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THYMIDINE KINETICS IN HUMAN LYMPHOCYTE TRANSFORMATION: DETERMINATION OF OPTIMAL LABELLING CONDITIONS

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SUMMARY

The optimal conditions for assessing the rate of DNA synthesis by the incorporation of labelled thymidine in PHA-stimulated human lymphocytes were determined. Optimal labelling conditions could be sustained for only 3–4 hr. The exposure time was limited by both the failure to maintain saturating concentrations of exogenous thymidine and the deleterious affect of internal radiation. A linear incorporation of the label with time was not found to be a reliable criteria of optimal labelling conditions.

INTRODUCTION

Of the various morphological, proliferative, and metabolic changes that occur during *in vitro* lymphocyte transformation, the rate of DNA synthesis as measured by the incorporation of radioactively labelled thymidine is the cellular event most frequently quantitated to monitor the response. Several factors affect the accuracy with which the rate of incorporation of the label reflects the rate of DNA synthesis: (1) The dilutional effect of endogenous thymidine affects the rate of incorporation of exogenous label (Adams, 1969); (2) thymidine is continuously lost by degradation (Marsh & Perry, 1964; Cooper & Milton, 1964; Milton, Cooper & Halle-Panneko, 1965); (3) the incorporated label may cause radiation damage to the cells (Painter, Drew & Hughes, 1958; Drew & Painter, 1962); and (4) high concentrations of thymidine can inhibit further synthesis of DNA (Bootsma, Budke & Vos, 1964; Xeros, 1962; Hartog, Cline & Grodsky, 1967).

Despite the fact that these critical factors have long been recognized, the labelling parameters have varied markedly in studies utilizing the incorporation of labelled thymidine by human lymphocytes (Schellekens & Eijsvoogel, 1968; Oppenheim, 1968; Boylston, Guttmann & Merrill, 1968; Sorensen, Anderson & Giese, 1969; Tormey, Kanin & Fudenberg, 1967; Daguillard *et al.*, 1969). There have only been a few attempts to optimize the

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accuracy of the labelling methods in this biological system (Hartog *et al.*, 1967; Schellekens & Eijsvoogel, 1968; Bain, 1970).

The purpose of this study was to develop a method of determining the optimal concentration of exogenous thymidine and duration of exposure for incorporation studies in human peripheral lymphocytes. Tritiated thymidine was used as the DNA precursor and the kinetics of incorporation were studied during maximal PHA stimulation. The four factor model of thymidine metabolism introduced by Quastler (1963) was used in the design and interpretation of the experiments. The model was reviewed by Cleaver (1967) and has given reasonable agreement with experimental results in several culture systems.

According to this model, when labelled exogenous thymidine is introduced into the intracellular pool, the rate of incorporation of the label into DNA will be effected by the amount of endogenous thymidine and the rate of thymidine degradation. Because of the dilutional effect of endogenous thymidine, as the amount of exogenous thymidine with a constant specific activity is increased, the rate of incorporation of the label will also increase even though the rate of DNA synthesis is not changed. The system is flooded, or saturated, when additional exogenous thymidine does not increase the rate of incorporation of the label. Under these conditions, the rate of incorporation of the label will be less affected by the other parameters in the system and will more accurately reflect the rate of DNA synthesis.

MATERIALS AND METHODS

Preparation of cultures

Blood was obtained from healthy volunteers and placed in Falcon No. 2070 disposable plastic tubes containing preservative free heparin (Fisher Scientific), 10 units/ml of whole blood. 6% dextran in normal saline (70,000 mol wt, Cutter Laboratories) was added 1 : 2 by volume and the mixture was allowed to sediment at 37°C at an angle of 60° to horizontal for 45 min. The leucocyte-rich plasma was removed; the cells were collected by centrifugation at 500 **g** for 10 min and washed twice in Roswell Park Memorial Institute (RPMI) 1640 media containing 100 units of penicillin, 100 μg of streptomycin, and 300 μg of glutamine/ml (Moore, Sandberg & Ulrich, 1966). The cells were resuspended in RPMI 1640 media and leucocyte counts were performed in Cetrimide (Eastman Organic Chemicals) diluent in a model B Coulter Counter (Hatch & Balazs, 1961). Differential counts, which were prepared with Wrights stain, ranged from 25 to 50% mononuclear cells; and viability, which was determined by trypan blue exclusion, was 99–100%. The leucocyte suspension was diluted with RPMI 1640 media and autologous serum (inactivated by heating at 56°C for 30 min) to a final cell concentration of $0{\cdot}5 \times 10^6$ mononuclear leucocytes/ml and 20% serum. 4 ml aliquots of the final cell suspension were cultured in 16 × 125 mm Falcon No. 3033 polystyrene screw cap tubes. Phytohemagglutinin-P (Difco Laboratories) was reconstituted in 0·14 M NaCl and added to the cultures in a concentration of 6·25 μg protein/ml. Preliminary experiments showed that this dose provided maximal stimulation. The cultures were incubated stationary and upright at 37°C in a humidified 95% air–5% Co_2 atmosphere. Cultures for each experimental point were prepared in triplicate and controls without PHA were always included.

Quantitation of [^{3}H]thymidine incorporation

In general, the 48–72 hr culture interval was monitored. [^{3}H]thymidine uniformly labelled in the methyl group was obtained from New England Nuclear. Preliminary investigations

showed that a specific activity of 300 mCi/mM gave satisfactory counting efficiency with reasonably short counting times. In experiments designed to assess the radiation effects, a specific activity of 10 mCi/mM was also used. Concentrations of [^{3}H]thymidine varied from 1×10^{-7} M to 1×10^{-4} M and the duration of exposure varied from 1 to 18 hr as indicated in the results.

At the conclusion of the labelling period, the cultures were harvested immediately by a technique similar to Dutton & Page (1964). After washing the cells once in ice-cold 0·14 M NaCl, nuclear protein was precipitated by ice-cold 5% TCA. The precipitate was washed once in 5% TCA and once in 95% methanol and dried for 1 hr at 60°C. One millilitre of Hyamine (Packard) was added for 12 hr at 37°C to solubilize the precipitate. The contents were transferred to scintillation counting vials by three washes with 5 ml aliquots of a toluene-Liquiflor (New England Nuclear) scintillation fluid. The vials were counted in a model 3003 Packard Tri Carb scintillation counter. The results were corrected for quench using an external standard and expressed in disintegrations/min.

RESULTS

Determination of the concentration of exogenous thymidine that produces saturating conditions

The rate of incorporation of the label was determined after exposing leucocyte cultures to a range of [^{3}H]thymidine concentrations of constant specific activity for various time periods. The results of one of five such experiments, all of which gave similar findings, are summarized in Fig. 1. With short pulses (1–4 hr), the amount of label incorporated increased linearly with increasing concentrations of thymidine up to $0{\cdot}5 \times 10^{-4}$M. At higher concentrations of thymidine there was no additional incorporation of label and the incorporation curve 'plateaued'. According to the four factor model, this indicated that the system was saturated with exogenous thymidine. With longer pulses, the amount of label incorporated did not plateau.

Duration of optimal labelling conditions

The duration of optimal labelling conditions was investigated by comparing the continuous incorporation of the label resulting from exposure to a single pulse of [^{3}H]thymidine with an accumulative incorporation based on the summation of hourly incorporations independently determined under known flooding conditions. Fig. 2 shows the results of one of four such experiments which gave similar patterns. The continuous incorporation of the label in the 2–4 hr pulsed cultures did not differ significantly from the accumulative incorporation. After 4 hr, however, the incorporation of label in the continuously pulsed cultures was progressively less than the summation of the hourly incorporations in identical cultures monitored over the same time period ($P<0{\cdot}01$). Even though the incorporation of the label was not maximum after 4 hr in the continuously labelled cultures, the incorporation was linear for 10 hr.

The effect of supplemental [^{3}H]thymidine on incorporation of the label in continuously labelled cultures

The continuous and accumulative incorporation of label was again measured in identical cultures. In addition, to some of the cultures which received a single pulse of

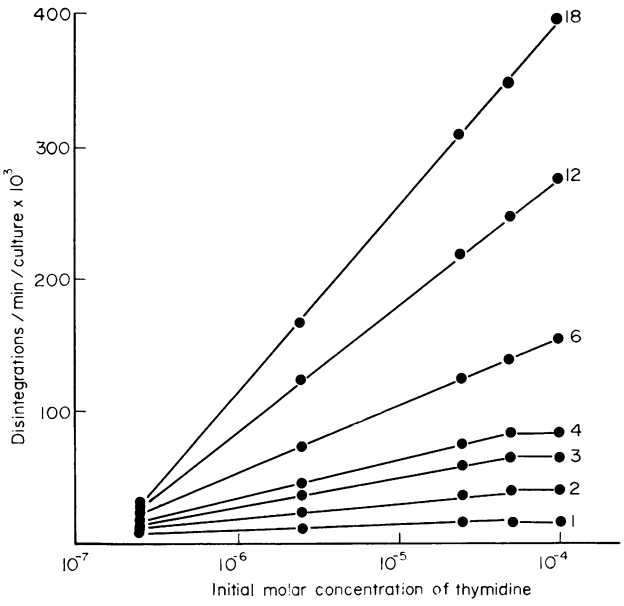

FIG. 1. Effect of concentration of thymidine and pulse duration on the rate of incorporation of label by PHA-stimulated leucocyte cultures. 48 hr after stimulation with 6·25 μg PHA protein/ml of culture [^{3}H]thymidine (specific activity 300 mCi/mM) was added to identical cultures to make the initial molar concentrations indicated on the horizontal axis. Each point represents the mean of triplicate determinations. Each line represents a different exposure time indicated at the end in hours.

[^{3}H]thymidine at 48 hr, supplemental [^{3}H]thymidine was added at 54 and/or 57 hr and the accumulative incorporation determined at 60 hr (Fig. 3). The results showed that the discrepancy between the continuous and accumulative incorporation patterns was partially eliminated by supplementing the continuously labelled cultures with additional [^{3}H]thymidine. Thus, the decline in incorporation of the label after 4 hr in continuously labelled cultures was due in part to a failure to maintain saturating concentrations of exogenous thymidine.

The effect of internal radiation on the continuously labelled cultures

According to the four factor model, with a given rate of DNA synthesis, an increase in the specific activity of labelled exogenous thymidine will result in a corresponding increase in the amount of label incorporated unless there is a deleterious effect from the increased internal radiation. The incorporation patterns of two different specific activities of [^{3}H]thymidine (10 mCi/mM and 300 mCi/mM) at the same starting concentrations (1×10^{-4} M) were determined in identical cultures (Fig. 4). Continuous and independent hourly incorporation were determined as in the previous two experiments. After 4 hr there was a discrepancy between the continuous and accumulative incorporations in the cultures pulsed with the low specific activity [^{3}H]thymidine. When identical cultures were pulsed with [^{3}H]thymidine having a specific activity thirty times greater, the accumulative incorporation was thirty times that observed with the low specific activity [^{3}H]thymidine. However, in identical

cultures exposed to a single pulse of [^{3}H]thymidine at the higher specific activity, the continuous incorporation was progressively less than that predicted from the results with the low specific activity [^{3}H]thymidine. This difference was significant with exposure periods longer than 6 hr.

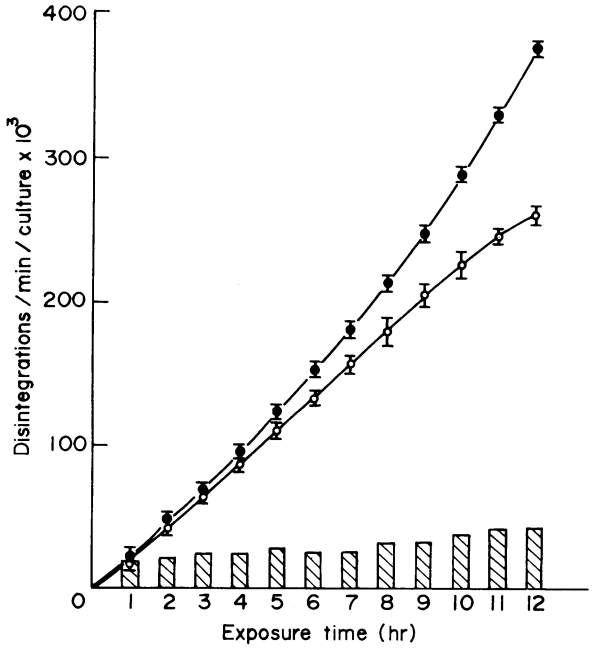

FIG. 2. Effect of exposure time to [^{3}H]thymidine on the rate of incorporation of the label by PHA-stimulated leucocyte cultures. From 48 to 59 hr after stimulation with 6·25 μg PHA protein/ml of culture, [^{3}H]thymidine (specific activity 300 mCi/mM) was added to identical cultures to make an initial concentration of 1×10^{-4}M. Hatched columns, hourly incorporation independently determined under known flooding conditions; ●, accumulative incorporation based on the summation of the hourly incorporations; ○, continuous incorporation with exposure to a single pulse added at 48 hr (time 0). Each represents the mean of triplicate determinations ± SEM.

DISCUSSION

When the radioactivity of an incorporated labelled precursor is used as an index of DNA synthesis by cells *in vitro*, ideally, the intracellular specific activity of the precursor should be constant throughout the exposure period. Furthermore, if the DNA synthesis by different populations of cells is to be compared, the same intracellular specific activity of the precursor must be achieved in each population. When the labelled precursor enters an intracellular pool, the resulting intracellular specific activity depends not only upon the concentration and specific activity of the exogenous supply, but the size of the exisiting endogenous pool and the rate of endogenous production, utilization, and degradation of the precursor during the labelling period.

According to the four factor model (Quastler, 1963; Cleaver, 1967), optimal stability of the *in vitro* labelling conditions is achieved when exogenous thymidine floods the intracellular

pool. With flooding conditions, the effect of the other factors on intracellular specific activity will be minimized and differences in incorporation of the label will accurately reflect differences in DNA synthesis. Flooding concentrations are determined for a given *in vitro* cell population by exposing identical cultures to increasing concentration of an exogenous precursor of constant specific activity for short exposure periods. A flooding concentration is indicated by a 'plateauing' of the amount of radioactivity incorporated (Fig. 1).

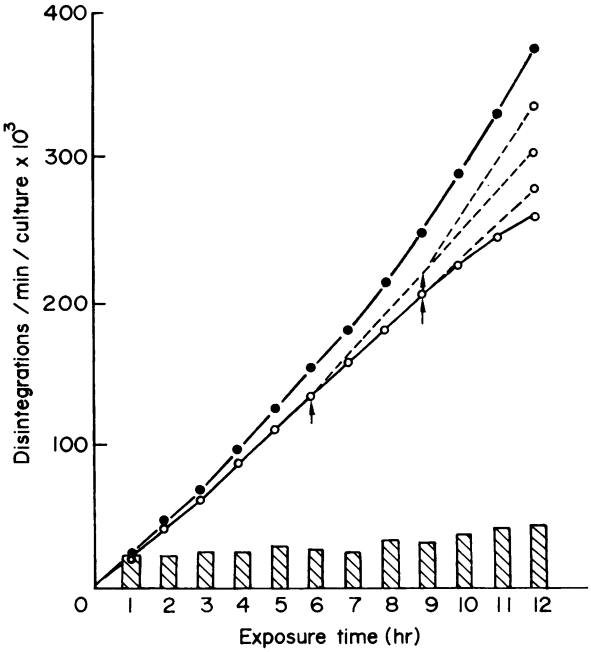

FIG. 3. Effect of supplemental [^{3}H]thymidine on the incorporation patterns in PHA-stimulated leucocyte cultures. From 48 to 59 hr after stimulation with 6·25 μg PHA protein/ml of culture [^{3}H]thymidine (specific activity 300 mCi/mM) was added to identical cultures to make an initial concentration of 1×10^{-4}M. Hatched columns, hourly incorporations independently determined under known flooding conditions; ●, accumulative incorporation based on the summation of the hourly incorporations; ○——○, continuous incorporation with exposure to a single pulse of [^{3}H]thymidine added at 48 hr (time 0); ↑ , 6 μg of [^{3}H]thymidine added/ml of culture; - - - o, additional incorporation at 60 hr resulting from supplemental [^{3}H]thymidine. Each point represents the mean of triplicate determinations.

In our studies of PHA-stimulated human leucocytes, relatively high concentrations of exogenous thymidine were required to attain flooding conditions (24·2 μg/ml of culture). To determine the duration that these optimal conditions could be maintained, the continuous incorporation of radioactivity resulting from the exposure to a single pulse of [^{3}H]thymidine was compared to the hourly accumulative incorporation under flooding conditions in identical cultures. The results showed that optimal labelling conditions could not be maintained for more than 4 hr with continuous exposure to a single pulse of [^{3}H]thymidine (Fig. 2). In addition, linear incorporation of radioactivity did not indicate that optimal labelling conditions were present.

Optimal thymidine concentrations and exposure times have been determined for human leucocyte cultures by several other investigators. Hartog *et al.* (1967), investigated PHA-stimulated leucocyte cultures and found 21·3 μg/ml to be optimal for 24 hr. In a study of PHA-stimulated lymphocyte cultures, Schellekens & Eijsvoogel (1968) found 2·9 μg/ml to

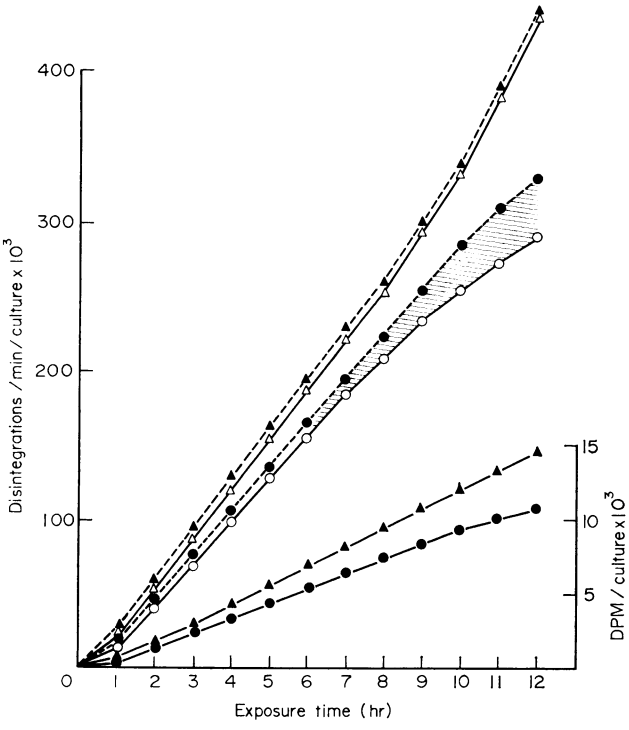

FIG. 4. Effect of increased specific activity of [^{3}H]thymidine on the incorporation patterns of PHA-stimulated leucocyte cultures. From 48 to 59 hr after stimulation with 6·25 μg PHA protein/ml of culture [^{3}H]thymidine was added to identical cultures to make an initial concentration of 1×10^{-4}M. Bars indicating hourly incorporation independently determined under known flooding conditions have been eliminated. The triangles indicate the accumulative incorporation based on the summation of the hourly incorporations: ▲——▲, measured at specific activity of 10 mCi/mM; ▲ - - - - - - ▲, predicted for a specific activity of 300 mCi/mM (30 × ▲——▲); △——△, measured at a specific activity of 300 mCi/mM. The circles indicate the continuous incorporation resulting from exposure to a single pulse of [^{3}H]thymidine added at 48 hr (time 0): ●——●, measured at a specific activity of 10 mCi/mM; ●- - - - - -●, predicted for a specific activity of 300 mCi/mM (30 × ●——●); ○——○, measured at a specific activity of 300 mCi/mM. Each point represents the mean of triplicate determinations.

be optimal for 24 hr. Bain (1970) examined mixed leucocyte cultures and found 0·25 μg/ml satisfactory for 4 hr. The results of the different investigations are difficult to compare because of the use of different isotopes, different types of stimulation, and different culture preparations. In two studies, both specific activity and starting concentration of thymidine were varied and the calculations of uptake did not take into account the dilutional effects of the endogenous pool (Hartog *et al.*, 1967; Bain, 1970). In two experiments, linear

incorporation was assumed to indicate continuous flooding conditions (Schellekens & Eijsvoogel, 1968; Bain, 1970), a contention that was not supported by our results.

The failure to maintain optimal incorporation conditions for long exposure periods is in part due to a failure to sustain flooding concentrations of thymidine. In the present experiments this was indicated by the partial reversal of the progressively diminishing rate of continuous incorporation by periodically supplementing the continuously labelled cultures with additional [^{3}H]thymidine (Fig. 3). Thymidine concentration can be significantly reduced either by utilization or by degradation to products not incorporated into DNA. Relevant to the latter mechanism is the fact that granulocytes as well as lymphocytes have been shown to contain the enzymes necessary to degrade thymidine (Marsh & Perry, 1964; Cooper & Milton, 1964) and PHA has been shown to augment the rate of breakdown (Milton, Cooper & Halle-Panneko, 1965). Furthermore, in human mixed leucocyte cultures, significant thymidine degradation rather than utilization was demonstrated (Bain, 1970).

The intranuclear disintegrations resulting from the incorporation of labelled precursor can cause radiation damage (Painter *et al.*, 1958). The extent of damage is a function of the specific activity of the labelled precursor and the duration of exposure (Drew & Painter, 1962). Although the specific activity of the [^{3}H]thymidine in these experiments was not high, the relatively high concentrations of thymidine necessary to maintain flooding concentrations increased the possibility of deleterious radiobiological effects with prolonged exposure. In the last experiment (Fig. 4), since all parameters used in the cultures exposed to the low specific activity [^{3}H]thymidine were the same as those used in the cultures exposed to the high specific activity [^{3}H]thymidine, the deleterious effect observed after 6 hr of continuous exposure to the high specific activity [^{3}H]thymidine was due to the increased internal radiation. The reduction in incorporation with the higher specific activity [^{3}H]thymidine was in addition to that resulting from the failure to maintain flooding concentrations of thymidine (Fig. 2).

Exogenous thymidine in high concentrations, may completely inhibit *in vitro* cellular DNA synthesis (Bootsma *et al.*, 1964; Xeros, 1962). The concentrations of exogenous thymidine that produced flooding conditions in this culture system were not found to be inhibitory and were less than the concentrations previously reported to be inhibitory in human leucocyte cultures (Hartog *et al.*, 1967).

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