Dye Uptake Methods for Assessing Viral Cytopathogenicity and their Application to Interferon Assays

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(Accepted 24 June 1969)

SUMMARY

When neutral red is added to the liquid medium in tissue cultures, more dye is taken up by healthy cells than by damaged cells. This forms the basis of a method for assessing the relative extent of cell damage in different cultures, the amounts of bound dye being determined directly by inspection of the stained cell sheets, or indirectly by estimating extracted dye colorimetrically. This procedure can be used to measure the effects of a cytocidal virus and can also be applied to the assay of interferons. Here it provides a precise and reproducible method which is unusually sensitive for the assay of mouse interferon, and has also been used to assay rat, chick, rabbit and human interferons.

INTRODUCTION

This paper describes a new general method for measuring the damage caused to cell cultures by the growth of a cytocidal virus or the action of another noxious agent. Its principle is that only healthy cells can take up a vital dye, such as neutral red, added to the culture medium. Thus after surplus dye has been washed away, the extent of cell damage in different cultures is shown by the relative amounts of dye bound in the cell sheets and these can be judged directly or measured colorimetrically. We have used this method particularly to assay mouse and other interferons, but it can also be used in virus infectivity and neutralization assays and virus growth studies, and in cytotoxicity tests (Finter, 1969).

METHODS

Tissue Cultures. Mouse L cells were grown in Eagle’s (1959) medium with 8% calf serum and 50 u./ml. penicillin. Cells were grown in rubber stoppered 4 in. × ½ in. test tubes, seeded with 10⁶ cells in 1 ml. growth medium. Alternatively, cells were grown on the bottom of vials, as suggested by Baron, Buckler & Takemoto (1966). Six Universal wide mouth one-ounce screw cap bottles (United Glass, London) without caps were grouped around a central bottle with elastic bands. Each bottle was seeded with 10⁶ cells in 2 ml. medium, and all seven were covered with a Petri dish lid. These ‘clusters’ were incubated in an atmosphere of 5% CO₂ in air. Cells were used when confluent, 3 to 5 days after seeding.

Virus. Semliki forest virus (the MB strain of Finter, 1964a) was used in the form of a 10% (w/v) mouse brain suspension, stored at −20°.

Neutral red. Dye (Gurr, London) was ground with alcohol to give about a 20% (w/v)
solution, diluted in water to 1%, filtered through paper, and autoclaved at 10 lb/in² for 20 min. One such batch diluted 1/2000 had an optical extinction of 0.84 at 540 nm in a 1 cm. light path cell. Other batches were adjusted to match this reading. The amounts for use in tests were determined empirically (see Results), but usually a final concentration of 1.5 x 10⁻⁵ g./ml. of medium was suitable.

*Interferons.* These were obtained from the brains of mice infected with West Nile virus (Finter, 1964b), and from the serum of mice injected intravenously with Newcastle disease virus (Baron & Buckler, 1963), the inducing virus being destroyed by treatment at pH 2 for 2 days and 5 days respectively.

![Diagram](image)

**Fig. 1.** Assay of an interferon preparation. (A) The appearance of the stained cell sheets (grown on the bottoms of one ounce bottles in a ‘cluster’). (B) The dye solutions extracted from the same cultures. The relative amounts of stain or extracted dye are indicated by the cross-hatching. The 50% dye uptake end point as estimated visually (shown by the arrow in A and B) was 2.75 log. DU 50 units per ml.

**Mouse interferon assay method.** Interferon samples were diluted in Eagle’s medium with 2% calf serum, and 1 ml. samples of each dilution were added to groups of two or three tube cultures. After overnight incubation at 37°, the cultures were drained, washed twice with 2 ml. of medium, and infected with an appropriate amount of Semliki Forest virus, usually about 1000 TCD50, diluted in Eagle’s medium with 2% calf serum. Control cultures treated with diluent only were included in each test, half being infected (virus controls) while the remainder served as cell controls. The cultures were reincubated for about 42 hr until there was marked destruction of the cells in the virus control tubes. The dye uptake method was then used to assess the extent of cell damage in the different cultures. Interferon activities were calculated as described below.

**Dye uptake method.** Neutral red (0.4 ml.) at a suitable dilution was added to the
CPE measured by dye uptake

medium in each culture. After 2 hr incubation, the medium was decanted and the cell sheets were drained by inversion and washed twice with 2 ml. of saline. When cells were grown on the bottom of bottles, as in the 'clusters' described, the relative amounts of dye bound by the cells in each bottle were easily judged if the cell sheets were viewed from above against a white background. Fig. 1 A depicts results with five twofold dilutions of an interferon preparation and cell and virus controls in a 'cluster'. There was an obvious difference between the deep staining of the cell sheet in the cell control culture (scored as 4) and the nearly colourless cell sheet in the virus control culture (scored as 0), and intermediate degrees of staining could easily be distinguished. In Fig. 1 A, the relative amounts of staining are indicated by the cross-hatching. With test tube cultures, it was not feasible to assess dye uptake in this way. Instead, the dye was extracted into ethanol (buffered with an equal volume of Sorensen citrate buffer, pH 4·2, so that the full acid colour of the dye was developed), and uptake was judged by the depth of colour of the solutions obtained (Fig. 1 B). When quantitative measurements of cell damage were required, the dye was extracted from bottle or tube cultures. Dye solutions from replicate cultures were measured individually or as a pool, as desired, as optical extinctions measured at 540 nm., using a Unicam SP 700 spectrophotometer, equipped with an Autochanger automatic sample changer.

RESULTS

Selection of neutral red concentration

The amount of neutral red suitable for use in dye uptake tests was determined empirically. Thus 1 % neutral red was diluted in twofold steps from 1/100 to 1/1600, and 0·4 ml. amounts of each dilution were added to groups of six L cell tube cultures. After incubation for 2 hr, the bound dye was extracted into 2 ml. of buffered alcohol per tube and estimated colorimetrically. In three such experiments progressively larger amounts were extracted from the cultures treated with increasing concentrations of dye, and solutions with a convenient $E_{540}$ value of about 0·7 were obtained from those to which the 1/200 dilution had been added, giving a final dye content of $1·5 \times 10^{-5}$ g./ml. of medium. This concentration of dye was therefore used in most subsequent tests, though the actual amounts of dye bound varied to some extent from one occasion to another, presumably depending on the physiological condition of the cells concerned.

In another experiment, the rate of dye uptake was measured. Groups of nine L cell cultures with dye added at a final concentration of $0·9 \times 10^{-5}$ g./ml. medium were incubated in the dark for 1, 2, 4 and 20 hr. The dye extracted at these times gave $E_{540}$ readings of 0·380, 0·457, 0·490 and 0·440 respectively. Thus little additional dye was taken up by the cells after incubation for more than 2 hr. Other groups of cultures in the same experiment were incubated for 1, 2, 4 and 20 hr exposed continuously to light from fluorescent tubes, and these provided dye solutions with $E_{540}$ values of 0·363, 0·457, 0·510 and 0·475. Thus this concentration of neutral red did not photosensitize the cells during the 20 hr period.

Assay of interferons by the colorimetric dye uptake method

Two interferon preparations, A and B, and our laboratory mouse serum standard preparation (MSS) were assayed together. The mean $E_{540}$ readings obtained for the
resulting dye solutions were plotted against the corresponding log. dilutions of interferon (Fig. 2). Statistical analysis of this and many similar assays confirmed the sigmoid shape of the full curve relating dye uptake to the log. interferon dilution, with an intermediate linear portion over a range from about 25 to 75% dye uptake: when different preparations are titrated in parallel, these linear portions have slopes which do not differ significantly, though the slopes on different occasions are not necessarily the same. Thus a common slope was used for the data obtained on each occasion, as in Fig. 2. From these dose-response lines, the activity of an interferon preparation

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\begin{align*}
\text{Dye uptake (mean } E_{480} \text{)} & \quad \text{Cell control} \\
\text{Log}_{10} \text{ dilution of interferon} & \\
\text{Virus control} & \\
\end{align*}
\]

*Fig. 2.* An assay of three mouse interferon preparations, A, B and MSS by the colorimetric dye uptake method.

(in 50% dye uptake units [DU 50] per ml.) was obtained from the reciprocal of the dilution giving dye uptake midway between that in the virus and cell control cultures. Thus the activities of A, B and MSS were 2.37, 3.28 and 4.27 log. DU 50/ml. (Fig. 2).

The assays of Fig. 2 were from a standardization experiment, and since rather precise estimates of relative activities were required, the three preparations were diluted in 0.2 log. steps. Each dilution was tested in nine tube cultures, the resulting dye solutions being pooled in groups of three. Mathematical analysis of the results showed that A was 79 times (95% confidence limits, 62 to 102 times) more active than MSS, and B was 9.8 times (95% confidence limits, 7.5 to 13.0 times) more active than MSS. For most purposes, however, we have used 0.4 or 0.6 log. dilutions of interferon preparations and four or six tubes to test each dilution. In consequence we have needed to make only a few colorimetric estimations for each interferon sample assayed, since these are only useful if the dye solutions give readings falling on the linear portion of the dose-response curve.

We have usually determined end points graphically, but mathematical methods can
be used. Mr C. J. Clarke of our Statistical department has devised a computer program providing a full statistical analysis of the results, and this has proved very convenient especially when the colorimeter readings are entered directly on to punched tape.

Assay Reproducibility

Samples (0.2 ml.) of a pool of mouse-serum interferon, used as a laboratory standard, were stored at -20°C. To guard against the chance occurrence of abnormally sensitive or insensitive conditions, this standard, diluted in 0.2 log. steps, was included in every set of assays. In 79 successive assays during a 44-week period (Fig. 3), the mean activity was 3.97 log. DU50 and the standard deviation of the individual activities was 0.18 log. units (coefficient of variation 0.163%).

![Fig. 3. Incidence of particular values for the activity of a standard mouse interferon preparation obtained by the dye uptake colorimetric method in 79 consecutive assays.](image)

Sensitivity

Results of assays of mouse interferon by the dye uptake method using Semliki Forest virus and L cells were about 0.2 log. units (1.6 times) higher than those obtained in the same system by estimating the 50% cytopathic effect–inhibition endpoints. Both these methods seem to be highly sensitive for the assay of mouse interferon. The dye uptake method was 25 times more sensitive than the plaque reduction method with the same cells and virus, and 200 times more sensitive than the quantitative haemadsorption method (Finter, 1964c) with L cells and Sendai virus. In collaborative studies with other workers, the dye uptake method was about 10 times more sensitive than a yield reduction method with vesicular stomatitis virus and primary mouse embryo cells, and about 400 times more sensitive than a plaque reduction method with the same cells and virus.
Amount of challenge virus

For each virus cell culture system, the time when cultures infected with about 1000 TCD50 of virus no longer bound any neutral red was first determined. Then groups of cultures infected with increasing amounts of virus were incubated for this time. The smallest amount of virus leading to nearly complete cell destruction in terms of dye uptake was chosen for use in interferon assays. Thus from the amounts of dye taken up by L cells 42 hr after infection with different amounts of Semliki Forest virus in two experiments (Fig. 4), a dose of 1000 TCD50 was selected for use in this system.

As shown by dye uptake (Fig. 4), and also by microscopy, there was incomplete destruction of L cells when large amounts of Semliki Forest virus were inoculated. This may have been due to interferon present in the mouse brain extracts used as a source of virus, since there was no such prozone if virus was used after one passage in chick embryo fibroblasts.

A mouse-serum interferon preparation was assayed using five different amounts of Semliki Forest virus. Endpoints were obtained at log. dilutions of 4.28, 4.30, 4.23 and 4.47 when respectively 10^6, 10^4, 10^3 and 10^2 TCD50 of virus were used. No result was obtained with 10 TCD50, as this amount of virus did not produce sufficient cell damage in 42 hr.

Some other applications of the dye uptake method.

Dye uptake can be used for other purposes to show if there has been damage to cultured cells; for example, in cytotoxicity tests (Finter, 1969), and in measurements of the TCD50 of a virus suspension or the neutralizing activity of an antiserum. The
CPE measured by dye uptake

Method is easier than direct microscopy if there are many cultures to be examined, or if conditions for microscopy are poor. Thus, optical distortions make it difficult to see the cells in the one-ounce bottles used in our 'clusters'.

Dye uptake can be used to follow the rate at which cells are damaged in cultures infected with a cytocidal virus, e.g. the growth of Semliki Forest virus in normal and interferon-treated L cell cultures (Fig. 5). Cultures were chilled at 4 ° at various times after infection, and at the end of the experiment they were all warmed for one hour at 37 ° before neutral red was added. Control L cells took up a normal amount of dye even when they had been held at 4 ° for 48 hr.

![Graph](image_url)

**Fig. 5.** Growth of Semliki Forest virus in control (O-O) cells and cells pretreated with 10^{-5} (□-□) or 10^{-4} (△-△) mouse interferon, as determined by infectivity (p.f.u. in L cells) measurements, by dye uptake, and by visual recording of CPE. ◆ = Dye uptake in uninfected cell controls. L cells (8 x 10^3 per culture) were infected at a multiplicity of 0.03 p.f.u. per cell and incubated at 37 °.

**DISCUSSION**

The measurement of the extent of damage to cells in tissue culture by the dye uptake methods has the following advantages. First, instead of viewing the cells under the microscope, the stained cell sheets are examined by eye. This is quicker and more convenient, and the eye can grade quite small differences in the extent of dye uptake. Second, quantitative measurement of the extent of cell damage can be obtained by colorimetric estimation of the extracted dye solutions. Third, the amount of dye extracted from a cell sheet gives an integrated response for the cells in that culture. Although when viewing under the microscope the observer attempts similarly to
integrate observations from a number of fields, scoring is inevitably arbitrary, particularly if there are only localized patches of cell destruction. The dye uptake method is therefore more reliable for assessing minor degrees of cell damage. A further practical advantage is that dye uptake may give results when extensive non-specific degeneration of the cells makes recognition of specific cytopathogenicity difficult or impossible: under such circumstances, cell controls may still take up substantial amounts of neutral red. The methods have however some minor disadvantages. Additional handling of cultures is required, though the labour can be greatly reduced by appropriate mechanical devices; we have used a seven jet manifold to add dye, saline or buffer simultaneously to the seven bottle 'clusters' described. Another point is that, once processed, cultures can yield no further information, and hence are wasted if processed before there is adequate growth of virus. However, if one or two virus control cultures are viewed under the microscope, the appropriate time to add dye to the test may be chosen.

We have made particular use of dye uptake methods to assay interferons. When results accurate to within about fourfold were adequate, we have assessed dye uptake visually, but we have used the colorimetric method extensively, and this has proved convenient and reliable. The reproducibility of results obtained with the colorimetric assay compares favourably with most of the results quoted in the literature for plaque reduction and other interferon assay methods (Finter, 1966), the standard deviation of the results of 79 consecutive assays of a single preparation being 0.18 log. units. Quite precise estimates of the relative activities of preparations can be obtained, since increasing the number of replicate cultures used to test each interferon dilution leads to very little extra work. To distinguish twofold differences in activity we have assayed interferons using twofold or fourfold dilution steps and two replicate cultures per dilution, but in standardization experiments we have used up to nine replicate cultures and 0.1 or 0.2 log. dilution steps.

Since the slopes of the linear portions of the dose-response curves for different preparations tested on one occasion are parallel, a large number of interferon samples can be screened quickly using fivefold, or even greater, dilutions. Provided that one response is obtained within the linear portion for each preparation, its activity can be calculated relative to that of a standard preparation which should be assayed with several replicates and small dilution steps, so that the linear portion of its dose-response curve is well defined.

As there are still no international reference activity standards for interferons from different species, no categorical statements concerning the sensitivity of assay methods can be made. Nevertheless, the dye uptake method is more sensitive for the assay of mouse interferon than any other method with which it has been directly compared, though this is mainly due to the particular combination of Semliki Forest virus and L cells with a fluid overlay: the 50% end-point activities obtained were only 0.2 log. greater than those obtained by the cytopathic effect–inhibition method in the same system. Probably an even more sensitive assay could be developed by testing other viruses systematically.

The two methods most frequently used for the assay of interferons are the cytopathic effect–inhibition method and the plaque reduction method. The dye uptake method described here is in some ways a hybrid of these methods, and has the simplicity, convenience, reliability and high sensitivity of the former method, together with the
reproducibility and precision which plaque reduction assays offer potentially but not always in practice (Finter, 1966).

The dye uptake interferon assay has also been used successfully with the following combinations of interferon, cell and challenge virus: rat interferon–secondary rat embryo cells–Sindbis virus; chick interferon–chick embryo fibroblasts–Semliki Forest virus; human interferon–human embryo lung cells–Sindbis virus (R. A. Bucknall, personal communication); rabbit interferon–suckling rabbit kidney cells–vaccinia virus.

I wish to thank Mr R. Simms and Miss B. Brocklehurst for their expert technical assistance.

REFERENCES


(Received 14 February 1969)