Studies of the Incorporation of [5-³H]Uridine during Activation and Transformation of Lymphocytes Induced by Phytohaemagglutinin

DEPENDENCE OF THE INCORPORATION RATE ON URIDINE CONCENTRATION AT CERTAIN CRITICAL CONCENTRATIONS

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1. Partially purified pig blood lymphocytes were stimulated to transform in culture with phytohaemagglutinin. Initial cell activation was assessed by measuring the increase of uridine incorporation into RNA induced by phytohaemagglutinin. The phytohaemagglutinin/serum ratio for this effect was similar to that required for transformation; however, no inhibition at high phytohaemagglutinin/serum ratios was found during cell activation. 2. Without replenishment of medium the pool of competitors with added uridine for incorporation fell to zero during 2 days of culture. At certain critical pool concentrations uridine itself could stimulate the rate of uridine incorporation. 3. Most of the tritium from [5-³H]uridine added at the initiation of culture had been incorporated into RNA by the end of the second day of culture; the subsequent loss of radioactivity preceded a fall in the total RNA content of cultures. 4. RNA was qualitatively examined on sucrose density gradients. In the first 30 min. after the addition of phytohaemagglutinin, increased labelling occurred predominantly between the 28s and 4s regions of the gradients. On the second day of culture with phytohaemagglutinin mainly RNA sedimenting beyond the 28s region of gradients was labelled in 30 min.

When a bean extract, PHA,* is added to lymphocytes cultured *in vitro* many of them are activated to transform into large pyronine-staining 'blast' cells, which divide (Nowell, 1960; Johnson & Roberts, 1964; Marshall & Roberts, 1965). By using the quantitative increase of RNA in cultures as an objective index of transformation, it has been shown that the response is proportional to the ratio of PHA to the serum in the culture medium (Forsdyke, 1966a, 1967c). However, quantitative nucleic acid measurement is not suitable for examining the initial activation of lymphocytes, or for detecting the small degrees of transformation occurring in antigen-stimulated cultures (Pearmain, Lycette & Fitzgerald, 1963; Cowling & Quaglini, 1965) and in 'mixed-cell' cultures (Bain, Vas & Lowenstein, 1964). The need for a sensitive and early assay of the transformation response to PHA became apparent during studies designed to identify the serum factor influencing the PHA requirement (Forsdyke, 1966c). Serum fractions added to cultures could produce effects both on the PHA concentration needed for the induction of a given transformation response and on the subsequent growth of cells during that response.

* Abbreviation: PHA, phytohaemagglutinin.

Shortly after addition of PHA to cultures an increased incorporation of radioactive RNA precursors is found (McIntyre & Ebaugh, 1962; Cooper & Rubin, 1965; Pogo, Allfrey & Mirsky, 1966; Mueller & Le Mahieu, 1966; Kay, 1966). The present paper reports studies of some of the factors affecting the incorporation of [5-³H]uridine in the PHA-lymphocyte system. The use of [5-³H]uridine to investigate the mechanism of initial activation of lymphocytes in the presence of PHA is described elsewhere (Forsdyke, 1967d). Preliminary accounts of this work have been presented (Forsdyke, 1966b,c, 1967a,b,d).

MATERIALS AND METHODS

[5-³H]Uridine (5–31 c/m-mole), [*Me*-³H]thymidine (3 c/m-mole) and [5-³H]orotic acid (5 c/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. *m*-Hyamine hydroxide [*p*-(di-isobutylcresoxy-ethoxyethyl)dimethylbenzylammonium hydroxide] in methanol was obtained from the Packard Instrument Co. Inc., La Grange, Ill., U.S.A. Other materials and details of culture techniques and nucleic acid extraction and assay have been described by Forsdyke (1967c).

Suspensions of pig cells in autologous serum were diluted with 2 vol. of medium 199 and immediately fractionated into culture vessels. PHA was usually added before the

cells had had time to settle. It was found that permitting cells to settle before adding PHA caused a 20% (S.E.M. $\pm$ 7.5) increase in the initial rate of labelling of RNA with [5-³H]-uridine over that found in cultures that had been gently shaken to resuspend cells (four experiments; $P=0.02$ by Wilcoxon signed ranks test; Siegel, 1956). Control cultures without PHA were not appreciably affected by the shaking.

Determination of tritium. (a) Counting cell extracts in aqueous scintillator. RNA- and DNA-hydrolysis products extracted from cells were in acid solution. No attempt was made to improve the counting efficiency by elevating the pH. A sample (0.3 ml.) of the solution to be counted was added to 3 ml. of scintillation fluid (Bray, 1960). Counting efficiency was approx. 0.6%.

(b) Counting acid-precipitable material in whole cells by using Hyamine and toluene scintillator. Cells were harvested (1500g for 3 min.) while still warm. The pellets were stored in ethanol at -10° . After extraction with 0.5N-HClO₄ at 0° for 10 min., the pellets were extracted and dehydrated by centrifugation first in 5 ml. of ethanol-ether (1:1, v/v) and then in 5 ml. of ether. The residues were dissolved in 0.2 ml. of Hyamine hydroxide by heating the stoppered tubes to 60° for 30 min., and then washed into a vial with 3 ml. of toluene scintillator containing 2,5-diphenyloxazole (3.5 g./l.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (50 mg./l.). Counting efficiency was approx. 10%. This was determined by adding a small volume of [5-³H]uridine to an ether-dried cell pellet and redrying the pellet in an oven at 60° before dissolving in Hyamine.

Correction of rate of uridine incorporation and determination of pool size by isotope dilution. Incorporation of an added labelled precursor into a macromolecule *in vitro* may be represented in the simplest possible terms as a one-step process of transfer from a single pool into a stable product. Assuming that equilibrium with the pool is rapid, and that the rate of incorporation is uninfluenced by the concentration of the precursor, a relationship between the pool size and the extent of labelling of the macromolecule can be derived.

With a precursor of known specific radioactivity, a maximum number of counts (n) should appear in the macromolecule in a given time and give a true index of its rate of synthesis provided that there is not an unknown pool of similar compounds within the system competing for incorporation. If the size of this pool happens to equal the quantity of added precursor (dilution $p=1$), the observed counts (x) should equal $n/2$. Thus $x=n/(p+1)$. If in addition to the labelled precursor an extra known quantity of unlabelled precursor is added, giving a further dilution y , then $x=n/(p+y+1)$. ($y+1$) is the 'added pool'. p is the combined extracellular and mean intracellular pool, which is termed the 'intrinsic pool'. On rearrangement the expression $y+1=(1/x)n-p$ is obtained. If the observed counts (x), which have been obtained in a given time in the presence of various dilutions of added precursor y , are expressed as a reciprocal and plotted against the added pool, a linear relationship should be obtained with a slope of n , and an intercept at the y -axis of $-p$. In practice, instead of dilutions being plotted, the actual quantities of uridine added to the system were used. Dilution values were used when calculating n .

Phenol extraction of RNA and sucrose-gradient centrifugation. Cultures of 2×10^8 – 4×10^8 cells were used. When rat liver RNA was added as carrier 5×10^6 cells were used.

Cultures were harvested (400g for 2 min.) and cell pellets washed into a final concentration of 0.05% sodium dodecyl sulphate [0.05 ml. of a 10% (w/v) solution] with 10 ml. of 0.14M-NaCl at 0° . After a brief shake, 10 ml. of cold freshly redistilled phenol saturated with 0.14M-NaCl and containing 8-hydroxyquinoline (0.1%, w/v) was added. The mixture was shaken for 3 min. either at between 0° and 10° (cold extraction) or at 65° (hot extraction). The mixture was then rapidly cooled in ice and carrier RNA was added where necessary; a further short period of shaking followed. After centrifugation (1500g for 15 min. at 0°), the upper aqueous phase was carefully pipetted off and extracted once more at low temperature with phenol. Finally the aqueous phase was made 2% (w/v) with respect to sodium acetate, pH 5.2, and, after the addition of 2 vol. of ethanol, the RNA precipitate was left to sediment at -10° . The precipitate was washed once by dissolving in 0.01M-sodium acetate buffer, pH 5.2, containing 0.1M-NaCl, and then was reprecipitated with ethanol. After centrifugation the tubes were inverted for 10 min. to drain off all but a trace of residual ethanol. The RNA was dissolved in 0.2 ml. of acetate buffer and layered on to a linear 5–20% (w/v) sucrose gradient made up in the above acetate buffer. The 5 ml. gradients were spun for $3\frac{1}{2}$ –4 hr. at 105 000g at approx. 6° . Gradient fractions (3 drops) (Munro & Korner, 1964), were collected and diluted to 3 ml. with acetate buffer for E_{260} measurement, and then the RNA was precipitated by the addition of 3 ml. of 0.5N-HClO₄ at 0° for 5 minutes. The precipitates were collected on Oxoid membrane filters (Oxoid Ltd., London, S.E. 1), and then washed once with 2% acetic acid and dried at 60° for 1 hr. The filters were counted in toluene scintillator. [5-³H]Uridine spotted alone on the filters was counted with an efficiency of 10%. This may not reflect the efficiency of counting of tritium in RNA precipitated on to such filters (Furlong, Williams & Willis, 1965).

Approx. 50% of the total RNA present in cells was recovered. Omission of detergent lowered the yield. Extraction by cold or hot procedures did not influence the quantitative yield of RNA, but hot extraction decreased the height of the heavier lymphocyte ribosomal RNA peak relative to the lighter ribosomal peak. Liver cytoplasmic RNA extracted by using the hot extraction technique behaved similarly (Kirby, 1965; Smith, 1967). Hot extraction was needed to increase the yield of rapidly labelled material and obtain appreciable labelling in regions other than those attributed to ribosomal RNA and transfer RNA.

RESULTS

Labelling of different macromolecules, by different precursors, at different times of culture. On the first day of culture the labelling of acid-precipitable material by tritium from uridine and leucine, but not thymidine, was stimulated by PHA. On the second day labelling with [³H]thymidine began. This is in agreement with other reports (Cooper, Barkham & Hale, 1963; Epstein & Stohlman, 1964; Pogo *et al.* 1966; Mueller & Le Mahieu, 1966; Kay, 1966). Table 1 shows the relative incorporation of tritium from uridine, thymidine and orotic acid into cell RNA and DNA fractions on the second day of culture with PHA. Only [5-³H]uridine labelled the

Table 1. Incorporation over a 3 hr. period of various nucleic acid precursors into pig lymphocyte RNA and DNA fractions

A 1 μ C sample of each tritium-labelled precursor was added to 5×10^6 cells that had been grown for 36 hr. with PHA (Burroughs Wellcome and Co., London, N.W. 1) (0.6 mg. of PHA/ml. of serum), in 5 ml. of culture medium 199 (67%), autologous serum (25%) and 0.14 M-NaCl (8%). RNA-hydrolysis products were extracted from cells with 1 ml. of 0.3 N-NaOH (37° for 50 min.). Then 1 ml. of N-HClO₄ was added. DNA-hydrolysis products were extracted from the resulting precipitate with 2 ml. of 0.5 N-HClO₄ (70° for 15 min.). The $E_{260m\mu}^{1cm}$ value of the extracts was recorded, and 0.3 ml. taken for determination of radioactivity in aqueous scintillator (see the Materials and Methods section). Each value is the mean of duplicate cultures. Numbers in parentheses refer to the total radioactivity (counts/min.) incorporated into each nucleic acid fraction/unit of total $E_{260m\mu}^{1cm}$ material in that fraction.

Precursor used	Time ...	Radioactivity incorporated (counts/min.)					
		RNA			DNA		
		1 hr.	2 hr.	3 hr.	1 hr.	2 hr.	3 hr.
Orotic acid (5c/m-mole)		2	5	2	—1	0	0
Thymidine (3c/m-mole)		7	6	1	35 (347)	46 (450)	88 (829)
Uridine (23c/m-mole)		80 (1190)	140 (2050)	240 (3200)	9	4	12

Table 2. Time-course of the incorporation of [³H]uridine added at the initiation of culture into pig lymphocytes stimulated by PHA

Cells were grown in the conditions described in Table 1. [³H]Uridine (5c/m-mole) was added at the initiation of culture and cells were harvested at various times thereafter. In Expt. (a), cells were in 1 ml. cultures containing 0.067 μ C/ml. Acid-precipitable material was solubilized in Hyamine and counted in toluene scintillator. Each value is the mean of triplicate cultures. The total theoretically achievable radioactivity is approx. 14700 counts/min. In a separate experiment, Expt. (b), cells in 5 ml. cultures containing 0.1 μ C/ml. were extracted with NaOH to obtain RNA-hydrolysis products. After acidification and centrifugation, the supernatants were taken for total $E_{260m\mu}^{1cm}$ measurement and counting in aqueous scintillator (Bray, 1960). Each value is the mean of duplicates. The total theoretically achievable radioactivity is approx. 6600 counts/min.

Expt. (a)			Expt. (b)					
			PHA present			PHA absent		
Duration of culture (hr.)	Total radioactivity (counts/min.)		Duration of culture (hr.)	Total radio-activity (counts/min.)	Sp. radio-activity (counts/min./ $E_{260m\mu}^{1cm}$)	Total radio-activity (counts/min.)	Sp. radio-activity (counts/min./ $E_{260m\mu}^{1cm}$)	
	PHA present	PHA absent				Total		
5	950	290	0				0.276	
19	3800	470	24	4598	0.384	11970	0.209	3792
29	11230	520	48	5474	0.558	9305	0.118	3160
43	12400	580	72	3598	0.570	6311	0.104	3077
55	10520	830	96	2265	0.447	5067	0.109	1715

RNA fraction during a 3 hr. period. [³H]Orotic acid labelled neither fraction.

Extent of incorporation of [³H]uridine added at the initiation of culture. Tritium was detected either in acid-precipitable material in Hyamine-solubilized whole cells, or in RNA-hydrolysis products extracted from cells (see the Materials and Methods section). Table 2 shows two separate experiments in which tritium incorporated from [³H]uridine that had been added at the initiation of culture was measured by the two methods. In the absence of

PHA, incorporation rose but fell after a few days of culture. The fall was greater than the fall in total RNA. In the presence of PHA, much more tritium was incorporated, reaching a peak at about 48 hr. Calculation showed that most of the tritium added to the system had been quantitatively incorporated into RNA by this time. After 48 hr. the total radioactivity declined. This decline preceded a fall in total RNA. The specific radioactivity of the RNA rose dramatically during the first day of culture, and then declined on the second day at a

Fig. 1. PHA dose-response curves for initial cell activation: incorporation of $[5\text{-}^3\text{H}]$ uridine into pig lymphocytes during the first 4 hr. of culture in the presence of various concentrations of PHA, at three different serum concentrations. The 1 ml. cultures contained 2×10^6 cells, $1 \mu\text{C}$ of $[5\text{-}^3\text{H}]$ uridine (26 c/m-mole), 8% 0.14M-NaCl, PHA ('PHA-P'; Difco Laboratories, Detroit, Mich., U.S.A.), and various proportions of autologous serum and culture medium 199: ●, 18% (v/v) serum; ■, 36% (v/v) serum; ○, 54% (v/v) serum. Acid-precipitable material was solubilized in Hyamine and the radioactivity determined in toluene scintillator. In (a) the radioactivity incorporated is plotted against the PHA concentration expressed as $\mu\text{g./ml.}$ of total medium. In (b) the same results are expressed as a percentage increase over the radioactivity obtained in the absence of PHA at a given serum concentration. In (c) these percentage increases are plotted against the PHA concentration expressed as $\mu\text{g./ml.}$ of serum in the culture medium. Each point is the mean of duplicates.

time when total radioactivity incorporated and the total RNA were still rising. Similar results were obtained with human leucocyte cultures. The Hyamine method, which was technically simple and gave a relatively high counting efficiency, was used in subsequent experiments.

PHA dose-response curve for initial cell activation. Fig. 1(a) shows the extent of incorporation of $[5\text{-}^3\text{H}]$ uridine into cell RNA over a 4 hr. period in

Fig. 2. PHA dose-response curves for lymphocyte transformation: quantitative increase of RNA in cultures of pig lymphocytes grown for 43 hr. in the presence of various concentrations of PHA, at two different serum concentrations. The 5 ml. cultures contained 2.5×10^6 cells, PHA (the same batch as in Fig. 1), and either 25% (v/v) serum (●) or 50% (v/v) serum (■). Each point is the mean of duplicates.

the presence of various concentrations of PHA at three different serum concentrations. The PHA concentrations are expressed as $\mu\text{g.}$ of PHA/ml. of total medium. With increasing serum concentration the total radioactivity incorporated decreased. With increasing PHA concentration the radioactivity increased until a plateau was approached. No inhibition at high PHA concentrations was found. Fig. 1(b) shows the same results plotted as the percentage increase in incorporation over that found in the absence of PHA at the same serum concentration. As the serum concentration increased the minimal PHA requirement for cell activation increased. Fig. 1(c) shows the same results plotted with the PHA concentration expressed as $\mu\text{g.}$ of PHA/ml. of serum. The curves became approximately superimposable. The same batch of PHA gave an optimum increase in total RNA over 43 hr. of culture when used at a concentration of 40 $\mu\text{g./ml.}$ of serum (Fig. 2). At higher PHA concentrations an increasing inhibition of the RNA increase was found at 43 hr.

Extent to which the incorporation of $[5\text{-}^3\text{H}]$ uridine can be interpreted in simple terms. Uridine incorporation into RNA within cells was treated in terms of a simple model system from which an equation was derived (see the Materials and Methods section). This predicted that a plot of the reciprocal of the radioactivity incorporated when $[^3\text{H}]$ uridine diluted with various quantities of unlabelled uridine was used against the total concentration of added

Fig. 3. Isotope-dilution plot (see the Materials and Methods section) of the incorporation of $[5\text{-}^3\text{H}]$ uridine into pig lymphocytes. The 1 ml. cultures containing 10^6 cells were incubated for 30 min. with $[5\text{-}^3\text{H}]$ uridine ($2\mu\text{C}$) that had been diluted with various quantities of unlabelled uridine (0–200-fold). (a) $[^3\text{H}]$ uridine with a specific radioactivity undiluted of 23 c/m-mole, added to freshly isolated cells; (b) a separate experiment in which $[^3\text{H}]$ uridine with a specific radioactivity undiluted of 25 c/m-mole was added to cells grown with PHA (Burroughs Wellcome) (0.6 mg./ml. of serum), for 22 hr. Broken lines illustrate an interpretation of the data presented in the text.

uridine should result in a linear relationship. Fig. 3 shows plots obtained under such circumstances in two separate experiments. Incorporation was permitted to proceed for a 30 min. period either into freshly isolated cells (Fig. 3a), or into cells that had been grown with PHA for 22 hr. (Fig. 3b). Within a certain range of concentrations a linear relationship was obtained. This line could be projected to the ordinate to obtain a value for the pool present in cultures at the time. The slope of the line gave a value for the true rate of uridine incorporation at the time.

As the uridine concentration was increased, however, discontinuities in the original linear relationship were revealed. A new relationship was found at higher concentrations. This could be extended linearly to meet the projection of the original linear relationship at the ordinate. The new relationship had a greater slope than the original relationship.

In this manner it was possible to construct plots at various times of culture with PHA and obtain values for the intrinsic pool and the incorporation rate corrected for dilution by this pool. However, as the value for p fell, evidence of another departure from the linear relationship appeared. Fig. 4 shows a plot obtained at 54 hr. of culture with PHA. Addition of increasing quantities of uridine increased the observed radioactivity and hence lowered the reciprocal of the radioactivity. The data were consistent with the pool at that time being zero.

Corrected rates of incorporation and pool changes

Fig. 4. Isotope-dilution plot of the incorporation of $[5\text{-}^3\text{H}]$ uridine (31 c/m-mole, diluted 0–100-fold) into pig lymphocytes grown for 54 hr. with PHA. Details are as given in Fig. 3.

throughout the culture period. Fig. 5 shows the observed rates of uridine incorporation and pool changes occurring over a 3-day culture period. Rates of uridine incorporation corrected for pool dilution are also plotted. The pool fell over 2 days of culture, then rose briefly, fell again and rose once more. The observed rate of uridine incorporation rose over 2 days and then suddenly declined. The rate of uridine incorporation, corrected for dilution by the intrinsic pool, changed only slightly over 43 hr. of culture, and then after a sudden rise at 48 hr. declined to almost zero.

Fig. 5. Data derived from isotope-dilution experiments performed at different times of culture with PHA. Pig lymphocytes were incubated for 30 min. with $[5\text{-}^3\text{H}]\text{uridine}$ (31 c/m-mole) diluted with various quantities of unlabelled uridine (0–100-fold). Details are as given in Fig. 3. ●, Observed radioactivity corrected only for background; ○, combined extracellular and intracellular pool ('intrinsic pool') of competitors with added $[^3\text{H}]\text{uridine}$; ■, uridine incorporation corrected for dilution by intrinsic pool; □, uridine incorporation corrected for dilution by pool, at a constant pool size of $6.6\text{ }\mu\text{M}$ (for explanation see the text).

From data such as shown in Figs. 3 and 4 it is possible to obtain the rates of uridine incorporation that might be found at any given concentration of the total pool (intrinsic and added). Values along the ordinate from the intercept upwards are summed, and the corresponding slope of the graph is measured. A pool value of $6.6\text{ }\mu\text{M}$ -uridine was chosen arbitrarily. At the initiation of culture this pool was contributed mainly from the intrinsic pool and not the added pool. After 2 days of culture the situation was reversed. Corrected rates of incorporation in the presence of this pool were determined and plotted in Fig. 5. It was found that the actual potential for uridine incorporation increased in an approximately linear manner.

Initial stimulation of uridine incorporation. Fig. 6 shows two isotope-dilution plots of uridine incorporation over a 30 min. period. PHA was added to one series of tubes at the same time as the uridine. The plot of data obtained from cultures with PHA had a greater slope than that obtained from cultures without PHA. The plots could be extrapolated linearly to meet close to the ordinate.

Qualitative nature of RNA labelled at different times of culture. Cells were incubated with $[5\text{-}^3\text{H}]\text{uridine}$ for 30 min. in the presence or absence of PHA, which was added at the same time as the $[5\text{-}^3\text{H}]\text{uridine}$. The RNA was extracted with rat liver RNA as carrier and the profiles of radioactivity from sucrose density gradients were compared (Fig. 7). The

results were variable. In general, incorporation into material sedimenting mainly between the 4s and 28s E_{260} peaks was found at this time of culture. Relatively little radioactivity sedimented beyond the 28s peak. A prominent peak of radioactivity between the 4s and 18s regions was usually seen only in cultures with PHA. The total yield of radioactivity in the presence of PHA was more than that obtained from cultures incubated without PHA. The difference was greater than was found under similar circumstances when radioactivity was measured in acid-precipitable material or in RNA hydrolysis products extracted from cells (Forsdyke, 1967d).

A 30 min. exposure to $[5\text{-}^3\text{H}]\text{uridine}$ on the second day of culture with PHA gave labelled material with entirely different sedimentation characteristics (Fig. 8). Most radioactivity was found consistently in two clearly separate regions of the gradient in material sedimenting beyond the 28s peak.

DISCUSSION

The data of Tables 1 and 2 indicate that $[5\text{-}^3\text{H}]\text{uridine}$ is mainly utilized for RNA synthesis and is stable under culture conditions. The finding that the fall in total radioactivity after 48 hr. of culture with PHA preceded the fall in total RNA may mean that RNA-breakdown products do not share this

Fig. 6. Isotope-dilution experiment performed during the first 30 min. of culture in the presence (●) or absence (○) of PHA. Pig cells were incubated with [³H]uridine (31 c/m-mole) diluted with various quantities of unlabelled uridine (0–100-fold). Details are as given in Fig. 3.

stability, or that they are sequestered within an intracellular compartment or a cell population no longer active in RNA synthesis. RNA-breakdown products might be used for DNA synthesis with uridyate and cytidylate losing tritium in the 5-position on conversion into thymidylate. Evans (1966) has pointed out that little is known about the radiation damage caused by the high-specific-radioactivity tritiated compounds now available. It is possible that the cell population most actively incorporating [³H]uridine might be selectively destroyed by the radiation.

Neither [³H]uridine nor [³H]orotic acid was directly utilized for DNA synthesis, even though it could be predicted from current knowledge of the metabolic pathways concerned (Reichard, 1959; Magasanik, 1962; Cooper, Perry & Breitman, 1966) that deoxycytidylate might be labelled. Inhibition of the enzymes of orotate utilization by a pool of phosphorylated uridine or cytidine derivative (Appel & Pettis, 1967) might contribute to the poor incorporation of this compound. This would be in keeping with the preferential utilization of uridine

Fig. 7. Sucrose-density-gradient profile of RNA extracted from pig lymphocytes after labelling with [³H]uridine for 30 min. in the absence (a) or presence (b) of PHA (0.6 mg./ml. of serum) added at the same time as the [³H]uridine. In this experiment 25×10^6 cells were grown in 5 ml. of medium containing 37.5% serum and $10 \mu\text{C}$ of [³H]uridine (31 c/m-mole). RNA was extracted at 65° with 0.05% sodium dodecyl sulphate and phenol, and rat liver RNA was added as carrier. Linear gradients of 5–20% sucrose were spun for 4 hr. at 37000 rev./min. in the SW 39 rotor of the Spinco model L ultracentrifuge. Direction of sedimentation is from right to left. —, E_{260} ; ●—●, radioactivity.

Fig. 8. Sucrose-density-gradient profile of RNA extracted from pig lymphocytes after labelling with [³H]uridine for 30 min. after 28 hr. of culture with PHA. In this experiment 5×10^6 cells were grown in 5 ml. of medium containing 25% serum; $5 \mu\text{C}$ of [³H]uridine (14 c/m-mole) was added. Linear gradients of 5–20% sucrose were spun for $3\frac{1}{2}$ hr. at 37000 rev./min. —, E_{260} ; ●—●, radioactivity. Other details are as given in Fig. 7.

for RNA synthesis at the initiation of culture (Table 2) when the pool of uridine competitors is high (Fig. 5). Appreciable incorporation of orotic acid has been found in the human PHA-lymphocyte system by Lucas (1967).

The relative contributions of the extracellular and intracellular compartments to the total pool of compounds competing with added uridine for incorporation are uncertain. Incorporation was decreased under conditions of increasing serum concentration and decreasing concentration of medium 199 (Fig. 1a). This could mean that an appreciable extracellular pool is contributed by serum, or that medium 199 stimulates incorporation, or that serum contains an inhibitor (such as cytidine; Lucus, 1967). Both the data of Fig. 1(a) and the results presented by Stansly & Schiop (1967) may be interpreted in terms of the isotope-dilution model presented in the Materials and Methods section, with serum being considered to contain a considerable pool of unlabelled uridine.

Extent to which the incorporation of [5-³H]uridine can be interpreted in simple terms. The incorporation of an added labelled low-molecular-weight precursor into macromolecules within cells *in vitro* is likely to be influenced by a variety of factors (Graham & Rake, 1963; Evans, 1966). These factors include the following: the rate of breakdown of the precursor both extracellularly and intracellularly; the size of extracellular and intracellular pools of similar compounds competing for incorporation; sequestration within these pools without incorporation; the position of the rate-limiting step on the particular metabolic pathway concerned, and whether it lies distal or proximal to the point of entry of the precursor; the rates of synthesis and breakdown of the macromolecule; the availability of its breakdown products for reutilization. In addition, the precursor may itself have physiological effects on cells that may influence the above factors. For example, thymidine has been shown to exert profound stimulatory or inhibitory effects on cells depending on the particular concentration employed (Greulich, Cameron & Thrasher, 1961; Morris, Reichard & Fischer, 1962; Painter & Rasmussen, 1964; Cooper *et al.* 1966).

The incorporation of [5-³H]uridine is linear in time over a period of a few hours with time-points extrapolating back close to zero (Forsdyke, 1967d). This suggests that equilibration with diluting pools is rapid. In terms of a simple model system (see the Materials and Methods section) it was predicted that there would be a linear relationship between pool size and the reciprocal of the observed radioactivity incorporated. To test this experimentally, incorporation periods of only 30 min. were used in the hope that RNA synthesis, and not turnover, would be under study. Although in general the prediction was found to be true, at certain critical pool concentrations discontinuities in the linear relationship were found. Such discontinuities occurred both at low uridine concentrations (Fig. 4) and at one or more higher concentrations (Fig. 3).

Within the theoretical framework proposed, the discontinuities indicate that the rate of uridine incorporation had been stimulated by uridine. When the concentration of uridine in the system was low the rate-limiting step in RNA synthesis would lie distal to the point of entry of uridine into the metabolic pathway concerned; then a stimulation of RNA synthesis would be produced by uridine (Steinberg & Nichols, 1967). At intermediate uridine concentrations, increasing the uridine concentration had no effect on the rate of incorporation (linear relationship). This suggests that under these conditions the rate-limiting step was proximal to the point of entry of uridine into the pathway. The stepwise stimulation of uridine incorporation occurring at high uridine concentrations would in such circumstances be produced by an actual stimulation, either directly or indirectly, of this rate-limiting step, by uridine. That such stimulation occurred without demonstrable change in pool size must be taken into account when possible explanations of the phenomenon are being considered. These explanations include the possibilities that at high uridine concentrations existing metabolic pathways are extended (i.e. uridylylate to cytidylate), or new loci of nucleic acid synthesis are activated, or already functioning loci are stimulated.

Corrected rates of incorporation and pool changes throughout the culture period. When the potential rate of incorporation, corrected for pool dilution, was determined for the presence of a constant pool arbitrarily set at 6.6 μ M, an almost linear increase in the ability to incorporate uridine during 2 days of culture was obtained. However, the actual condition present in cultures in which total RNA doubled in 2 days (Table 2) was that of a varying intrinsic pool. In such circumstances the rate of incorporation, corrected for dilution, showed little change during 2 days of culture. The burst of incorporation at 48 hr. possibly reflected the temporary increase in pool size caused by RNA breakdown in some cells. The subsequent fall in incorporation rate after this burst may indicate cell death (Fig. 5).

It is probable that an important factor governing the rise in total RNA in cultures is the increased formation of a stable RNA species such as ribosomal RNA. If uridine is incorporated mainly into uridylic acid residues, which comprise 20% of total RNA (Cooper & Rubin, 1966), and if all radioactive components are stable, it may be calculated from the corrected counts/min., adjusted for counting efficiency, that 0.05 $E_{260\text{m}\mu}^{1\text{cm}}$ units of hydrolysed RNA would be synthesized/day/ 10^6 cells. This is of an order required to explain the observed RNA increase. From the same assumptions, the fall in pool size is in slight excess of that required to explain the RNA increase.

Qualitative examination of RNA on density gradients. Discussions of some of the limitations of sucrose-density-gradient analysis of RNA have recently appeared (Bramwell & Harris, 1967; Peacock & Dingman, 1967). Differences between radioactivity profiles under different experimental conditions may mean that different RNA species have been synthesized, or that the extractability of labelled RNA has altered. Other factors, such as differences in the activity of ribonuclease under different circumstances, must also be considered.

Although profiles were variable, a peak between the 4s and 18s regions of the gradient was generally found to be dependent on the addition of PHA to cultures (Fig. 7). Increased labelling in this region has been demonstrated with cultures of human lymphocytes during the first 2 hr. of culture (Cooper & Rubin, 1965; Rubin & Cooper, 1966; Kay, 1966). With HeLa cells, material with these sedimentation characteristics has been considered to have the properties of messenger RNA (Latham & Darnell, 1965).

During the first day of culture there was an alteration in the labelling pattern of RNA so that by the second day predominantly rapidly sedimenting material was labelled in 30 min. (Fig. 8). In cultures of human lymphocytes this material is less obvious (Rubin & Cooper, 1966; Kay, 1966). There is evidence that in HeLa cells 'ribosomal precursor' RNA has similar sedimentation characteristics (Penman, 1966). Mach & Vassalli (1965) were able to extract such material from antigen-stimulated rat lymph-nodes after a 20 min. 'pulse' of [³H]-uridine *in vivo*; 3 hr. later the radioactivity was localized in 28s, 18s and 4s regions of the gradients. In 'chase' experiments on the second day of culture it has been demonstrated that radioactivity incorporated in 30 min. into PHA-stimulated lymphocytes becomes localized similarly (Forsdyke, 1967d).

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