eighth day. Kidneys of two day old rabbits were not trypsinized by the cold method.

Experiments were carried out in growing monolayer cultures of kidneys of adult rabbits in nutritive media with pH = 7.2; 7.4; 7.5 and 7.6 without change of nutritive medium.

For this purpose media with pH 7.5-7.6 proved to be best, however, monolayer cultures were obtained only on the eighth to eleventh day, and with pH 7.2-7.4 monolayer cells obtained failed because of rapid souring of the medium, which led to degeneration of the cells. In order to grow monolayer cultures of rabbit kidneys with positive results, nutritive medium was used which was prepared on a base of Hottinger muscle hydrolysate (according to the protocol of G. Ye. Pankova and D. A. Tsuverkalov). In this medium monolayer kidney cells also grew on the fourth to fifth day, as they did in lactalbumin hydrolysate.

Conclusions

1. For trypsinization of baby rabbit and adult rabbit kidneys, it is best to use the hot (37°) single-step method, which is superior to the others which we used in yield of cells in 1 g of tissue and consumption of trypsin; it is simpler and more economical.
2. The use of Hottinger muscle digest as the nutritive medium for cultivation insures great availability of rabbit kidney cultures for institutes and practical laboratories.
3. Use of monolayer cultures of rabbit kidneys in veterinary virology is promising because of the possibility of creating standard conditions for obtained the tissue.

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