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TRYPSINIZATION OF MONKEY-KIDNEY TISSUE: AN AUTOMATIC METHOD FOR THE PREPARATION OF CELL SUSPENSIONS *

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SYNOPSIS

A study of some of the factors which influenced the rate and amount of cells released from tissue fragments during trypsinization led to a revision of the method described by Youngner for monkey kidney. The revision includes the use of a glass mixing-chamber and magnetic stirrer in place of the Waring blendor. Simpler to use, the revised method has been found to yield, consistently, about 7×10^7 cells per g of kidney tissue, or from two to three times more than that obtained by the earlier method.

The revised method may be done either manually or automatically. A simple glass apparatus which automatically regulates the continuous addition of trypsin and removal of cell suspension during trypsinization has been developed. It operates reliably over a threefold volume range and a varying flow rate. The yield of cells per gram of tissue treated in the automatic trypsinizer is about 30% greater than when the change of fluids is done manually.

Introduction

The dissolution of tissue fragments into cell suspensions by trypsin was first demonstrated in 1914 by Rous & Jones.[5] Dulbecco & Vogt [1] reintroduced the method for the preparation of chick-embryo and monkey-kidney cell suspensions. Their work demonstrated the fundamental importance of this technique for studies of animal virology. Youngner [8] recently revised the trypsinization procedure with attention directed to the production of large quantities of monkey-kidney cell suspensions.

The Youngner modification of the Dulbecco-Vogt procedure has been widely used. The method, however, requires the full attention of a technician, and thus the scope of experimental studies has been limited by the time required in preparing cell suspensions.

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This paper describes an automatic method for trypsinization. A flask has been designed by which trypsin solution can be added continuously and cell suspension removed automatically as it is formed during the mechanical stirring of tissue. Other modifications of the Youngner method have been made, based on a study of some of the factors affecting the rate of cell release and number of cells obtained during trypsinization. The revised method yields from two to three times more cells than the earlier method.

Materials and Methods

Solutions: trypsin solution (after Dulbecco & Vogt [1])—0.25% Nutritional Biochemical Co. " 1:300 " trypsin in phosphate buffered saline made as follows: NaCl, 8.0 g per l; KCL, 0.2 g per l; Na_2HPO_4, 1.15 g per l; $MgCl_2$, $6H_2O$, 0.1 g per l; $CaCl_2$, 0.14 g per l.

Growth medium: 0.5% lactalbumin enzymatic hydrolysate and 2% calf serum in Hanks' balanced salt solution (after Melnick & Riordan [3]).

Cell counts: One part of cell suspension was stained with two parts of a stain (after Sanford et al.[6]) of 0.1% crystal violet in 0.1 M citric acid. The stained suspension was mixed thoroughly and counted, using a haemocytometer. All the cells falling in the four large squares of the haemocytometer, as used in a white cell count, were counted and averaged. The cell count per ml equals the average count per square $\times$ 10 000 (haemocytometer volume) $\times$ 3 (dilution factor). Only cells showing both nuclei and cytoplasm were counted. These have been called " whole " cells. The whole cell count approaches the number of viable cells, although it is certainly not to be assumed that every " whole " cell is viable. The quality of the suspensions was also judged by the absence of clumps and tubules. However, our best suspensions always contained some clumps. The following convention was used in counting clumps: clumps in which the individual nuclei were surrounded by a large volume of cytoplasm were treated as aggregations of single cells and every visible cell was counted. When the clump showed the nuclei close together with little visible cytoplasm, the whole clump was counted as only one cell. This method requires some evaluation by the counter. It has been found, however, that counts obtained by a given worker on the same preparation, even after an interval of several days, are within 5% of each other. The total error in the method is due to variation in sampling and in charging the haemocytometer. For these studies, three aliquots of the suspensions to be counted were taken and stained. The counts on each aliquot were made in duplicate (and the haemocytometer re-charged) and averaged. The counts were found to have a mean arithmetic variation of $\pm 15\%$.

Growth tubes: The success of each trypsinization procedure was evaluated by the yield of whole cells and by the quality of outgrowth. The cell suspension was diluted into the growth medium to a concentration of 300 000 cells per ml and 0.5 ml seeded into 16×150 mm test-tubes and incubated in stationary racks at 37°C. This concentration was used because it had been found in previous work by the present author that growth is optimal when 125 000-200 000 cells are added per tube. Thus, even if there had been an error of 25% in the cell counts the cells were seeded in the range of optimum concentration. Cell suspension was considered satisfactory if clear confluent sheets were obtained in 4-5 days.

Monkeys: Rhesus monkeys weighing from 5 to 7 pounds (about 2.2 kg to 3.2 kg) were used.

Experimental

Several factors which might influence the yield of cells obtained during trypsinization were investigated. The two most important were found to be the time of incubation with respect to time of stirring and the centrifugation speeds used to harvest the cells from the trypsinization fluid.

For these experiments, two sets of kidneys from healthy rhesus monkeys were removed under aseptic conditions. A kidney of each set was paired with a kidney of the other set. One of the paired sets served as control and was trypsinized according to the Youngner method. The other set was trypsinized by a modification of his method. The total number of whole cells obtained and the quality of outgrowth were compared for the two preparations.

In order to follow the changes which were made, a résumé of the Youngner method is given. Kidney cortex and medulla are minced into pieces 2 mm-4 mm in size and stirred in a trypsin solution in a Waring blendor. After 10 minutes of stirring, the fluid, now containing single cells, is decanted. Fresh trypsin solution is added to the residual tissue and the stirring is repeated. Each period of stirring and decanting is called a " run ". Runs are repeated until the tissue is " exhausted ", i.e., until no more pink tissue remains. The fluids from the combined runs are collected by centrifugation at 1000 revolutions per minute (r.p.m.) for five minutes.

Effect of incubation of tissue with trypsin

The effect of preliminary incubation of the minced tissue in trypsin without stirring was determined. The number of cells in each run during trypsinization by the Youngner method was determined and compared with the number obtained when tissue was allowed to incubate for 45 minutes in trypsin before stirring in the Waring blendor. The results are shown in Table I.

TABLE I. EFFECT OF INCUBATION WITH TRYPSIN ON RATE OF RELEASE OF CELLS FROM MONKEY-KIDNEY TISSUE DURING TRYPSINIZATION *

Controls		Pre-incubated with trypsin for 1 hour at 37°C	
run no.	cells recovered ** $\times 10^7$	run no.	cells recovered ** $\times 10^7$
	Experiment 1		
1	<0.3	1	6.0
2	1.8	2	6.3
3	4.2	3	4.0
4	6.3	4	2.6
5	3.3	5	1.0
6	2.1		
7	<0.3		
Yield: total cells	18×10^7	Yield: total cells	19.1×10^7
weight of tissue	4.3 g	weight of tissue	2.8 g
cells/g	4.2×10^7	cells/g	7.1×10^7
	Experiment 2		
1	<0.3	1	18.7
2	0.9	2	11.0
3	7.5	3	9.6
4	9.6	4	3.6
5	4.5	5	3.0
6	3.8	6	1.0
Yield: total cells	27.7×10^7	Yield: total cells	46.9×10^7
weight of tissue	4.9 g	weight of tissue	4.0 g
cells/g	5.3×10^7	cells/g	12.0×10^7

* Tissue stirred in Waring blendor with 100-ml aliquots of trypsin solutions
** Number of "whole" cells per ml $\times$ 100

It is seen that when tissue is trypsinized the successive runs become richer in cells. After about four runs the rate of release of cells is maximal. Under the conditions of trypsinization, the four runs require about an hour. After the fourth or fifth run, the number of cells released into the trypsin fluid declines.

The rate of release of cells is different if the tissue is allowed to incubate in trypsin before the runs are begun. After incubation, the number of cells in the first run is equivalent to the number of cells after four runs

without incubation. The total number of cells obtained after four runs with incubation is almost twice that obtained in four runs without incubation.

The experiment was modified by carrying two sets of tissue through several trypsinizations without preliminary incubation. When the number of cells started to decline after five runs, one set was incubated with trypsin for 20 minutes and the other was trypsinized with successive 10-minute runs as before. A comparison of the number of cells obtained (Table II)

TABLE II. EFFECT OF INCUBATION DURING TRYPSINIZATION ON RELEASE OF CELLS *

Run No.	Total cells recovered × 10^8	
1	<0.03	
2	0.18	
3	1.45	
4	1.80	
5	0.90	
	Control (without incubation)	Incubated for 20 minutes at 34°-37°C
6	0.38	0.61
7	0.13	0.50
8	0.03	0.10
		Re-incubated for 20 minutes at 34°-37°C
9	0.045	0.240
10	0.030	0.11
11	0.015	0.03
Total cells from runs nos. 6-11	0.63	1.59

* 7.0 g of monkey-kidney tissue trypsinized with 100-ml aliquots of trypsin solution for 5 runs. Tissue divided into two equal portions and trypsinization continued with and without incubation.

shows that after the 20 minutes of incubation, one run was equivalent to two or three runs without incubation.

The favourable effect of incubation may be due to the fact that trypsin only activates intracellular proteases which cause the dissolution of the tissue mass. Two observations may be mentioned in this connexion: (1) cells are released after incubation in the absence of trypsin and subsequent stirring in a trypsin-free salt solution, although the rate is too slow to be practical; and (2) although cutting the tissue finely would be expected to facilitate trypsinization, since a greater surface would be exposed to

trypsin, it has been found that this actually decreases the yield of cells. A favourable effect of cutting the tissue finely observed at the beginning of these studies proved to be due to the fact that a longer time was taken in the preparation of the tissue. After incubation, large fragments (1 cm) of kidney disintegrate more quickly and with a greater yield of free cells than smaller pieces.

Regardless of the mechanism of the breakdown of tissue into cell suspensions, advantage may be taken of the fact that the trypsinization requires a certain amount of time. The number of runs, and hence the number of manipulations and the volume of fluid which must subsequently be centrifuged to obtain a given number of cells, may be greatly reduced by allowing for this time.

It has been shown that an increase in time of incubation with respect to time of stirring increases the number of cells obtained by trypsinization. This may be due not only to the time required for the action of trypsin, but also, since the cells are very fragile as will be shown below, to the decrease in the amount of mechanical agitation.

Centrifugation

Freshly prepared suspensions of trypsinized cells were found to be very fragile and were destroyed by manipulations such as simple pipetting or centrifugation at forces which are without effect on most cells. The effect of centrifugation at different speeds on the number of cells harvested from trypsinization fluids is shown in Table III. For these studies an International Centrifuge, model PR-2, was used. This centrifuge does not set up resonance at low speeds. Many centrifuges do so in the range 200-300 r.p.m. and the resonance may be sufficient completely to disrupt sedimented pellets of monkey-kidney cells.

It is seen in Table III that at speeds above 400 r.p.m. there is a significant loss in the number of cells. In some cases the total number of cells in both the supernate and the sediment after centrifugation at these speeds was less than 50% of the original number. In these counts only cells showing both nuclei and attached cytoplasm were counted. The number of free nuclei in the supernatant after centrifugation was noticeably higher than before, although accurate counts were not made. The number of cells recovered after centrifugation at 300 r.p.m. approached, within the errors of counting, the number before centrifugation. The number actually sedimented, however, increased with length of time of centrifugation, but 30 minutes of centrifugation at 200 r.p.m. ($11 \times g$) were sufficient to sediment all the cells into a firm pellet. The pellet is firm enough to permit removal of the supernatant fluid by aspiration.

The cells released by trypsin proved unexpectedly fragile, and even after standing several days in the cold in the growth medium substantial

TABLE III. FRAGILITY OF FRESHLY TRYPSINIZED MONKEY-KIDNEY CELLS IN CENTRIFUGATION *

Trypsinization fluid cells/ml ± 15%	Centrifugation		Cells recovered/ml × 10^6 ± 15%		
	r.p.m.	minutes	sediment	supernatant	total
0.95 × 10^6	500	5	0.31	<0.03	0.31
		5	0.61	<0.03	0.61
	400	5	0.45	<0.03	0.45
		10	0.51	<0.03	0.51
	300	5	0.45	0.31	0.76
		10	0.72	0.10	0.82
		20	0.68	0.11	0.79
	200	10	0.41	0.55	0.96
		20	0.85	<0.03	0.85
		30	0.91	<0.03	0.91

* 100-ml trypsinization fluids centrifuged in International PR-2 centrifuge in 250-ml bottles; sediments resuspended in 100 ml.

loss may be entailed if they are centrifuged above 400 r.p.m. In contrast, cells which have grown in culture and have then been removed from the glass by trypsin or Versene can withstand centrifugation up to 1000 r.p.m.

Trypsin solution

The concentration of trypsin was found to affect the time required for the dissolution of the trypsin mass. The rate was maximum at 0.2% trypsin, and increasing the concentration of trypsin did not substantially increase the rate of release of cells (Table IV).

TABLE IV. EFFECT OF TRYPSIN CONCENTRATION ON RATE OF CELL RELEASE *

Trypsin solution (% concentration)	Total cells recovered × 10^8	Tissue weight (g)	Yield (cells/g × 10^8)
0.03	0.59	4.2	0.14
0.09	1.05	5.0	0.21
0.18	1.41	3.7	0.38
0.25	1.92	4.8	0.40

* Kidney tissue incubated for 40 minutes and trypsinized for 4 runs by the revised manual procedure.

The effect of pH or ionic strength was not investigated. The pH of 7.4 recommended by Dulbecco & Vogt [1] was considered a satisfactory com-

promise between the pH optimal for trypsin (pH 8.2) and that which is physiological for cells. The high phosphate concentration in the trypsin solution has been found to be useful not so much because of buffering action but because it prevents irreversible clumping of the cells.

The effect which incubation was found to have on trypsinization suggests that intracellular factors may determine the rate of release of cells. If this were the case, the rate would be determined by the concentration at which tissue extracts were maintained during trypsinization, i.e., by the rate of change of trypsin fluid above the tissue mass. It was not possible to investigate this when fluids were added and decanted manually. Using the automatic trypsinizer, where a constant volume in the mixing chamber may be maintained with a continuous change of fluid, it has been found that the number of cells present in the mixing chamber determines the rate of release of cells from the tissue mass.

Efforts to substitute Versene for trypsin were unsuccessful. Versene has been used successfully for preparation of liver cells by several workers,[2,7] and also for the removal of kidney cells from tissue-culture monolayers (M. Vogt—personal communication). The release of cells from the fresh kidney tissue was found to be too slow to be practical. Further, the cells obtained from the Versene-treated tissue had to be harvested and re-suspended before they would grow.

Revised Trypsinization Procedure

The observations presented indicate that successful trypsinization depends on maintaining a proper relationship between time of incubation and time of mechanical stirring and observing precautions made necessary by the extreme fragility of the cells. With these requirements in mind, it was possible to simplify the trypsinization procedure.

The first and most desirable item was replacing the Waring blendor with a mixing chamber which would be easier to handle. An Erlenmeyer flask was modified so that a magnetic stirrer could be used in place of a Waring blendor. Using this flask, kidneys were trypsinized with systematic modification of the Youngner method. Each modification was judged by: (1) the number of whole cells, and (2) the ease of operation.

The revised procedure to be given has been in routine use for over a year in the Yale laboratory. It may be done by adding and decanting fluids manually or the fluids may be changed mechanically by use of the automatic trypsinizer. The revised procedure yields two to three times more cells per gram of tissue trypsinized than the earlier method. A step-by-step procedure, with manual changes of fluids, will be given first. The preparation of the tissue and the harvesting of the cells, steps 1-3 and steps 8-10, are the same for both the manual and automatic methods.

A description of the automatic trypsinizing flask and notes on its use will then be given.

Trypsinization flask [a]

A few simple modifications of an ordinary Erlenmeyer flask will permit one to use a magnetic stirrer instead of the Waring blendor. The sides of a 500-ml, or 250-ml, Erlenmeyer flask have been indented at right angles to the bottom surface in four places. They should extend to about two-thirds of the height of the flask and cut into the bottom surface about 2.5 cm. This gives the bottom of the flask the shape of a Maltese cross. This modification permits good mixing with cavitation at certain critical volumes. Maximum mixing over a wider range of volumes may be obtained if the flask is further modified by accentuating the curvature between the bottom and the sides of the flask. This is done by compressing the glass in this region inwards and upwards.

During trypsinization it is necessary to add and decant fluids many times and therefore it is convenient to have an inlet for trypsin from a reservoir and a side-arm for decanting fluids incorporated into the flask. The side-arm for decanting should be placed so that the tissue tends to be trapped in the bottom when the flask is tilted for decanting. The efficiency of trapping may be increased by enlarging the flask just under the side-arm. This is particularly useful when large volumes of tissue are trypsinized. The flask assembled for use is shown in Fig. 1.

A 500-ml flask modified in this manner is large enough to trypsinize an initial load of 80 g of tissue. If less than 30 g of tissue are to be trypsinized, a 250-ml flask should be used, thus reducing the amount of trypsin solution required.

The volume of trypsin required during stirring is determined by the size and shape of the flask and not by the amount of tissue. In the 500-ml flask, maximum stirring efficiency is obtained at a total volume (including magnet and tissue) of about 150 ml. This is determined for any given flask and tissue load by the volume which allows rapid rotation of fluid and tissue fragments up and around the sides of the flask with slight cavitation and no foaming. The flask should be marked at this level and trypsin added from a reservoir to this mark for each run.

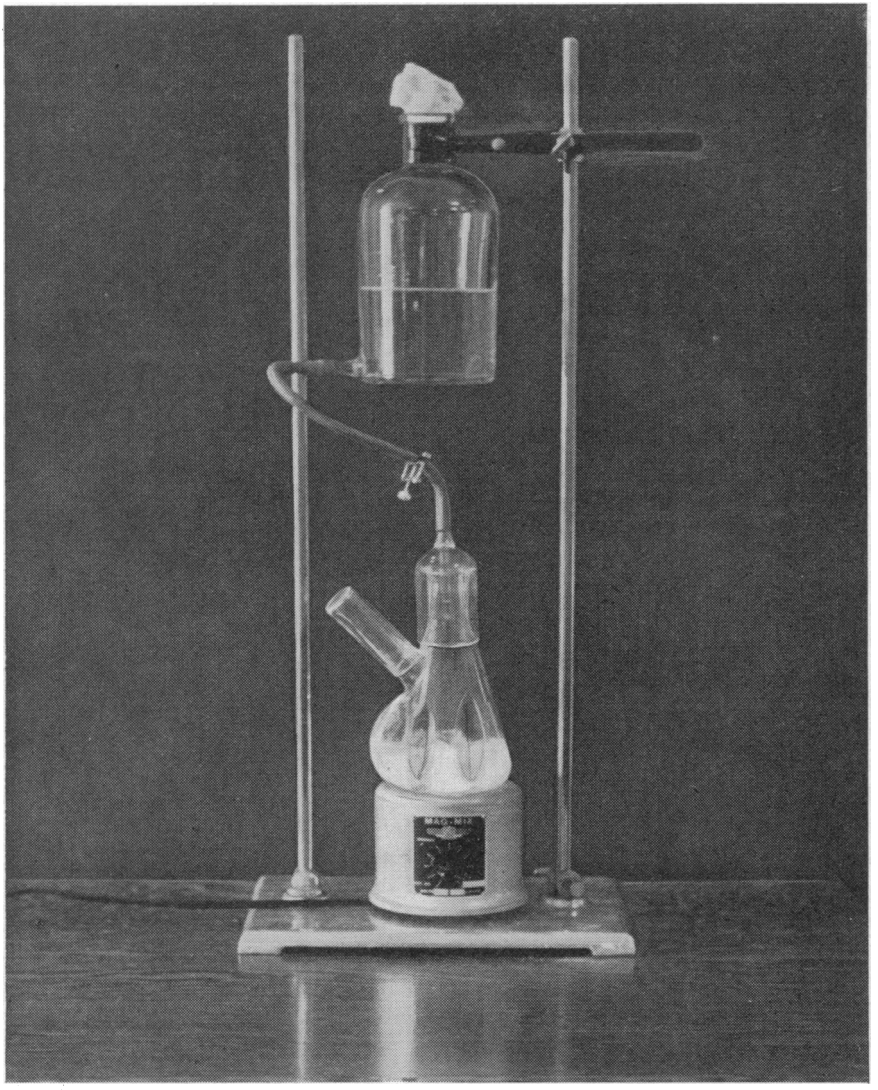
Trypsinization of monkey kidneys

Step 1. Kidneys are removed from freshly killed and exsanguinated monkeys and are placed in a sterile Petri dish. They are decapsulated and cut in half. This is best done by holding the kidney firmly with forceps and

[a] The trypsinization flask and the automatic trypsinizer (with magnets) described in this paper are available at the Macalaster-Bicknell Co., New Haven, Conn., USA.

making a cut through the kidney horizontal to the flat surface with uterine scissors, exposing the central pelvis. The pelvis is removed by cutting around its outer edge, lifting it with forceps to free it from the underlying tissue. Any adhering connective tissue is also discarded.

FIG. 1. SPECIAL TRYPSINIZATION FLASK USED WITH MAGNETIC STIRRER, ASSEMBLED FOR TRYPSINIZATION BY MANUAL METHOD



Step 2. The tissue is cut into pieces at least 1 cm × 1 cm. It is important to cut through the tissue because strands of connective tissue may interfere with proper mixing.

Step 3. The cut tissue is transferred to the trypsinization flask and covered with trypsin pre-warmed to 37°C.

Steps 1-3, the preparation of one kidney, should take about 7-10 minutes. The process is repeated for each of the remaining kidneys. After each kidney is prepared it is added to the others, covered with trypsin, and incubated. Thus, 6-8 kidneys may be conveniently prepared in the hour required before stirring starts.

Step 4. One hour after trypsin was added to the first kidney the incubation fluid is decanted into a sterile flask which is kept at 4°C. Fresh pre-warmed trypsin is added to flask to a level equal to a total volume of about 150 ml.

Step 5. Tissue and trypsin are stirred with a magnetic stirrer for 7 minutes. The speed of stirring should be sufficient to allow rapid mixing with cavitation and no foaming.

Step 6. After 7 minutes of stirring, the magnetic stirrer is turned off, the tissue allowed to settle, and the fluid decanted into the receptacle containing the incubation fluid.

The remaining tissue is washed clean of free but adhering cells by adding 50-100 ml of trypsin solution, swirling by hand and decanting. If the tissue has been disintegrated significantly during a run or wash, the decanted fluids when held to the light will be seen to be a fine suspension of tissue fragments and cells. After the third or fourth run, it may be necessary to wash the tissue 2-4 times before the washings are clear.

This step, decanting, washing, and re-filling, should be done without haste and may take as much as 5-10 minutes.

Steps 5 and 6 (mixing with trypsin, and decanting and washing) are repeated 6-8 times or until there is a noticeable decrease in the rate of dissolution of tissue as indicated by a clearing of the fluids.

Step 7. After 6-8 runs, when the fluids have noticeably cleared, the tissue is removed to a small beaker. Considerable connective tissue has usually become visible during trypsinization at this point and may interfere with mixing. The connective tissue is cut, and if necessary the remaining tissue should be cut into pieces of 0.5 cm in size. The tissue is covered with trypsin and incubated at 37°C for 20 minutes.

Repeat steps 5 and 6 after the second incubation until tissue is exhausted. The number of runs required to exhaust tissue varies with the mass of tissue. A total of 8 runs is usually sufficient for 4 kidneys and 10-13 for 8 kidneys.

Step 8. The combined fluids from all the runs are centrifuged at 200 r.p.m. ($11 \times g$) for 30 minutes.

Step 9. The supernatant fluid is removed from the packed cells by a vacuum aspirator. The fluid can be removed to within 1-2 ml without removing cells. This residual fluid has not been found to affect the quality or viability of the suspension in any way. There should be approximately 3 ml of packed cells for each kidney trypsinized.

Step 10. The packed cells are resuspended in about 100 ml of the growth medium, pre-warmed to 37°C. If cold solution is added, the cells may clump badly. The suspension is filtered through 2 layers of sterile cheese cloth. The gauze filter is washed free of adhering cells with about 100 ml of nutrient solution.

Step 11. The number of whole cells, i.e., those showing both nuclei and attached cytoplasm, are counted in a haemocytometer.

Step 12. The suspension is diluted in the nutrient medium to give 300 000 cells per ml, and 0.5 ml of this suspension is seeded into 16 mm × 150 mm tubes. After 4-5 days of incubation, a confluent sheet of cells is present.

Step 13. If the suspension is to be kept several days before use,[4] it should be stored at 4°C at a concentration of not more than 600 000 cells per ml. The stored suspension should be centrifuged after the first 10-24 hours and resuspended in fresh growth medium for the remainder of the storage period. The suspension is again centrifuged and again resuspended in fresh medium just before use. This procedure is necessary for maximum survival and growth, because it has been found by the author that freshly trypsinized kidney-cell suspensions liberate a heat-labile toxin which kills slowly at 4°C and may destroy up to 90% of the cells in a few hours under growing conditions at 37°C.

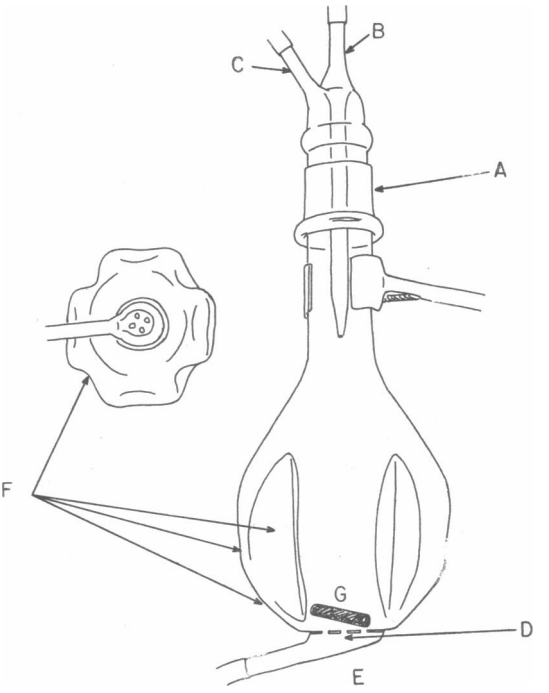
The preparation and trypsinization of 8 kidneys takes about 4-5 hours. An average yield is between 0.6×10^8 and 0.8×10^8 cells per g of "dressed" tissue.

Continuous automatic trypsinizing flask [a]

A flask permitting the continuous and automatic addition of trypsin and withdrawal of cells is shown in Fig. 2. The main problem in designing a suitable flask was the position of the outflow valve. The vigorous stirring during trypsinization required to sheer the cells from the tissue mass keeps the tissue in suspension and tends to force tissue into an outflow valve. This difficulty could be overcome if the valve were arranged so that the tissue could settle before the fluid flowed out. However, a system such as this could probably be operated at only one volume with maximum efficiency. It was considered advantageous to have a flask that could operate at several different volumes.

[a] We are indebted to Mr. Frank Lynsky of Macalaster-Bicknell Co. for advice and work involved in the development of this flask.

FIG. 2. AUTOMATIC TRYPSINIZER



The diagram shows the side and bottom of a 500-ml Kjeldahl flask modified for use as a mixing chamber in the continuous trypsinization procedure. A = Ground-glass bacteriological joint. B = Inlet tube for trypsin from reservoir. C = Air valve. D = Flat glass disc, 2 inches (50 mm) in diameter, with 6 bored holes, 1.5 mm in diameter. E = Sloped outlet drain. F = Indentations and modifications at sides and bottom of flask to increase mixing efficiency. G = Plastic-covered magnet, $1^5/_8 \times {}^3/_8$ inches (about 41 mm × 9.5 mm).

The apparatus shown in Fig. 2 consists of a glass mixing-chamber closed by a ground-glass joint containing two openings. Through one, trypsin can be admitted from reservoir. The other is a valve which can admit air when desirable. In the bottom centre of the flask holes have been drilled so that fluid can drain from the mixing chamber into a receiving jar.

The flask takes advantage of the fact that a magnetic stirrer can mix liquids in a chamber placed at some distance from the surface of the stirrer. Thus, when tissue fragments are stirred in the mixing chamber, the motion of the magnet sweeps the fragments from the drain and permits only the cell suspension to pass through into the receiving jar.

The mixing chamber and magnetic stirrer should be arranged so that trypsin is admitted by siphon action through a gum-rubber lead from a reservoir. The flow of trypsin is thus virtually independent of the amount of trypsin in the reservoir and can be controlled simply by a pinch clamp on a gum-rubber lead into the mixing chamber. After equilibration is

established, the flow of trypsin into the mixing chamber regulates the outflow of liquid through the drain. The system can never overflow or drain dry.

It is important to realize that the action of the magnet is indispensable for the flow of liquid through the system. The drain valve must never be opened unless the magnet is in motion. If it is opened and the magnet is not turning, the tissue will settle and clog the pores. In this case, the cells are not recovered as soon as they are released and they may be lost by excessive digestion with trypsin.

The size of the magnet is important. It should be heavy enough so that it is not deflected by the tissue and yet not so heavy that it cannot be turned smoothly by the magnetic stirrer. A magnet $1^5/_8 \times {}^3/_8$ inches (about 41 mm $\times$ 9.5 mm), weighing at least 10 g, has been found satisfactory for loads of tissue up to 30 g and the usual size of stirrer.

The bottom of the flask has been designed so that the magnet tends to recentre itself if deflected by tissue or by the usual changes in the line voltage. It may be worth while mentioning the points which were considered in developing the flask. First, the bottom is blown so that the inside surface is flat. Secondly, the flat portion of the bottom is only ¼ inch (about 6 mm) greater than the length of the magnet. Thus, if the magnet is displaced during stirring it will necessarily return to the centre. Thirdly, the indentations and modifications on the side of the flask were selected from several trial flasks because they gave efficient mixing over a wide range of volumes and particularly because the flow of liquid during mixing was itself a centring force.

The self-regulating capacity of the flask is not sufficient to withstand marked changes in the rate of stirring due to large fluctuations in line voltage. If the apparatus is to be left unattended on a voltage source which may change suddenly, it is advisable to use a constant voltage transformer. The smallest constant voltage transformer, capacity 15 amperes, commercially available is more than adequate for this purpose. Operated at proper speeds and on a steady line the trypsinizer has proved very reliable and has been left trypsinizing for more than 24 hours unattended.

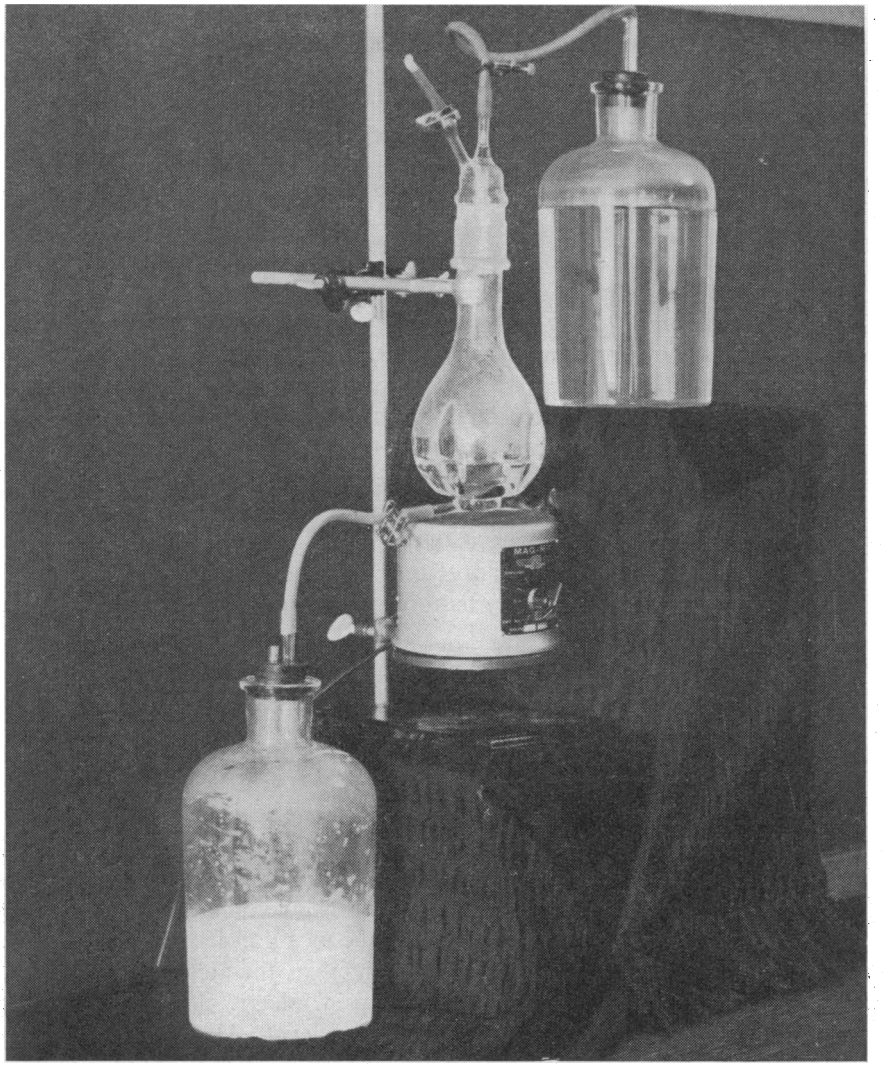
Operation of flask. The flask and rubber tubing leading from the reservoir and to the receiving vessel are sterilized by autoclaving. The air outlet should have a short rubber tube attached, which is plugged with cotton before sterilization. Pure gum rubber is recommended for all connexions, because some of the synthetic rubbers or plastics may be distorted enough by autoclaving to allow air to leak in. A plastic-covered magnet $1^5/_8 \times {}^3/_8$ inches (about 41 mm $\times$ 9.5 mm) is sterilized by boiling just before use.

Kidney fragments of not less than 1 cm $\times$ 1 cm are prepared as already described. It is advisable to cut the kidneys in a beaker or Petri dish,

because if the tissue is minced in a test-tube the cutting is uneven and some very small pieces are produced.

The tissue and magnet are placed in the flask, of which the outlet drain has been closed by a pinch clamp; 100 ml-150 ml of trypsin solution, pre-warmed to 37°C are also added. The trypsin reservoir is placed so that trypsin can be admitted into the mixing chamber by siphon action through a drop of at least 1 foot (30 cm). The reservoir should be kept at 37°C.

FIG. 3. AUTOMATIC TRYPSINIZER, ASSEMBLED



This may be done by placing a microburner under the reservoir or by setting up the entire apparatus in a " walk-in " incubator. The outlet drain should feed into a suitable receptacle, placed at least 1½ feet (about 45 cm) lower than the mixing chamber. If it is desired to tap the trypsinizer for cell suspension throughout the day, it will be found convenient to use a bell-cap on the outlet tube for the receiving flask. The bell-cap can be changed easily from one receptacle to another with a minimum chance of contamination. If the cells are to be kept several hours before harvesting, the receptacle should be kept in an ice-water bath. The apparatus assembled for use is shown in Fig. 3.

The magnetic stirrer is started and flask centred so that the magnet operates smoothly in the centre of the flask. This may be adjusted by the sound of the magnet rotating, as well as by the appearance. When properly centred, the rotation of the magnet is almost noiseless.

The tissue is incubated with gentle stirring for 30-45 minutes. After incubation, the speed of stirring is increased until there is rapid swirling of tissue up and around the sides of the mixing chamber. There should be slight cavitation but no foaming.

At this time, with the air valve closed, the drain valve is opened slowly. Trypsin will flow from the mixing chamber into the receiving jar and prime the siphoning of trypsin from the reservoir. When trypsin starts flowing into the mixing chamber, the inlet valve should be closed gradually until, with the drain valve completely open, the rate of flow through the flask is regulated by the pinch clamp on the inlet from the trypsin reservoir.

During trypsinization, small pieces of tissue may escape through the drain into the receiving jar. If the tissue has been cut as directed and if the speed of the magnet is properly adjusted, the tissue actually lost in this way is negligible. However, if the flow of fluid is regulated by a pinch clamp on the drain valve instead of on the trypsin inlet as indicated, the constriction will tend to trap any tissue escaping and may block the outflow of fluid.

Volumes and loads. The flask is marked on the outside at three different levels, indicating a total volume (from the mark to the pinch clamp on the outlet drain) of 60 ml, 100 ml, and 150 ml. These levels have been found satisfactory for trypsinization of 13, 30, and 50 g of tissue respectively. The constant volume in the flask, or " working volume " at which it is desirable to run a trypsinization, depends on the liquid volume which will permit adequate stirring and smooth rotation of the magnet in the flask, and not on the amount of tissue. Thus, the working volume may be changed if the amount of tissue is changed or if tissue of a different density is used.

To change the volume in the mixing chamber, the air outlet is opened. If the volume is to be decreased, the trypsin inlet is closed and fluid is allowed to drain out until it is at the desired level. The air valve is then

closed and the flow of trypsin regulated. To increase the volume in the mixing chamber, the drain valve is closed, the air valve is opened and trypsin is allowed to run in until it is at a level equivalent to about 30 ml more than the desired volume. On closing the air outlet, and opening the drain valve, the volume will adjust itself at the desired level with the previous rate of trypsin inflow.

Rate of flow. The rate of flow into the mixing chamber is regulated so that the fluid which is drained off contains from 5×10^5 to 10×10^5 cells per ml. At this concentration, the fluid is turbid and when held to the light will be seen to be a suspension of material. The regulation may be made satisfactorily without counting, by adjusting the rate so that the outflow is always turbid but fast enough to prevent the cells from settling out along the drain tube. Increasing the rate of flow results in an extravagant use of trypsin and seems to decrease the number of cells obtained (see Table V).

When the flask is at working volume of 60 ml (including magnet and tissue) with loads up to 15 g, a rate of flow of 1-2 drops per second has been found to be satisfactory for the main portion of the trypsinization. This is a " clear-out " time of less than 20 minutes. For loads of tissue of 30 g and a working volume of 100 ml, a rate of 3-4 drops per second is sufficient.

TABLE V. EFFECT OF FLOW RATE DURING AUTOMATIC TRYPSINIZATION ON RATE OF CELL RELEASE *

Tissue weight (g)	Flow rate (drops/ second)	Cell count × 10^6 in outflow at			Total ml of trypsin used	Yield (cells/g)
		1 hour	2 hours	3 hours		
8.0	1.2	0.51	1.1	0.83	610	0.6×10^8
7.8	3.4	0.05	0.08	0.11	1750	0.2×10^8

* Paired kidney tissue incubated for 40 minutes and trypsinized for 3 hours in automatic trypsinizers at 100-ml working volumes.

Towards the end of trypsinization—or at the very beginning, when the release of cells is slow—the rate may be considerably reduced with a significant saving in trypsin and in the volume which must subsequently be centrifuged. If the apparatus is to be left unattended for the entire trypsinization, however, the rates suggested are adequate for the whole procedure.

Performance. The kidneys are usually dressed and placed in the flask with trypsin at 9.30 in the morning. After 30-45 minutes' incubation the continuous flow system is started. Viable cell suspensions are available an hour later and are harvested and used throughout the day. Fresh kidney tissue may be added 1-2 hours later and again after 4-5 hours, and trypsinization may be continued for 24 hours. Kidney tissue trypsinized overnight

and harvested the following morning has been found satisfactory for both growth and biochemical studies and has been found to compare favourably with cells which are harvested an hour or two after leaving the tissue mass.

The yield of cells per gram of kidney has been found to be somewhat greater than by the manual method, i.e., 10^8 cells per g as compared to 6×10^7 to 8×10^7 per g obtained by the manual method.

The amount of trypsin required is considerably less with the continuous flow system than by the manual method. Two to three litres of trypsin are sufficient for four kidneys even when trypsinized overnight, where only one flow rate is used for the entire procedure. The manual method requires 3-4 litres for four kidneys if the tissue is exhausted.

After trypsinization of 30 g of tissue, it is advisable to remove the connective tissue remaining in the mixing chamber since it may interfere with the rotation of the magnet. In practice it is usually easier to substitute another mixing chamber when the trypsinization is to be continued.

Discussion

It has been found that the rate of release of cells from tissue fragments by trypsin depends largely on a favourable time of incubation with trypsin. The dissolution of the tissue mass into a cell suspension is a complex process apparently dependent on intracellular factors as well as on external trypsin and mechanical agitation. The release of cells requires time, and hurrying the procedure only increases the number of manipulations required to obtain a given number of cells. On the other hand, since the free cells are unusually fragile they should be removed from the mixing chamber as soon as they are released to protect them against mechanical damage or excessive digestion with trypsin. The manual method giving the highest yields was one that alternated periods of incubation with trypsin with periods of stirring during which the fluid was changed every 3-10 minutes. By this method two to three times more cells per gram of kidney tissue trypsinized are obtained than by the Youngner method.[8] The favourable effect of incubation and of frequent fluid changes during stirring suggested that the most successful method would be one where the tissue was stirred gently for many hours with a continuously renewed trypsin solution and continuous withdrawal of the cell suspension.

The automatic method presented is a continuous flow system using a specially designed mixing chamber. The fact that the yield of cells per gram of tissue trypsinized in the automatic trypsinizer is consistently about 30% greater than with the manual method is probably due to the fact that, with a continuous flow system, the concentration of factors derived from the tissue itself and regulating the dissolution of tissue mass may be maintained throughout the procedure at the optimal level.

The automatic trypsinizing apparatus was developed because it was felt that the time and attention required for preparation of cell suspensions, although not a serious factor for certain kinds of studies, prohibited investigations requiring small amounts of fresh material frequently. With this in mind, it was considered that simplicity, inexpensiveness, and ease of handling were important factors in the choice of design. An apparatus operating under a wider variety of conditions than the one described here and with complete automatic control could certainly be developed, but it would be more complicated and more expensive.

The automatic trypsinizer which has been developed is a simple glass flask to be used with a magnetic stirrer. The flask is inexpensive, may be sterilized by autoclaving, and is easily operated. It can trypsinize efficiently a constant load of 30 g of tissue. It operates over a threefold volume range and a varying flow rate.

Two simple precautions should be observed when the apparatus is to be left for many hours unattended. First, the voltage supply should be relatively constant as indicated by a constant rate of rotation of the magnet, or a constant voltage transformer should be used. Secondly, each set-up should be checked for leaks, indicated by a discrepancy between the rate of inflow of trypsin and the rate of outflow. If the system is not closed, it will eventually overflow or drain dry. When these two precautions are observed, the apparatus will work reliably for many hours without adjustment.

RÉSUMÉ

La méthode de résolution de fragments tissulaires en suspensions cellulaires par action de la trypsine s'est révélée très féconde en virologie. Introduite en 1914 par Rous et Jones, reprise par Dulbecco et Vogt, cette méthode a été récemment adaptée par Youngner à la production de suspensions de cellules d'embryon de poulet et de rein de singe. Telle qu'elle était pratiquée jusqu'à maintenant, cette méthode avait l'inconvénient de nécessiter une surveillance constante et de se prêter mal aux recherches demandant fréquemment de petites quantités de matériel frais.

L'auteur décrit une modification qui rend cette méthode automatique. Grâce à une chambre de mélange spécialement conçue à cet effet et remplaçant le Waring blendor, l'appareil assure l'afflux constant de trypsine et l'élimination automatique des cellules mises en suspension par l'agitateur magnétique. Cet appareil qui fonctionne à trois volumes différents et avec une vitesse d'écoulement réglable, peut traiter de façon constante 30 g de tissu. Il donne un rendement en cellules deux à trois fois supérieur à celui des autres méthodes. Moyennant certaines précautions — voltage constant assurant la rotation régulière de l'agitateur et suppression des causes de fuites et de pertes dans l'appareil — le système fonctionne sans surveillance pendant plusieurs heures.

Les diverses étapes de la méthode et l'utilisation de l'appareil sont décrites en détail. L'effet de certains facteurs sur le rendement en cellules y est discuté sur la base de données expérimentales; ce sont en particulier la durée de la trypsinisation, la centrifugation, la concentration de la trypsine, la relation entre la durée de l'incubation et celle de l'agitation.

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