for human liver cell growth. The substance in the material from mouse lung cell growth fluid is heat labile. The crude material was heat coagulable with loss of growth promoting activity for human liver cells. A graded dose response of growth of liver cells was obtained.

1. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

2. Merchant, D. J., Rahn, R. H., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 359.

3. Waymouth, C., *J. Nat. Cancer Inst.*, 1956, v17, 315.

4. Jerade, H. W., Jones, M. J., *J. Biol. Chem.*, 1953, v201, 553.

5. Lieberman, I., Ove, P., *ibid.*, 1958, v233, 637.

6. Kutsky, R. J., *Science*, 1959, v129, 1486.

7. Pumper, R. W., *ibid.*, 1958, v128, 363.

8. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.

9. Mcrgan, J. F., Morton, H. J., Parker, R. C., *ibid.*, 1950, v73, 1.

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Centrifugation of Rickettsiae and Viruses Onto Cells and Its Effect on Infection. (25637)

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Chick embryo entodermal cells have been extensively used in investigations of viruses of the psittacosis group and rickettsiae(1-4). During attempts to improve and simplify the basic procedure, it was found that centrifugation of the infectious agents directly onto the cells greatly enhanced infection. Preliminary results also indicated that the centrifugation technic might be profitably applied to other host cells. These studies were prompted by the report by Macdougald *et al.*(5) that chick embryo explants can withstand exceedingly high centrifugal forces and by the studies by Gey *et al.*(6) who effectively sedimented eastern equine encephalomyelitis virus onto HeLa cells.

Materials and methods. Microorganisms. The following strains were used: *Rickettsia prowazekii* (Madrid E), *Coxiella burnetii* (California), psittacosis virus (6 BC), and trachoma virus (TW 29, isolated in the laboratory of Dr. J. T. Grayston, Naval Med. Research Unit No. 2, Taiwan). Pools of these microorganisms were prepared from infected yolk sacs and diluted in Bovarnick's isotonic sucrose solution(7). Purified suspensions of typhus rickettsiae and trachoma virus were prepared by minor modifications of commonly used procedures(8,9) which included the following steps: 1. Treatment with trypsin ("Tryptar" Armour), 0.25% final dilution, for 1 hour at room temperature; 2. Centrifugation at 2,500 g for 45 minutes at 2°C in an angle centrifuge (Spinco); 3. Suspension of pellet in sucrose solution containing 0.6% bovine plasma albumin. The suspension was cleared with an amount of celite (Johns Manville) corresponding to 10% of weight of the yolk sacs and by centrifugation in an horizontal centrifuge at 500 g for 10 minutes at room temperature. The slightly opalescent supernatant was saved and used directly or subjected to step 4, centrifugation at 11,000 g for 20 minutes and suspension of pellet in tissue culture medium. Q fever rickettsiae were partially purified by a somewhat simpler procedure, while psittacosis virus was used as a crude suspension. All 4 agents were titrated in eggs, viruses by the routine serial 10-fold dilution method, rickettsiae by the single dilution method as described previously (9,10), or as explained in a later section on *C. burnetii*. *Entodermal cell cultures.* Explants were obtained from the avascular membranes of the 4-day-embryo as described previously (1-3). When the experiments did not require microscopic observation, explants were placed in standard round-bottomed 16 × 125 mm screw-cap tubes. The nutrient, 0.6 ml per tube, consisted of 20% rooster serum diluted in Hanks'(11) balanced salt solution. Tubes were incubated at 35°C in an upright position

and monolayers were formed on lower surface by the 3d day. For microscopic observations the same procedure was used, except that explants were deposited on 12 mm round coverslips resting on the flat bottom of special tubes, prepared from standard 16 × 100 mm test tubes by Mr. A. D. Mack of Naval Med. Research Inst. Volume of the nutrient in these tubes was 0.5 ml. The cultures were usually inoculated on the 3d day after explantation by replacing the fluid with the inoculum diluted in fresh medium. To test the effect of centrifugation on infection, the cultures were centrifuged in a refrigerated horizontal centrifuge (International) for 1 hour. Because of the relatively long radial distance to the lower 5 mm of the tubes containing the cells and their fluids, centrifugal forces averaging 1600 and 1800 g were attained in the round- and flat-bottomed tubes, respectively. Temperature was maintained at 20°C during the centrifugation procedure or carefully matched to temperature of the control stationary cultures, which were maintained at 22-26 C. The cultures were then incubated at 35-37 C, depending on infectious agent used. The fluids were not changed during an experiment. For determination of total infectious titer of a group of cultures, the fluids were carefully removed and saved. The cells were disintegrated by rapid shaking for 1 hour at room temperature in a volume of 0.25% trypsin in isotonic sucrose solution equivalent to the nutrient. The cell brei, containing no microscopically recognizable cells, was added to the nutrient, and mixture was appropriately diluted in isotonic sucrose solution for titration. Coverslips were fixed in methanol and stained with May-Gruenwald-Giemsa. *Human skin cells* were prepared by Dr. N. H. Wiebenga, in our Division. They were obtained from the Tissue Culture Section, Lab. of Biology, Nat. Cancer Inst., N.I.H., and Tissue Bank Dept., Nat. Naval Medical Center. They were designated HuS 2806 and had been adapted to 10% horse serum in 10% NCTC 109 medium(12). Prior to their use in these experiments they were maintained for one month in a medium free of antibiotics. Following infection they were maintained on a medium containing only 2% horse serum.

TABLE I. Sedimentation of Typhus Rickettsiae Suspended in Tissue Culture Nutrient.*

Rickettsial suspension	No. of infectious rickettsiae: Upper .3 ml	Lower .3 ml	% in upper layer
Stationary	2.4×10^4	3.8×10^4	38.7
	2.6 "	1.8 "	59.1
Centrifuged	3.2×10^3	6.9 "	4.4
	3.9 "	16.2 "	2.4

* Rooster serum, 20%, in Hanks' balanced salt solution.

Results. Experiments with entodermal cells. R. prowazekii was used for some of the preliminary experiments. Table I presents results of a test of sedimentation of these microorganisms by the centrifugal force used in subsequent experiments. Purified rickettsiae were suspended in tissue culture medium and distributed in 0.6 ml volumes in screw-cap tubes, as in the routine tissue culture procedure. Six tubes were left in an upright stationary position, while another 6 tubes were being centrifuged, as described previously. The upper 0.3 ml of each tube was then carefully pipetted out, pooled in groups of 3, and titrated. The lower 0.3 ml was similarly treated. Rickettsiae remained approximately equally distributed in the 2 layers in the stationary tubes. In the centrifuged tubes the lower layer contained 95-98% of the rickettsiae, which would suggest that sedimentation was effective, but, possibly, not to the same extent as in the routine purification procedure.

Table II presents results of a similar experiment in which entodermal cell monolayers were used. Following centrifugation, or incubation at room temperature, the cultures were incubated for 1 hour at 35°C, to permit any additional interaction between microorganisms and host cells to take place. Nutrient fluids were then carefully removed and titrated, and the unwashed cells were broken

TABLE II. Sedimentation of Typhus Rickettsiae onto Entodermal Cells.

Cultures	No. of infectious rickettsiae: Medium	Cells	% with cells
Stationary	3.2×10^4	2.8×10^2	.9
	2.3 "	8.1 "	3.4
Centrifuged	1.2 "	2.3×10^4	65.7
	6.9×10^3	2.0 "	74.3

up and titrated separately. While over-all titers in the 2 groups of cultures were approximately the same, there was a pronounced difference in content of rickettsiae in the cell fractions, about 2% of the total in stationary and 70% in centrifuged cultures.

Table III presents results of a typical titration of typhus rickettsiae in entodermal cell cultures. Each of 6 ten-fold dilutions of rickettsiae were injected into 8 cultures, 4 were kept in a stationary position, and 4 were centrifuged. Cultures of each group were harvested at 7 days and titrated. In this and in several other experiments consistent infection of stationary cultures was not obtained with dilutions of rickettsial suspensions much greater than 10^{-2}. When the rickettsiae were centrifuged onto the entodermal monolayer, the cells appeared to be as susceptible as in the intact embryo. Experiments not shown in Table III indicated that peak titers were usually reached before the 7th day and numbers of rickettsiae seldom exceeded 3×10^5 per culture. Therefore, an increment was not clearly demonstrated in the group of cultures which had received the highest concentration of rickettsiae, but 100-200-fold increases were obtained in other groups (Table III). In some cases the cultures proved to be infected, but less than 3 rickettsiae per culture were recovered. This finding cannot be simply explained.

Some attempts to pass rickettsiae serially in stationary cultures failed, because, as appears from Table III, infection of the cells required large inocula, which, eventually, were not provided by the harvests of these cultures. By centrifugation, serial passage could be carried out without any particular difficulty or loss in rickettsial titer. The harvest from the 10th passage, which represented a 10^{-15} dilution of the original yolk sac, had a titer (confirmed by cross-protection test in cotton rats) of 2×10^4 rickettsiae per culture.

TABLE III. Recovery of Typhus Rickettsiae from Entodermal Cell Cultures.

Inoculum No. rickettsiae	Yield of infectious rickettsiae at 7 days (per culture)	
	Stationary	Centrifuged
5×10^3	8×10^1	1×10^4
5×10^2	< 3	5 "
5×10^1	"	1 "
5	No	5×10^2
.5	evidence of	6×10^1
.05	infection	< 3

TABLE IV. Recovery of Q Fever Rickettsiae from Entodermal Cell Cultures.

Time of harvesting (hr)	Effect on chick embryos			
	Deaths/total		Mean survival time*	
	Stationary	Centrifuged	Stationary	Centrifuged
72	2/14	7/12	14.9	14.4
	3/14		14.8	
120	4/14	12/13	14.2	11.8
	8/14	15/15	13.4	12.3
166	12/14	13/13	12.4	11.5
	11/14	11/12	12.5	11.9

* Embryos surviving at 14 days were given 15 as day of death.

C. burnetii. Work with Q fever rickettsiae was complicated by the fact that they cannot be titrated by simple methods(13). Weiss and Pietryk(2) enumerated foci of infection formed on monolayers of entodermal cells. By application of this method to the current study, differences were not noted in number of foci of infection in stationary and centrifuged cultures. Table IV illustrates an experiment in which cultures were harvested at 3 intervals of time and the harvests diluted 1:10 and injected into eggs. The results, expressed in terms of relative numbers of embryos dying in each group, and their mean survival times, indicate that greater numbers of rickettsiae were harvested from the centrifuged than from the stationary cultures, especially at 120 hours. However, the differences are not as great as those obtained with typhus rickettsiae.

Psittacosis virus and related viruses, grown in entodermal cells, produce inclusion bodies which can be readily recognized with an 8 mm objective(1,4). Therefore, experiments with psittacosis virus were evaluated by microscopic examination of the cultures, and a number of observations were made that could not be accomplished with the rickettsiae. For example, examination of cultures fixed and stained during the first cycle of infection, at approximately 34 hours after inoculation, revealed that the inclusion bodies were more evenly distributed and of more uniform size in centrifuged than in stationary cultures.

Table V illustrates the difference in titers obtained in eggs and in entodermal cell cultures fixed and stained 88 hours after inoculation. The cultures listed as "shaken" were prepared in roller tubes with one flat side, just large enough to support an 11 × 22 mm coverslip. These cultures were maintained in a horizontal position and were shaken at 80 oscillations per minute for 1 hour at room temperature. This procedure has been shown to enhance infection(1), but did not prove to be superior to the stationary cultures used in these experiments. Entodermal cells were infected in centrifuged tubes almost as readily as in the intact embryo. The differences between stationary and centrifuged cultures (Table V) are significant, if some of the qualitative differences, noted above, are considered. For example, some of the stationary cultures listed as "infected" appeared to contain a single relatively small cluster of elementary bodies.

A more valid appraisal of the difference between stationary and centrifuged cultures is presented in Table VI. In this experiment the cultures were fixed at 34 hours and infected cells detected with an 8 mm objective were counted. The difference between the infected cell counts of the 2 types of cultures appeared to be approximately 100-fold.

Trachoma virus. Following successful cultivation of trachoma virus by Gordon *et al.* (14) in centrifuged entodermal cell cultures, a preliminary experiment was carried out to determine effect of centrifugation. The cultures were inoculated with a single dilution of virus and fixed and stained at 67 hours, at a time when inclusion bodies were most conspicuous and still represented the product of initial infection(14). Infected cells were not uniformly distributed in stationary or centrifuged cultures and far too numerous in the centrifuged cultures to provide more than a rough estimate of the difference between the 2 groups, which appeared to be at least 10-fold. In the centrifuged cultures, microscopic fields (covering an approximate area of 0.16 mm^2) containing 60-100 infected cells could readily be found. Equivalent fields in the stationary cultures contained 5-9 infected cells. Calculated ID_{50} for centrifuged cultures appeared to be of the same order of magnitude as that for eggs ($10^{-4.6}$).

TABLE V. Titration of Psittacosis Virus in Eggs and in Entodermal Cell Cultures.

Dilution of yolk sac suspension	Embryos: Deaths/total	Cultures: Infected/total		
		Shaken	Stationary	Centrifuged
10^{-5}	10/10	6/6	5/5	6/6
10^{-6}	"	4/6	4/6	"
10^{-7}	"	0/6	3/6	"
10^{-8}	5/10			0/4
ID_{50}	$10^{-8.0}$	$10^{-6.2}$	$10^{-6.7}$	$10^{-7.5}$

TABLE VI. Titration of Psittacosis Virus by Infected Entodermal Cell Count Method.

Procedure	Cultures No.	Virus dil.	Count ± S.E.	Calculated ID_{50}*
(Egg)				$10^{-8.2}$
Stationary	4	$10^{-3.3}$	338 ±116	$10^{-6.1}$
	4	$10^{-4.3}$	85 ± 22	$10^{-6.4}$
	4	$10^{-5.3}$	3 ± 1	$10^{-5.9}$
Centrifuged	17	$10^{-5.3}$	298 ± 40	$10^{-7.9}$

* ID_{50} was calculated on the basis that it represents approximately 0.7 particle.

Experiments with human skin cells. Suspensions of skin cells were inoculated with typhus rickettsiae, psittacosis and trachoma viruses, but only psittacosis virus appeared to be highly infectious. The effect of centrifugation on infection with this virus was tested in an experiment similar to that illustrated in Table V. The skin cells, 100,000 per 0.5 ml, were suspended in a mixture of nutrient medium and inoculum and placed in flat-bottomed tubes with coverslips. The skin cells formed monolayers on the coverslips and ID_{50} were determined by microscopic examination of the cultures fixed and stained 88 hours after inoculation. The ID_{50} for eggs, and for the centrifuged and stationary cultures were, respectively, $10^{-7.7}$, $10^{-7.2}$, and $10^{-4.6}$. Thus, centrifugation increased infection at least 100-fold and raised it to a level approaching that obtained in eggs.

Discussion. The variation in efficacy of the centrifugation procedure with the 4 infectious agents can in part be attributed to differences in thermostability. For example, with a thermostable agent, such as *C. burnetii*, centrifugation, possibly, only accelerates adsorption which will eventually take place in stationary cultures. In the case of a ther-

molabile agent, such as *R. prowazekii*, centrifugation plays the dual role of accelerating adsorption and, by this action, of preserving activity of a greater number of particles.

During development of the centrifugation procedure, a number of arbitrary choices were made which require further testing. For example, speed of centrifugation was the highest which could be conveniently employed, but the effect of higher or lower speeds has not been investigated. Temperature during centrifugation is another variable which was eliminated by selecting 20°C. This choice was made as a compromise to prevent both thermal inactivation of infectious agents and inhibition of host-parasite interaction.

The practical advantages of the procedure are obvious. It is simple and efficient and produces consistent results that closely resemble those obtained in eggs. It also offers an opportunity to separate adsorption from the other phases of intracellular growth of the infectious agents. Similarity of results obtained with 4 different agents and 2 unrelated host cells indicates that this method may have wide applications.

Summary. A method for effective inoculation of cultured cells with some rickettsiae and larger viruses is described. Chick embryo entodermal cell monolayers were prepared either in standard screw-cap tubes or, when microscopic examination was required, in flat-bottomed tubes containing 12 mm round coverslips. Following addition of inoculum diluted in fresh nutrient, the tubes were centrifuged horizontally at 1600-1800 g for 1 hour at 20°C. By this procedure infection of cells with *Rickettsia prowazekii* was greatly enhanced (about 10,000-fold) over that obtained in stationary tubes, and susceptibility of entodermal cells appeared to be as great in centrifuged tubes as in intact embryo. Good enhancement was also obtained with psittacosis (about 100-fold) and trachoma (at least 10-fold) viruses and moderate enhancement with *Coxiella burnetii*. The same procedure was successfully used to enhance infection of cells from human skin cell line (HuS 2806) with psittacosis virus.

1. Weiss, E., Huang, J. S., *J. Inf. Dis.*, 1954, v94, 107.

2. Weiss, E., Pietryk, H., *J. Bact.*, 1956, v72, 235.

3. Weiss, E., Dressler, H. R., *ibid.*, 1958, v75, 544.

4. Weiss, E., Gordon, F. B., *Fed. Proc.*, 1958, v17, 464.

5. Macdougald, T. J., Beams, H. W., King, R. L., PROC. SOC. EXP. BIOL. AND MED., 1937-38, v37, 468.

6. Gey, G. O., Bang, F. B., Gey, M. K., *Ann. N. Y. Acad. Sci.*, 1954, v58, 976.

7. Bovarnick, M. R., Miller, J. C., Snyder, J. C., *J. Bact.*, 1950, v59, 509.

8. Allen, E. G., Bovarnick, M. R., Snyder, J. C., *ibid.*, 1954, v67, 718.

9. Weiss, E., Dressler, H. R., Suitor, E. C., Jr., *ibid.*, 1957, v73, 421.

10. ———, *ibid.*, 1959, v77, 91.

11. Hanks, J. H., Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.

12. Perry, V. P., Evans, V. J., Earle, W. R., Hyatt, C. W., Bedell, W. C., *Am. J. Hyg.*, 1956, v63, 52.

13. Wagner, J. C., Gordon, F. B., *Bact. Proc.*, 1950, p92.

14. Gordon, F. B., Quan, A. L., Trimmer, R. W., *Science*, 1960, v131, 733.

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Effect of Cortisone on Liver Phosphorylase Activity.* (25638)

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Liver glucose-6-phosphatase activity, in adrenalectomized rats, is increased by cortisone(1,2). While this could explain increase in blood sugar induced by cortisone, it would not explain increased liver glycogen also induced. Phosphorylase is required for synthesis of glycogen in the glycolytic pathway. The effect of adrenalectomy and subsequent cortisone injection on rate of phosphorylase activity in rat liver has been determined.

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