THE PRODUCTION OF HIGH TITRE POLIOVIRUS IN CONCENTRATED SUSPENSIONS OF TISSUE CULTURE CELLS

J. C. N. WESTWOOD, G. APPLEYARD, D. TAYLOR-ROBINSON AND H. T. ZWARTOUW

From the Microbiological Research Establishment, Porton, Salisbury, Wilts.

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" MINIMUM requirements ", as laid down by the Ministry of Health for material to be used in the manufacture of poliomyelitis vaccine, demand a virus infectivity titre of at least 10^6/ml. Though the material used in practice normally exceeds this titre by about ten-fold, it seems likely that the borderline efficiency of the vaccine, and indeed of inactivated virus vaccines in general, is due to their low content of virus antigens. While high titre virus suspensions may be produced by physical or chemical concentration procedures, such methods entail unavoidable losses in virus and, at least with the smaller viruses, are too cumbersome for use on a large scale. In this laboratory, the possibility is being explored of producing high concentrations of poliovirus directly into the supernatant fluid of tissue cultures by the infection of concentrated suspensions of cells. The work reported here shows that the method is effective and simple.

Published results indicate that the average yield of poliovirus from single cells of serially cultivated cell lines lies between 100 and 500 infective units. (Girardi, McMichael and Henle, 1956; Darnell and Eagle, 1958; Dunnebacke and Reaume, 1958; Roizman, Hopken and Mayer, 1958). If this yield cannot be exceeded, an increase in the final virus concentration can be attained only by increasing the number of infected cells per unit volume of fluid. Churcher, Sheffield and Smith (1959) achieved this in monolayer cultures by growing cells on all six sides of hexagonal Pyrex bottles and infecting them in half the normal volume of medium. This method cannot, however, be easily adapted to large scale production whereas cell suspensions may be used at any desired volume and any desired concentration.

Pereira (1954) successfully infected suspensions of chick embryo cells with fowl plague virus and McLimans, Giardinello, Davis, Thomas and Kucera (1957) showed that the cells of the ERK1 cell line (Westwood, Macpherson and Titmuss 1957) would support the multiplication of poliovirus when grown and infected in suspension. Girardi *et al.* (1956), investigating the multiplication of polio and influenza viruses in suspensions of HeLa cells found that cell survival was optimal at a concentration of 5×10^5/ml. and concluded that, above this figure, exhaustion of essential nutrients led to early cell deterioration. This concentration of cells gave yields of about 100 plaque-forming units (PFU) per cell of poliovirus, but would clearly be inadequate for the production of virus in high titre. It seemed necessary therefore to investigate first the factors governing cell survival under the adverse conditions obtaining in high cell concentrations.

The work was originally undertaken, in parallel with that already reported from University College Hospital (Churcher *et al.* 1959) in order to investigate

methods of producing flocculating poliovirus antigen (Smith, Sheffield, Lee and Churcher, 1956) without the necessity for centrifugal concentration.

MATERIALS AND METHODS

Poliovirus.—Type 1 (Brunhilde) poliovirus, obtained through the courtesy of Professor Wilson Smith, was used throughout.

Tissue cell line.—The ERK1 cell line, derived from embryo rabbit kidney (Westwood *et al.* 1957) was used. For the sake of continuity with previous work at U.C.H., the "D" line of cells, originally sent to U.C.H. in February, 1957, was reintroduced into this laboratory in January, 1958, and was used for the present investigations.

Growth and preparation of cells.—Cells were grown in stationary Roux bottle cultures for 7 days without change of medium and were just confluent, without overcrowding, at the end of this time.

The growth medium was a modification of the high glucose, high bicarbonate medium described by Zwartouw and Westwood (1958) and consisted of:—calf serum, Seitz-filtered and inactivated at 60° for 120 min., 15 per cent; tryptic digest of lean beef, 5 per cent; yeast extract, 0·5 per cent; Fildes' peptic digest of sheeps' blood, 0·1 per cent; Earle's saline, modified to contain 4 g./l. of glucose and 3 g./l. of sodium bicarbonate, to 100 per cent.

Neomycin, 125 μg./ml.; Penicillin, 125 units/ml. and Streptomycin, 125 μg./ml. were included in the medium.

Cells were harvested with a mixture of 0·05 per cent trypsin (DIFCO 1/250) and 0·05 per cent EDTA (Ethylene-diamine-tetra-acetic acid, disodium salt; British Drug Houses Ltd.) in phosphate-buffered saline, pH 7·2, modified by the addition of sodium bicarbonate to a concentration of 0·1 per cent. After centrifugation from the trypsin-EDTA mixture the cells were resuspended in a small volume of medium, counted in a haemocytometer and diluted to the desired concentration.

Growth of poliovirus.—Cell suspensions were distributed into 250 ml. Pyrex feeding bottles, and virus inoculum added to bring the final volume in each bottle to 10 ml. Bottles were gassed with air containing the appropriate concentration of CO_2, stoppered and placed in a slow roller drum at 37°. After suitable time intervals, the cultures were cooled, cell débris removed by centrifugation at 2000 *g.* for 5 min., and the supernatant fluids stored at 2–4°. Samples were titrated within one week of harvesting.

The medium used for poliovirus replication was the same as that used for cell growth except that it contained only 10 per cent of calf serum and the glucose and bicarbonate concentrations were varied as described in the text.

Poliovirus titrations.—Infectivity titrations were carried out by Dulbecco's plaque technique as modified by Cooper (1955) and titres are reported as plaque-forming units (PFU) per ml.

Flocculation titrations.—Specific rabbit antisera, kindly supplied by Professor Wilson Smith, were absorbed to remove ERK1-cell precipitins and used in the micro-flocculation test described by Smith, Sheffield, Churcher and Lee (1956) to measure the flocculating potency of poliovirus preparations. Antigen dilutions were titrated against a fixed serum dilution, chosen to give optimal flocculation for the particular batch of serum. Antigen potency is reported as the highest initial dilution of antigen giving a positive reaction.

RESULTS

Maintenance of Viability in Cell Suspensions

It was assumed that the virus yield from cells infected in suspensions was likely to depend on the success with which cell viability was maintained. For the technique to be successful virus titres of at least 10^9 PFU per ml. should be obtainable. This would require cell concentrations of $5–10 \times 10^6$/ml. provided that the expected yield of virus per cell was attained. Experiments were therefore performed to determine the optimal conditions for cell survival at concentrations of this order. The two most obvious factors to be considered were the

rapidity with which glucose would be exhausted and the rate at which the pH would fall below tolerable limits.

In an experiment relating changes in pH, glucose exhaustion and cell viability, 3 cell concentrations and 2 levels of bicarbonate-CO_2 buffer were examined. From each set of replicate cultures in the roller drum at 37°, a sample bottle was withdrawn for assay at various time intervals. pH determinations were made at 37°, using an enclosed glass electrode, within thirty seconds of each bottle being opened. Exhaustion of glucose from the medium was detected by a colorimetric test using "Clinistix" (Ames Co., London). The medium contained initially 4 g./l. of glucose, the highest concentration found by Zwartouw and Westwood (1958) to be non-toxic, and a fall to 0·5 g./l. was taken to indicate imminent exhaustion. Positive staining with trypan blue was assumed to indicate loss of cell viability (Girardi *et al.* 1956, McLimans, Davis, Glover and Rake, 1958).

TABLE I.—*The Relationship of pH Fall and Glucose Utilization to Maintenance of Cell Viability at Various Cell Concentrations*

Buffer concentration	Incubation time in hr.	Cell concentration/ml. 5×10^6 pH	Glucose*	Per cent dead cells	10×10^6 pH	Glucose	Per cent dead cells	20×10^6 pH	Glucose	Per cent dead cells
$NaHCO_3$: 2 g/l. CO_2: 5 per cent.	0	7·65	++	3·5	7·65	++	3·5	7·65	++	3·5
	2	7·43	++	..	7·22	++	..	6·7	++	..
	5	7·3	++	21	6·99	++	23	6·27	++	29
	10	7·0	++	19	6·75	+	17	6·65	+	27
	24	6·7	+	37	6·75	−	35	6·8	−	52
$NaHCO_3$: 4 g/l. CO_2: 10 per cent.	0	7·54	++	3·5	7·54	++	3·5	7·54	++	3·5
	2	7·42	++	..	7·3	++	..	6·98	++	..
	5	7·36	++	18	7·18	++	17	6·9	−	23
	10	7·2	++	18	6·95	+	25	6·9	−	34
	24	7·0	−	40	7·1	−	53	7·08	−	69

* Glucose. ++ = > 1 g/l. + = 1·0 − 0·5 g/l. − = < 0·5 g/l.

The results (Table I) showed the better pH control obtained by doubling the normal buffer concentration and also indicated the rapidity with which glucose was exhausted at high cell concentrations when a rapid fall in pH was prevented. In other experiments carried out on both infected and uninfected cultures it was found that higher concentrations of glucose were tolerated provided that the buffer concentration was increased to compensate for the extra acid production. However, even when 8g./ l. of glucose was initially present, all glucose was exhausted within 16 hr. by cells at a concentration of 10^7/ml. The results shown in Table I suggest that the rate of cell death is not directly correlated with fall in pH and this conclusion was confirmed in other experiments. The fact that more cells died in those suspensions in which the pH was prevented from falling below 6·9 suggests rather that glucose exhaustion may be the more important factor. However the divergence in the rate of cell death occurred after 10 hr., at which time three quarters of the cells were still not stainable with trypan blue and glucose was still demonstrable in the cultures containing 10^7 cells per ml.

It was concluded that, at a concentration of 10^7/ml., about three-quarters of the cells could be maintained in a viable state for a ten hour period without exhaustion of glucose. Since it seemed probable that adequate control of the rate of fall of pH would favour optimal virus production, a medium containing 4g./l. of both glucose and sodium bicarbonate was adopted.

Virus Production from Concentrated Cell Suspensions

Virus inoculum

It was clear from the results of the experiments already discussed that virus replication must be restricted to a single cycle. The ten hour period during which cell viability could be maintained at a high level should then suffice for maximum virus replication. This conclusion was confirmed by experiments in which the multiplicity of infection was varied in cell suspensions containing 10^7 cells per ml. Table II shows the result of one such experiment.

TABLE II.—*Virus Yields at Various Multiplicities of Infection*

Inoculum PFU/cell	Virus titre at 24 hr. $\times 10^{-9}$	Virus yield PFU/cell
0·2	1·35	135
1·0	1·65	165
2·0	2·35	235
20·0	2·30	230

Cell concentration 10^7/ml.

Increasing the multiplicity of infection above 2 PFU per cell, at which level about 86 per cent of the cells should in theory be initially infected, did not improve the virus yield. This multiplicity was therefore adopted for all future experiments.

Effect of cell concentration on virus yield

In a number of experiments it was found that increasing virus titres were obtained as the cell concentration was increased up to 10^7 cells per ml. but at or above the 2×10^7 concentration an abrupt fall in virus yield occurred (Table III). From Table I it can be seen that control of the rate of fall of pH begins to fail at this level, even with the higher buffer concentration. That pH is in fact a contributory factor in determining the optimal cell concentration under these experimental conditions is suggested by Experiment 2 of Table III, in which two levels of buffer were compared. The effect of the better pH control at the higher buffer concentration is clearly shown. It may also be seen that, at both buffer levels, the highest virus yield in terms of PFU per cell occurred at the next lower cell concentration to that giving the highest virus titre.

Despite the poorer yield per cell, a concentration of 10^7 cells per ml. was selected for further work because it produced the highest virus titres.

Effect of pH

Investigation of the effect of pH on virus yield is complicated by the fact that the pH cannot be maintained at a constant value under the conditions of these experiments. From experiments in which the pH was followed under

TABLE III.—*Effect of Cell Concentration on Virus Yield*

Expt.	Buffer concentration	Cell concentration/ml. × 10^{-6}	Virus titre × 10^{-8}	Virus yield PFU/cell
1	$NaHCO_3$: 4 g./l. CO_2 : 10 per cent	2·5	0·45	18
		10	10	100
		20	7	35
		30	0.5	—
		40	0·5	—
2 A	$NaHCO_3$: 2 g./l. CO_2 : 5 per cent	2·5	8	320
		5	11·5	230
		10	7·1	71
		20	0·05	0·25
		40	0·05	0·1
2 B	$NaHCO_3$: 4 g./l. CO_2 : 10 per cent	2·5	2·8	112
		5	10.5	210
		10	13	130
		20	10	50
		40	0·22	0·6

various medium conditions, it was possible to conclude that the best yields were obtained only when the pH was not permitted to exceed the limits of 7·5–6·8 during the course of the experiment. The optimal pH range for poliovirus production cannot, however, be determined in the presence of glucose without continuous pH regulation. This problem is being further investigated.

Effect of time of incubation on infectivity titre

The intracellular development of infective virus was followed in one experiment only. The rate of appearance of virus was similar to that observed in monolayer cultures (Roizman *et al.*, 1958) and maximum titre was observed at 12 hr. The time relationships of virus release have been more closely followed and Table IV shows the results of three experiments in which sample cultures were harvested at the stated time intervals after infection. In all such experiments the highest infectivity titre in the culture fluid was obtained at 16–24 hr.

TABLE IV.—*Effect of Time of Incubation on Infectivity and Flocculation Titres*

Expt.	Hours after infection	Virus titre × 10^{-8}	Virus yield PFU/cell	Reciprocal of flocculation titre
1	8	<5	—	8
	16	23	230	16
	24	32·5	325	16
	48	16	160	16
	72	2·4	24	16
2	8	6·8	68	8
	16	28	280	16
	24	15	150	16
	48	26	260	16
	72	<0·1	—	16
3	8	<0·1	—	1
	16	16	160	8
	24	20	200	8
	48	4·5	45	16

A terminal fall in titre of about 1 log unit, due presumably to virus inactivation, was evident at either 48 or 72 hr. It was found that virus suspensions set up under the test conditions but in the absence of cells lost about 90 per cent of their infectivity titres within 48 hr.

Flocculating Activity of Virus Preparations

Since the original purpose of this work was to develop a method for producing flocculating antigen without the necessity for virus concentration, many of the preparations were titrated for flocculating potency as well as infectivity. The experiments presented in Table IV show that high infectivity titres in the supernatants of early harvests, up to 24 hr., are associated with satisfactory flocculating activity. It is also evident from this table that flocculating activity is more stable than infectivity at 37°. The antisera used in the flocculation tests (Belyavin, personal communication) contained antibodies against both the D and C components of the virus (Mayer, Rapp, Roizman, Klein, Cowan, Lukens, Schwerdt, Schaffer and Charney, 1957) and we have made no attempt to distinguish between the two activities. It is known (Roizman, Mayer and Roane, 1959) that D activity may be converted to C as a result of heat treatment, and it it possible that the retention of flocculating activity, despite loss of infectivity after 24 hr., may be due to this change. Although this would seem unlikely at the temperature and times involved, it was decided to harvest virus for flocculating antigen during the 16–24 hr. period when both infectivity and flocculating activity had reached their peak, so as to avoid the possibility of degradation of D component. Flocculation titres of between $\frac{1}{16}$ and $\frac{1}{32}$ are regularly obtained in this manner.

DISCUSSION

The experiments quoted in this paper show that high titre virus preparations may be obtained by infecting cell suspensions containing 10^7 cells per ml. The virus yields, in terms of PFU per cell, are comparable to those obtained in monolayer cultures. The work reported has been confined to Type 1 poliovirus and to the use of a fixed volume of 10 ml. of suspension agitated by rolling in a 250 ml. feeding bottle. The medium used was complex and contained a high proportion of serum. These conditions were imposed as a matter of convenience for early experimental investigations and subsequent work have shown that the method is applicable to the other poliovirus types, that larger volumes of suspension may be used if conditions are suitably adjusted and that similar virus yields may be obtained in a simple physiological salt solution in the absence of serum, nitrogenous nutrients and glucose. This work, which will be reported in detail, shows that the theoretical advantages of the method should be fully realisable.

The practical limitation to the use of this technique on a production scale is likely to be the supply of cells, but recent work (Cooper, Burt and Wilson, 1958) suggests that this problem may be solved by the growth of cells in suspension on the chemostat principle.

SUMMARY

The possibility has been investigated of obtaining high titre poliovirus preparations by infecting suspended tissue culture cells at high concentration in order to obtain the released virus in the smallest practicable volume of fluid.

It was found that three-quarters of the cells in a suspension containing 10^7 cells per ml. could be maintained in a viable state as judged by absence of staining with trypan blue, for a period long enough to allow a single cycle of virus replication.

If a multiplicity of infection of 2 PFU per cell or higher were used, maximum virus titres in the culture fluid were reached within 24 hr. and virus yields of up to 325 PFU per cell were obtained. Poliovirus preparations containing over 10^9 PFU per ml. could be obtained in this way.

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