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Application of the Isotope-Dilution Principle to the Analysis of Factors Affecting the Incorporation of [^{3}H]Uridine and [^{3}H]Cytidine into Cultured Lymphocytes

EVALUATION OF POOLS IN SERUM AND CULTURE MEDIA

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1. Rat lymph-node cells were incubated in serum and medium 199 with [5-^{3}H]uridine or [5-^{3}H]cytidine and acid-precipitable radioactivity was measured. Results were interpreted in terms of an isotope-dilution model. 2. Both serum and medium 199 contained pools that inhibited radioactive labelling in a competitive manner. The serum activity was diffusible and inhibited labelling with [^{3}H]cytidine more than with [^{3}H]uridine; in these respects the activity resembled cytidine (14μM). 3. The pools in serum and plasma were the same size; however, the rate of labelling was greater in plasma, owing to a diffusible factor. 4. Paradoxically, relatively simple media (Earle's salts and Eagle's minimum essential) appeared to have a larger pool than the more complex pyrimidine-containing medium 199; this suggests a contribution to the pool by cells in the simple media. 5. In the absence of pools the average cell was capable of incorporating 2000 radioactive nucleoside molecules/s.

Radioactively labelled nucleosides are rapidly incorporated into mammalian tissues and much of the label is found in nucleic acids (Feinendegen, 1967; Cleaver, 1967). These properties have prompted the wide use of nucleosides in studies in various biological systems to label nucleic acid molecules (Darnell, 1968) or to act as an early index of changes in cell activity (Forsdyke, 1968). Many of these systems have contained serum. However, serum inhibits the labelling of cells *in vitro* with the radioactive pyrimidine ribonucleoside uridine, indicating that serum might inhibit RNA synthesis (Stansley & Schiop, 1967), or contain a nucleoside pool that dilutes the added labelled uridine (Forsdyke, 1967*a*, 1968). The important biochemical role of nucleoside derivatives both as nucleic acid precursors and in intermediary metabolism (Leloir, 1964) indicated that an understanding of the effects of serum on labelling might be of general value.

In the present study the process of incorporation of [^{3}H]pyrimidine ribonucleosides from the culture medium into acid-precipitable material within cells *in vitro* has been interpreted in terms of a simple isotope-dilution model. Intracellular and extracellular pools of inhibitors of incorporation, and maximum incorporation rates, have been evaluated. The effects of serum, serum diffusate and plasma on radioactive labelling have been compared and culture medium 199 has been compared with other defined media. Preliminary accounts of this work have been presented (Forsdyke, 1969, 1970).

THEORY

General model. The incorporation of a labelled nucleoside into nucleic acid molecules within a biological system can be viewed in terms of the following general model. The labelled compound enters a network of branching and converging metabolic pathways. The compound passes along certain of these pathways, undergoing various chemical transformations until a labelled product is formed. The labelling of the product can be measured. If this measurement is made during the lifetime of the product further flow of label into catabolic pathways can be disregarded. If small changes in the concentration of the compound stimulate the rate of passage of the compound along the pathways, then the concentration of the compound is rate-limiting, and the normal rate-limiting step in the system lies distal to the site of entry of the compound into the reaction sequence. If the rate of passage of the compound along the pathway is independent of the concentration of the compound, then the normal rate-limiting step in the system lies on the pathway. By definition this step is operating at its maximum velocity ($V_{max.}$). The observed $V_{max.}$ of the labelling of the final product of the pathway will be the $V_{max.}$ of the rate-limiting step. In such circumstances a simple competitive isotope-dilution model (Forsdyke, 1968), rather than a combined isotope-dilution and

Michaelis–Menten model (Newsholme & Taylor, 1968; Brooker & Appleman, 1968; Gander, 1970), may be used to examine changes in labelling of the final product with a constant quantity of the labelled compound produced by various quantities of the corresponding unlabelled compound.

Isotope-dilution model. By using the isotope-dilution model it may be shown (Forsdyke, 1968) that $y+1 = (1/x)n-p$, where y is the number of times the labelled compound is diluted by the same added compound unlabelled, and p is the competitive inhibitor pool within the system expressed in the same units as y. In a system containing extracellular and intracellular compartments in equilibrium, p is the sum of the extracellular pool and the mean intracellular pool within cells that are incorporating the labelled compound into the end product of the pathway. This pool is termed the 'intrinsic pool' to distinguish it from $(y+1)$, which is the 'extrinsic' or 'added pool'. n is the theoretical maximum number of counts obtained when y and p are zero.

If the observed radioactivity (x c.p.m.) that has been incorporated in a given time in the presence of various dilutions of the added precursor y is expressed as a reciprocal and plotted against $(y+1)$, a linear relationship should be obtained with a slope of n and an intercept at the y axis of $-p$ (Fig. 1). If the concentration of the intrinsic pool is not limiting, the relationship should still be linear when y is zero. However, if the size of the intrinsic pool were decreased then the concentration of the compound would become limiting and linearity would be lost at low values of y. This condition may be produced by growing cells *in vitro* until the medium becomes exhausted (Forsdyke, 1968). Any loss of linearity at high values of y would indicate that the rate of incorporation of the compound had been influenced in a non-competitive manner (Forsdyke, 1968).

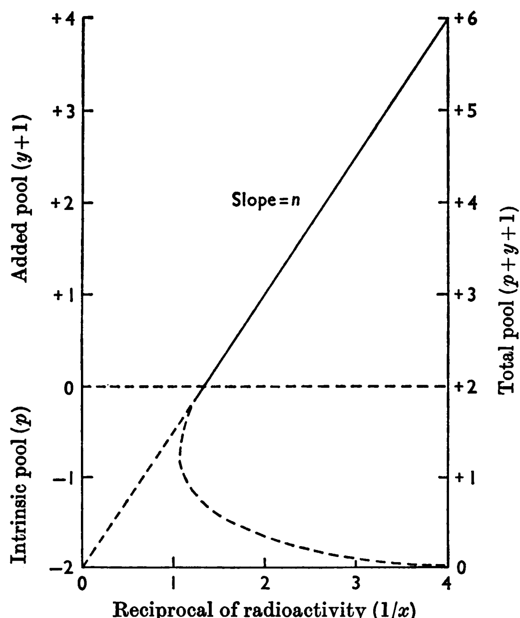

Fig. 1. Ideal isotope-dilution plot. The intercept on the y axis provides a measure of the intrinsic pool within the system. The slope of the plot (n) provides a measure of the rate of incorporation of the radioactive precursor corrected for dilution by this pool. The loss of linearity at low pool concentrations (----) represents the condition in which pool concentration limits the incorporation rate. For further details see the text.

This treatment, although permitting a rapid visual analysis of experimental results, is subject to errors inherent in the linearization of data by using reciprocals (Riggs, 1963). In practice values of n and p were obtained by using a non-linear regression computer program from the expression $x = 1/(\theta_1 + \theta_2 \cdot y')$ where $\theta_1 = p/n$, $\theta_2 = 1/n$ and y' is the added pool in dilution units. The rate of nucleoside incorporation by a given number of cells was calculated from the value of n by using the expression $100\ n/(D.E.R.C.T.)$ where D was the number of radioactive disintegrations corresponding to a Curie (2.22×10^{12}), E was the counting efficiency (approx. 34%), R was the specific radioactivity of the radioactive precursor, C was the cell number and T was the time of incubation. This equation assumes that radioactive labelling is a linear function of time and cell concentration under the experimental conditions employed. Results were calculated with the standard error of the mean.

Model applied to a system containing two extracellular pools. The analysis can be adapted for studying the effects on the labelling rate of media (i.e. serum) containing unknown competitive pools. The percentage (v/v) concentration of the medium is varied instead of the concentration of the unlabelled precursor (Fig. 2). To keep the total volume of the system constant, as the volume of one medium (S) increases, so the volume of another medium (M) must decrease. It is assumed that the pools contributed by the added labelled compound and cells are negligible compared with the pools in media S and M. The slope (N_S) of the isotope-dilution plot will be uninfluenced by the concentration of medium M if changes in the volume of M do not affect the metabolic pathway under consideration, and M contains no competitive pools itself; the plot will extrapolate to the ordinate at zero concentrations of medium S. If there is a competitive pool in M then this will influence the slope (N_{SM}) of the plot, which will extrapolate to the ordinate a distance (B) below the abscissa. Under such conditions the competitive ability (A) of M in terms of the percentage volume of S required to give the same competitive effect as 100% M, may be calculated from the value of the intercept (B) where $A = 100 \cdot B/(100+B)$. Similarly, the slope (N_S) of the plot that would be obtained if there were a pool only in medium S may be related to the slope N_{SM} obtained with pools in both media by the expression $N_S = 100 \cdot N_{SM}/(100+B)$.

MATERIALS AND METHODS

Details of materials and methods have been described previously (Forsdyke, 1968, 1969). Female white rats of an inbred strain were obtained from Woodlyn Farms Ltd., Guelph, Ont., Canada. Culture media were obtained from the Grand Island Biological Company, Grand Island,

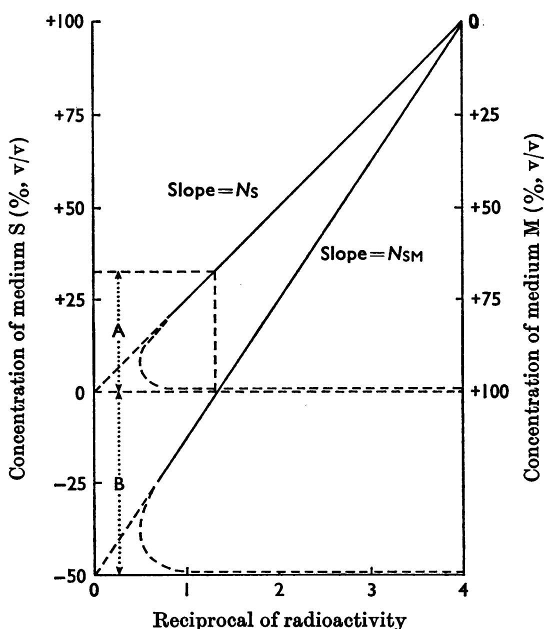

Fig. 2. Ideal isotope-dilution plots under conditions in which the relative proportions of two culture media are varied, the total culture volume being kept constant. For details see the text.

N.Y., U.S.A. Medium 199 (Morgan, Morton & Parker, 1950) contained an Earle's salts base (Earle, 1943) with $NaHCO_3$ (17mM). Minimum essential medium (Eagle, 1959) contained a similar base with 30mM-$NaHCO_3$.

Cell preparation and culture technique. A rat (approx. 200g) was decapitated and mesenteric and para-aortic lymph-nodes were removed. The nodes were cleaned of obvious fatty tissue, washed in medium 199 and then scissored for 2–3min in 4ml of medium 199 with fine-pointed scissors. The suspension was stood for 1min to permit lumps to settle and fatty tissue to rise and the intermediate layer of the suspension containing free lymphoid cells was pipetted off. The cells were pelleted by centrifugation (300g, 3min) and resuspended in fresh medium 199. To initiate radioactive labelling, samples of this suspension were added directly to an appropriate mixture of serum, medium 199 and a tritiated nucleoside at 37°C. Cultures (volume 1ml) were incubated at 37°C in an atmosphere of air+CO_2 (95:5). At appropriate time-points cells were pelleted by centrifugation (1200g, 4min), washed once in 4ml of 0.14M-NaCl (4°C) and finally stored in ethanol (95%) at −20°C. The samples were prepared for the determination of radioactivity in acid-precipitable material, by successive 5min (4°C) extractions of the pellets with 0.5M-$HClO_4$, ethanol–ether (1:1, v/v) and ether. The extracted pellets were solubilized in Hyamine (0.4ml) and mixed with a toluene-based scintillation fluid (10ml). Counting efficiency was approx. 34%. Most of the radioactivity was in RNA (Forsdyke, 1968).

Serum and plasma. Rats were bled from the aorta while under light ether anaesthesia. The blood samples (approx. 7ml) were left to clot at room temperature (10–20min), and then clots were loosened from the sides of the tubes and centrifuged down (1200g, 10min). Sera were pooled and re-centrifuged. Serum was stored at 4°C overnight in experiments involving dialysis; otherwise serum was used within a few hours of its preparation, being kept at room temperature until shortly before its inclusion in culture medium. Plasma was prepared in a similar manner by using 100μg of heparin per blood sample.

Dialysis tubing was boiled in 0.14M-NaCl for 10min. Serum in such tubing was placed in an equal volume of medium 199 at 4°C and left for approx. 18h. Time-courses of the equilibration of added tritiated nucleosides from serum across the membrane showed half equilibrium to be reached between 1.5 and 2h under these conditions; by 18h values for radioactivity on either side of the membrane were within 5% of each other, and no change in the percentage occurred on further incubation.

RESULTS

Variation of labelling with cell concentration. The radioactive labelling with [^{3}H]uridine of acid-precipitable material in suspensions of rat lymph-node cells varies with time and cell concentration (Forsdyke, 1969). In a typical experiment washed cells were incubated at 37°C for 3h in a medium containing fresh homologous serum (20%, v/v), medium 199 (80%) and a radioactive pyrimidine ribonucleoside (1μCi/ml). Fig. 3 shows the labelling of different concentrations of cells with either [5-^{3}H]uridine (Fig. 3a) or [5-^{3}H]cytidine (Fig. 3b) in the presence of different concentrations of the corresponding unlabelled nucleoside. Labelling was a linear function of cell concentration over the range of cell concentrations studied (0.3×10^6–3.0×10^6/ml) and plots extrapolated to zero c.p.m. at zero cell concentrations. As the concentration of unlabelled nucleoside increased the labelling was progressively depressed. At a given cell concentration more labelling was obtained with [^{3}H]uridine than with [^{3}H]cytidine of a similar specific radioactivity.

Figs. 3(c) and 3(d) show the results of Figs. 3(a) and 3(b) expressed as isotope-dilution plots (Fig. 1) at different cell concentrations. The concentration of unlabelled uridine is expressed in μmol/litre rather than in dilution units. All the plots were linear. As the cell concentration doubled, so the slopes of the plots doubled. Plots could be extrapolated to meet close to the ordinate. The mean value from six experiments of the ordinate intercepts for uridine was $3.08\pm0.25\mu$M and for cytidine $6.28\pm0.51\mu$M. From the premises of the isotope-dilution model (Fig. 1) it was inferred that the system contained an intrinsic competitor pool or pools for uridine and cytidine. The pool was

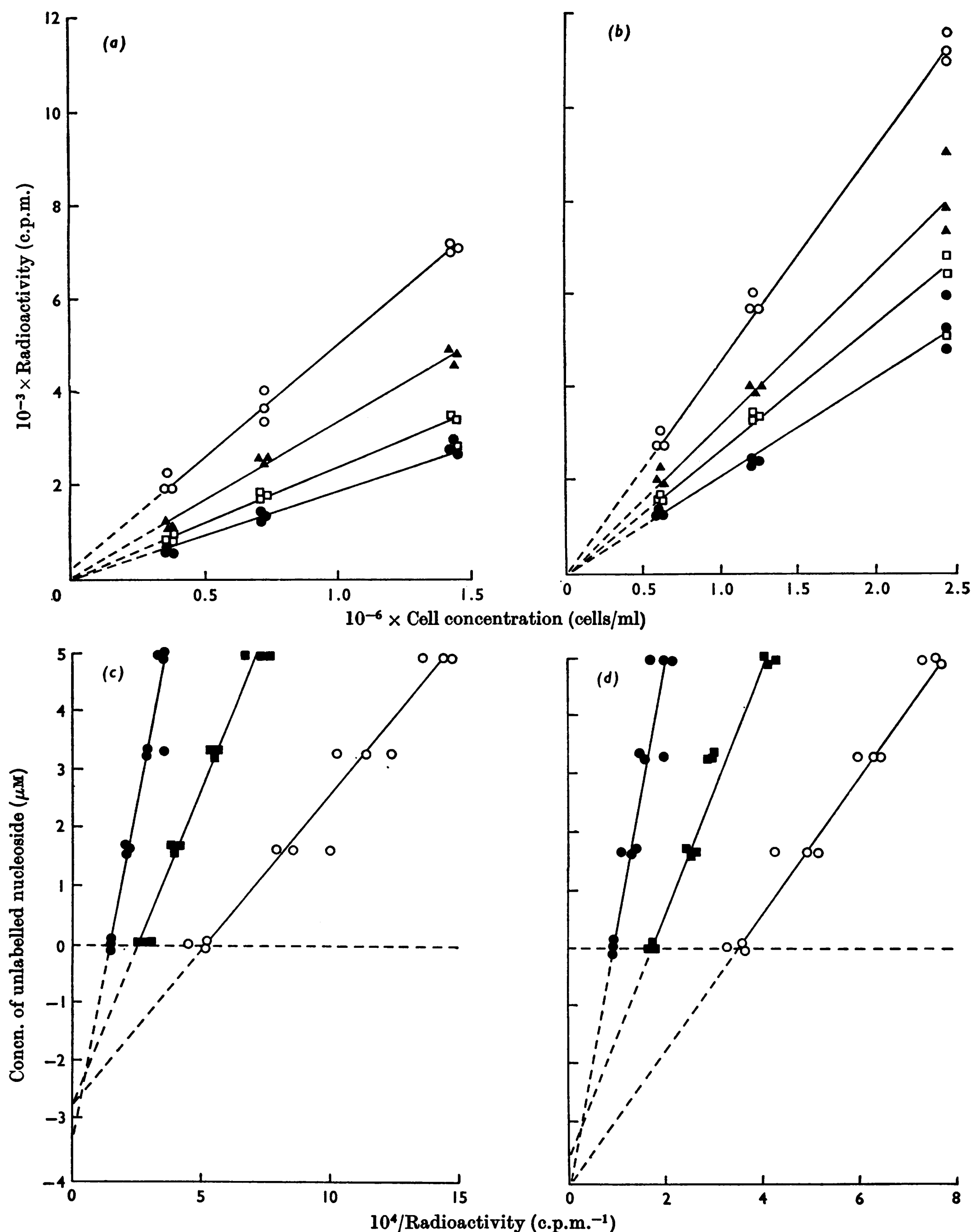

Fig. 3. Effect of different concentrations of cells and of unlabelled nucleosides on the labelling of rat lymph-node cells with [^{3}H]uridine (*a*) and [^{3}H]cytidine (*b*). Cells were incubated at 37°C in 1 ml of homologous serum (20%) and medium 199 (80%) with 1 μCi of [5-^{3}H]uridine (22.8 Ci/mmol) or [5-^{3}H]cytidine (24.7 Ci/mmol) and various concentrations of the corresponding unlabelled nucleoside. After 3 h cells were harvested for determination of acid-precipitable radioactivity. Figs. 3(*a*) and 3(*b*) are plots of radioactive labelling versus cell concentration at different concentrations of the corresponding unlabelled nucleoside; 0 μM (○), 1.64 μM (▲), 3.28 μM (□) and 4.92 μM (●). Figs. 3(*c*) and 3(*d*) are isotope-dilution plots (see Fig. 1) at different cell concentrations of the results in Figs. 3(*a*) and 3(*b*) respectively. Symbols for cell concentration are in ascending order of concentration, ○, ■, and ●; for [^{3}H]uridine these concentrations were, in millions per ml of culture, 0.36, 0.72 and 1.44, and for [^{3}H]cytidine, 0.6, 1.2 and 2.4.

independent of cell concentration indicating that the major if not the entire contribution to this pool was extracellular. In the absence of competitor pools the maximum rate of uptake of labelled nucleoside could be calculated (six experiments) as 11.4 ± 2.1 pmol of uridine/h per 10^6 cells and 11.5 ± 1.0 pmol of cytidine/h per 10^6 cells (see the appendix). A value of 11.5 pmol of nucleoside/h per 10^6 cells is equivalent to 1923 molecules of nucleoside/s per cell.

Variation of labelling with concentration of serum and medium 199. With the cell concentration constant the relative proportions of homologous serum and medium 199 in cultures were varied. As the serum concentration increased the labelling with [^{3}H]uridine and [^{3}H]cytidine was depressed. Fig. 4 shows that the reciprocal of the radioactivity incorporated was linearly related to the serum concentration employed. This linearity was retained at all serum concentrations examined (0–50%, v/v). The slope of the plot for uridine was greater than that for cytidine. The plot for uridine extrapolated to the ordinate below the abscissa; the plot for cytidine extrapolated to the ordinate even further below the abscissa. Similar linear plots were obtained with [^{3}H]uridine when cells from different rat lymphoid organs (Forsdyke, 1969) and pig blood leukocytes in autologous serum were used.

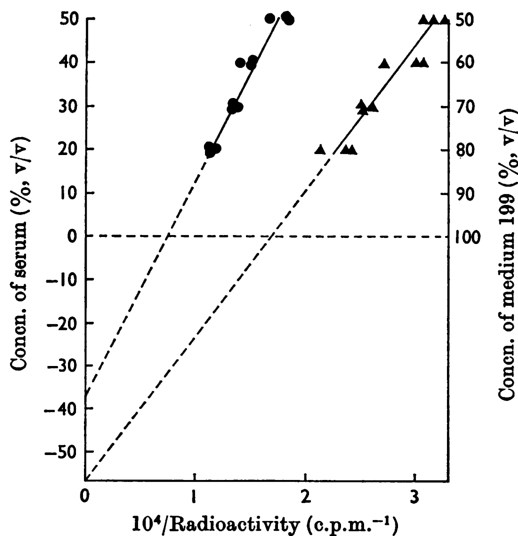

Fig. 4. Effect of various proportions of serum and culture medium 199 on the labelling of lymph-node cells with [^{3}H]uridine (●) and [^{3}H]cytidine (▲). Results are expressed in the form of the isotope-dilution plot of Fig. 2 where serum is medium S and medium 199 is medium M. Cultures (1 ml) containing 1.3×10^6 cells were incubated as described in Fig. 3.

In terms of the isotope-dilution model (Figs. 1 and 2) the linear relationship indicated (i) that serum was inhibiting the labelling in a competitive manner similar to that found with unlabelled nucleoside (Fig. 3) and (ii) that the rate of labelling was independent of serum concentration. If the extracellular pool in the system were contributed solely by serum then the plots should have crossed the serum axis at zero serum concentration. In the absence of an appreciable intracellular pool, intercepts at negative serum concentrations implied a competitive pool in medium 199. After correction for this pool the slope with [^{3}H]uridine, caused solely by the changing serum concentration, was found to be greater than the slope with [^{3}H]cytidine by a factor of 1.9 ± 0.1 (three experiments). From the intercepts it was calculated that medium 199 had a competitive value for uridine equivalent to 21.8 ± 2.6% serum (five experiments), and a competitive value for cytidine equivalent to 30.9 ± 3.1% serum (three experiments). These values permitted the calculation of the relative contributions of serum and medium 199 to the extracellular pool values provided by the negative ordinate intercepts in Figs. 3(*c*) and 3(*d*) (see the appendix). Serum possessed a competitive ability equivalent to 8.2 μM-uridine or 14.1 μM-cytidine, whereas medium 199 possessed a competitive ability equivalent to 1.8 μM-uridine or 4.3 μM-cytidine.

Fig. 5 shows the linear isotope-dilution plot for [^{3}H]uridine obtained with cells incubated in

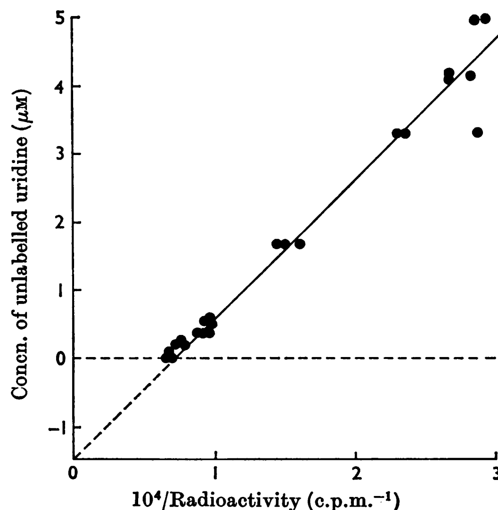

Fig. 5. Isotope-dilution plot showing the effect of various concentrations of unlabelled uridine on the labelling with [^{3}H]uridine of lymph-node cells (1×10^6) incubated in 1 ml of medium 199 without serum. Other details are as in Fig. 3.

medium 199 in the absence of serum. The mean intercept value of four experiments was 2.2±0.3 μM-uridine. The maximum rate of uptake of labelled uridine in the absence of competitor pools (calculated from the slopes) was 12.0±2.3 pmol of uridine/h per 10^6 cells.

Comparison of medium 199 *with other defined culture media.* The effects of three different types of defined culture media were compared in the presence of different concentrations of serum (Fig. 6). The slopes of the linear isotope-dilution plots were independent of the defined culture medium used; however, the plot with Eagle's minimum essential medium was displaced to the right of the plot with medium 199, and the plot with Earle's salts medium was displaced even further to the right.

Diffusible nature of the serum inhibitory activity. Serum was dialysed against an equal volume of medium 199 and the diffusate and dialysis residue were added separately in different concentrations to cultures containing 15% (v/v) serum. Both inhibited labelling with [^{3}H]uridine to similar extents. Assuming that diffusible molecules (micromolecules) were distributed equally on either side of the dialysis membrane, the diffusate and dialysis residue were accorded serum-volume values equal to that of half an equal volume of undialysed serum. Table 1 shows values of the radioactivity incorporated at different serum micromolecular volumes. Values were similar whether the serum micromolecules were provided by whole serum, whole serum plus diffusate, or whole serum plus dialysis residue.

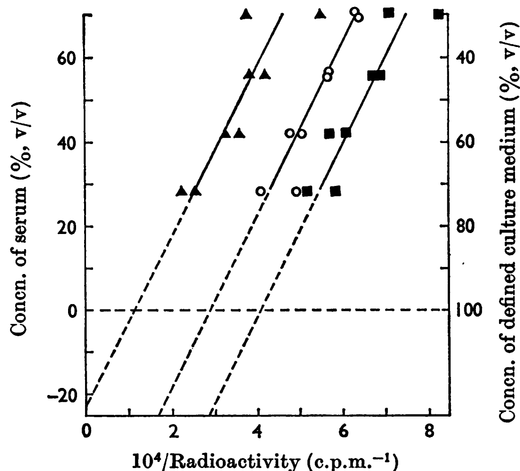

Fig. 6. Isotope-dilution plots showing the effects of various concentrations of different defined culture media on the labelling of lymph-node cells (1.3×10^6/ml) with [^{3}H]cytidine (1.4 μCi/ml). ▲, Medium 199; ○, Eagle's minimum essential medium; ■, Earle's salts medium. Other details are as in Figs. 2 and 3.

Inhibition of labelling with [^{3}H]*uridine by* [^{1}H]-*cytidine.* Fig. 7 is an isotope-dilution plot showing the effect of different concentrations of unlabelled uridine or cytidine on the labelling of cells with either [^{3}H]uridine or [^{3}H]cytidine. Labelling with [^{3}H]uridine was inhibited by [^{1}H]uridine more than by [^{1}H]cytidine, whereas labelling with [^{3}H]cytidine was inhibited equally by both unlabelled uridine and cytidine. The slopes of the plots of the dilution by [^{1}H]uridine of [^{3}H]uridine and [^{3}H]-cytidine of similar specific radioactivities were similar. If the slopes of [^{3}H]uridine–[^{1}H]uridine plots were considered as unity, the average slopes of other plots in four experiments were 2.59±0.21 ([^{3}H]uridine–[^{1}H]cytidine), 1.04±0.15 ([^{3}H]cytidine–[^{1}H]uridine) and 0.95±0.15 ([^{3}H]cytidine–[^{1}H]cytidine). The [^{3}H]uridine–[^{1}H]cytidine plots tended to extrapolate to the ordinate close to the extrapolation of the [^{3}H]cytidine plots. If the negative ordinate intercept values of the [^{3}H]-uridine–[^{1}H]uridine plots were considered as unity, the average intercepts of other plots (four experiments) were 2.51±0.20 ([^{3}H]uridine–[^{1}H]cytidine), 2.40±0.45 ([^{3}H]cytidine–[^{1}H]uridine) and 2.16±0.29 ([^{3}H]cytidine–[^{1}H]cytidine).

Comparison of serum and plasma. To prepare serum, blood was left at room temperature for

Table 1. *Effects of different concentrations of serum, serum dialysis residue and serum diffusate on the labelling of lymph-node cells with* [^{3}H]*uridine*

The dialysis residue and diffusate were prepared by dialysing serum for 18 h at 4°C against an equal volume of medium 199. The diffusible molecules (micromolecules) were assumed to distribute equally on either side of the dialysis membrane, and the dialysis residue and diffusate were accorded serum micromolecular volume values equal to that of half an equal volume of undialysed serum. Lymph-node cells (2×10^6) were incubated for 2 h in medium containing [^{3}H]uridine (1 μCi; 5 Ci/mmol), serum (15%), and different volumes of either dialysis residue or diffusate. The volume was made up to 1 ml with medium 199. Cells and serum were from rats of the strain maintained at the Institute of Animal Physiology, Babraham, Cambridge. The values of the mean radioactivity (c.p.m.) of quadruplet cultures are presented together with the S.E.M.

Source of serum micromolecules	Radioactivity (c.p.m.) at different concentrations (%, v/v) of serum micromolecules		
	15%	30%	45%
Serum	4476±73	3620±75	3037±41
Serum+dialysis residue	—	3709±82	2924±59
Serum+diffusate	—	3589±86	3207±84

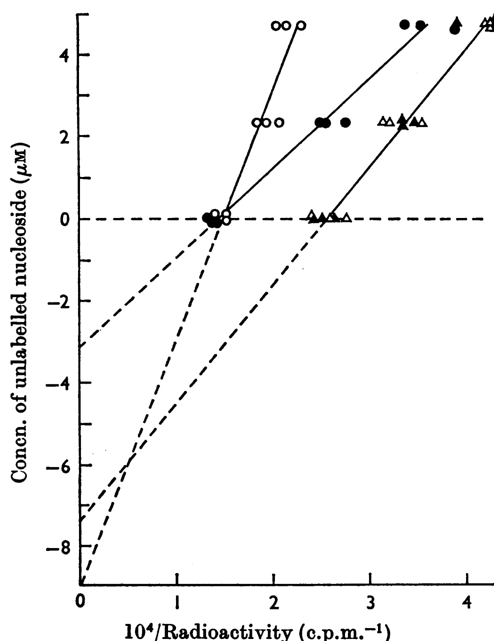

Fig. 7. Isotope-dilution plots showing the effects of various concentrations of unlabelled uridine and cytidine on the labelling with [^{3}H]uridine and [^{3}H]cytidine of lymph-node cells (1.2×10^6) incubated in serum and medium 199. ○, [^{3}H]Uridine–[^{1}H]cytidine; ●, [^{3}H]-uridine–[^{1}H]uridine; ▲, [^{3}H]cytidine–[^{1}H]cytidine; △, [^{3}H]cytidine–[^{1}H]uridine. Other details are as in Fig. 3.

10–20min to permit clot formation before the separation of cellular elements by centrifugation. Plasma was prepared by centrifuging heparinized blood immediately after removal from the animal. Table 2 shows the results of an experiment in which the duration of contact of the cellular elements of blood with serum or plasma after removal of the blood from the rat was varied; the effects of the serum and plasma (20%) on the labelling of lymph-node cells with [^{3}H]uridine were then compared. More labelling was obtained in plasma than in serum. The time between removal of blood and separation of cellular elements did not affect the labelling in any consistent manner. Heparin added to serum at concentrations between 14 and 140μg/ml was also without effect.

The effects of different concentrations of plasma and serum on [^{3}H]uridine labelling were compared (Fig. 8*a*). As with serum, there was a linear relationship between the plasma concentration and the reciprocal of the radioactivity (c.p.m.) except for a consistent decrease in labelling (increase in reciprocal of radioactivity) at the lowest plasma concentrations used. The plasma plot was steeper than the serum plot by a factor of 1.8 ± 0.1 (three experiments), and both lines met close to the ordinate. Plasma was dialysed for 18h against serum and the effects of different concentrations of the plasma and serum on labelling were again compared (Fig. 8*b*). Values of reciprocals of the radioactivity were intermediate between values obtained with equal concentrations of undialysed plasma and serum.

DISCUSSION

Scope and limitations of the isotope-dilution approach. Although the results derived from the system do appear to conform with the predictions of the isotope-dilution model, such conformity cannot be taken as necessarily showing that the model is a true description of the system. This is a limitation of the present approach that is common to all attempts to interpret the function of a complex system in terms of a simple model.

Another limitation is that the approach only shows the existence of pools of compounds in various compartments of the system that appear to inhibit radioactive labelling in a competitive manner. The approach cannot unambiguously identify the compounds or distinguish between the possibilities (i) that they inhibit but are not incorporated instead of the labelled compound, and (ii) that they inhibit and are incorporated at

Table 2. *Labelling of culture lymph-node cells in serum or plasma with [^{3}H]uridine: effect of varying the time between taking blood from the rat and separation of cellular elements from the serum or plasma*

A series of rats (E, D, C, B, A) were bled in turn and each blood sample (approx. 7ml) was divided between two tubes at room temperature, one of which contained heparin (100μg). After serum from the last rat (A) had clotted spontaneously the tubes were centrifuged to separate cellular elements. Thus serum and plasma from rat A had the shortest period of contact with the cellular elements of blood after removal from the animal. Lymph-node cells (10^6/ml) were incubated with [^{3}H]uridine (1μCi; 30.7Ci/mmol) in serum or plasma (20%) for 2h. Each radioactivity value is the mean of triplicate cultures ± S.E.M.

	Serum		Plasma	
Rat	Time (min)	Radioactivity (c.p.m.)	Time (min)	Radioactivity (c.p.m.)
A	12	5827±40	8	6279±49
B	17	5204±111	13	6234±60
C	23	6023±92	19	6496±45
D	31	5574±74	27	6628±31
E	41	6129±252	37	6550±110

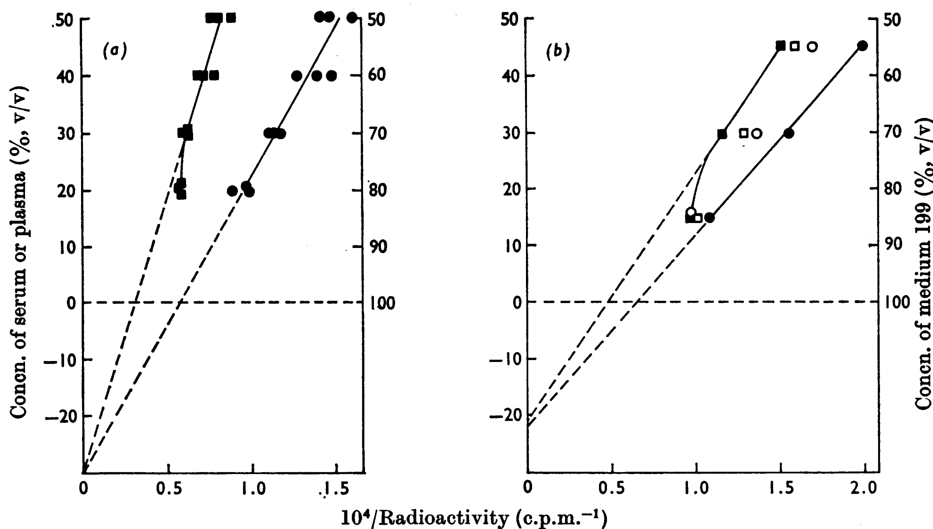

Fig. 8. Isotope-dilution plots showing the effects of various concentrations of plasma (■) and serum (●) on the labelling of lymph-node cells with [^{3}H]uridine. For the experiment shown in Fig. 8(*b*) serum and plasma were dialysed against each other for 18h at 4°C; □, plasma after dialysis against serum; ○, serum after dialysis against plasma. Lymph-node cells (1.6×10^6, Fig. 8*a*; 1.2×10^6, Fig. 8*b*) were incubated with 1 μCi of [^{3}H]-uridine (28Ci/mmol) in various concentrations of medium 199 and plasma or serum, under the conditions described in Fig. 3. In Fig. 8(*b*) each point is the reciprocal of the mean of triplicate radioactivity values.

the same rate as (or a greater rate than) the labelled compound. Between these extremes there is a spectrum of compounds that might be incorporated instead of the labelled compound but at a slower rate. Inhibitory activity can be measured in terms of the extent to which the inhibitor mimics the effect of a given quantity of unlabelled precursor identical with the labelled precursor under study. The extent of inhibition by any compound can be regarded as a dual function of its effective concentration and its affinity for the sites at which it inhibits. Further characterization of inhibitors present in any compartment of the system may be carried out by combining the isotope-dilution assay with conventional fractionation procedures; initially this characterization may be by dialysis to reveal the macro- or micro-molecular nature of inhibitory activity.

With isotope-dilution plots it is feasible to ask whether a change in the radioactive labelling of cells in response to a stimulus could be caused by a change in the activity of the rate-limiting step in labelling, or by a change in the pool size. For example, it has been shown that the early change in labelling with [^{3}H]uridine of leukocytes responding to phytohaemagglutinin is due to a change in the activity of the rate-limiting step (change in slope of isotope-dilution plot), rather than a change in pool size (no change in intercept at the ordinate); however, much of the subsequent increase in the labelling rate, over two days of culture, is caused by a decrease in the pool (change in intercept; Forsdyke, 1968).

Location, size and composition of competitive pools. (*a*) Cells. The isotope-dilution model is concerned with the rate-limiting step in labelling and not with a chemical equilibrium. Provided equilibrium is reached rapidly, factors altering the equilibria between pools before the rate-limiting step should not affect the rate of labelling. (If the equilibrium were not reached rapidly, then the step mediating the equilibrium would itself, by definition, be rate-limiting.) If the rate-limiting step were at the interface between intracellular and extracellular pools, then such pools would not be in chemical equilibrium. If the rate-limiting step were at some point within the cell, then extracellular pools and intracellular pools before this point would be in equilibrium. Both would contribute additively to the total pool in the system, and the latter would be a function of the cell concentration.

If there were a detectable intracellular pool within the system, then in terms of the isotope-dilution model (Fig. 1), ordinate intercepts of plots at different cell concentrations should permit the pool to be measured. No evidence for such a pool was obtained when cells were cultured in

serum and medium 199 with [^{3}H]uridine and [^{3}H]cytidine (Figs. 3*c* and 3*d*). This would imply either that the rate-limiting step was at the interface between extracellular and intracellular pools, or that the size of the intracellular pool is below the limits of sensitivity of the assay. The major pool was extracellular being contributed mainly by serum and partly by medium 199.

(*b*) Culture media. Analysis of results derived from cultures containing both serum and medium 199 (Figs. 3 and 4) gave a pool in medium 199 equivalent to 1.8μM-uridine. This was in quite close agreement with the analysis of results derived from cultures containing medium 199 only (Fig. 5), which gave a value of 2.2μM-uridine.

Medium 199 contains various purine and pyrimidine derivatives including uracil (2.6μM) and thymine (2.3μM). Although these bases alone are poorly incorporated into mammalian nucleic acids (Plentl & Schoenheimer, 1944; Friedkin & Wood, 1956; Weissman, Lewis & Karon, 1963) it is likely that they would inhibit labelling with nucleosides (Steck, Nakata & Bader, 1969). To study this further, medium 199 was replaced by simpler media not containing purine or pyrimidine derivatives (Earle's salts and Eagle's minimum essential medium). Paradoxically these media appeared to contain a greater pool of competitors than did medium 199 (Fig. 6). It is probable that in simpler media a competitive pool is formed by cells, either by RNA degradation (Caldwell & Chan, 1970), or by *de novo* synthesis.

(*c*) Serum. The difference between the slopes of the isotope-dilution plots for [^{3}H]uridine and [^{3}H]cytidine at different serum concentrations (Fig. 4) could mean either that the rate of uridine incorporation is greater than the rate of cytidine incorporation, or that a serum pool of activity competitive with [^{3}H]cytidine is greater than a serum pool of activity competitive with [^{3}H]uridine. The latter possibility was supported by calculations from the results in Fig. 3, which showed the rates of labelling with the two precursors to be similar. Combining the results of Figs. 3 and 4 it was found that serum had a competitive ability equivalent to 14μM-cytidine or 8μM-uridine; such activity was diffusible (Table 1). The difference could result from separate compounds in serum inhibiting the incorporation of the two radioactive compounds independently, or a single compound inhibiting the incorporation of the two labelled compounds to different extents. The results in Fig. 7 exclude the possibility that the serum inhibitory activity is due to a single pool of uridine in serum, since uridine inhibits the labelling of cells with [^{3}H]uridine and [^{3}H]cytidine to equal extents; however, serum and cytidine have in common the property of inhibiting labelling with [^{3}H]cytidine approximately twice as effectively as they inhibit labelling with [^{3}H]uridine.

By using a different approach Guri, Swingle & Cole (1968) have reported that rat plasma contains deoxycytidine (26μM) and an uncertain quantity of cytidine. That the inhibitory pool in the system does contain RNA precursors is suggested from previous work with pig leukocytes (Forsdyke, 1968) in which the uridine competitive pool decreased from an initial value of 7.1$\pm$1.3μM-uridine (four experiments) to zero over two days of culture. In this time there was an increase in RNA in the culture which could be quantitatively correlated with the decrease in the pool (see the appendix).

Physiological basis of the serum pool. The detection of an activity in serum may mean that the activity (i) is normally present in the extracellular fluid of blood, or (ii) is released into the blood during exsanguination, or (iii) is released from the cellular elements of blood before the separation of serum from cells. Sera separated at different times after collection of blood showed similar abilities to influence cell labelling, indicating that no competitor pool had been released from cells during the time-period studied. To shorten the period between collection of blood and separation of cellular elements, blood was collected into heparin-containing tubes, which were immediately centrifuged. The plasma obtained was found to inhibit labelling less than an equal volume of serum from the same animal, but this difference persisted when the period before separation of plasma from cells was prolonged (Table 2). Thus it appeared that the difference between plasma and serum could not be due to the extra period of contact of serum with cells necessary for spontaneous clotting to occur. Intercepts of isotope-dilution plots with serum or plasma were similar, whereas slopes with plasma were steeper than those with serum (Fig. 8*a*). The results indicated that plasma and serum pools were identical and the incorporation rate was greater in plasma than in serum; this difference was due to a diffusible factor (Fig. 8*b*). Losses of linearity at low plasma concentrations implied that the concentration of the diffusible factor was limiting labelling at such concentrations (Fig. 1).

The above considerations indicated that, in the absence of evidence that competitive activity was released into the blood during exsanguination, the serum pool could be regarded as normally representative of the extracellular fluid of blood *in vivo*. The concept of a physiological pool of a pyrimidine nucleoside such as cytidine is compatible with the results and would lead to the predictions (i), that hereditary disorders of the *de novo* pyrimidine biosynthesis pathway should not be manifest until

after birth (Becroft, Phillips & Simmonds, 1969), and (ii), that one tissue, such as the liver parenchyma, could supply the nucleoside needs of other tissues, which in turn would normally be expected to have rather quiescent *de novo* pathways.

Independence of labelling rate on serum concentration. Isotope-dilution plots at different serum concentrations (0–50%, v/v) were found to be linear (Figs. 4 and 6), indicating that serum does not stimulate the rate-limiting step in labelling. When cells were grown in serum-free medium (medium 199), the mean incorporation rate was 12.0 ± 2.3pmol of uridine/h per 10^6 cells (Fig. 5), whereas in serum-containing medium the rate was 11.4 ± 2.1pmol of uridine/h per 10^6 cells (Fig. 3). Medium 199 contains uracil, which, although unlikely to be a substrate, might be expected to help saturate the rate-limiting step and maintain the linearity of isotope-dilution plots as the serum concentration was decreased. In the absence of serum the rate did not increase, and this would appear incompatible with the hypothesis (Forsdyke, 1967*a,b*) that phytohaemagglutinin activates cells by removing a macromolecular serum inhibitor.

Rate of RNA synthesis. Nucleosides are incorporated into nucleic acids by way of salvage pathways. Initially the nucleoside probably enters the cell under the influence of a specific permease (Grenson, 1969; Plagemann & Roth, 1969); then it is phosphorylated to the mononucleotide. At this stage it is in the main pathway of pyrimidine trinucleotide biosynthesis *de novo* (Reichard, 1959; Tatibana & Ito, 1969).

The rate-limiting step of labelling might lie either before or after the point of entry of the labelled nucleoside into the *de novo* pathway. The position of the rate-limiting step would determine whether the dilution-corrected labelling rate reflected the true rate of RNA synthesis. If the rate-limiting step lay after the point of entry of the labelled nucleoside into the *de novo* pathway the products of this pathway would dilute the labelled nucleoside as an intracellular pool, thus influencing isotope-dilution plots. The slope of such plots would provide a measure of the rate of RNA synthesis. [As discussed above, the failure to detect an intracellular pool (Fig. 3) may imply that the size of such a pool is below the limits of sensitivity of our assay.] If the rate-limiting step lay before the point of entry of labelled nucleoside into the *de novo* pathway then products of this pathway would not directly influence isotope-dilution plots, and the slope of such plots might not reflect the rate of RNA synthesis. However, the flow of an RNA precursor along a salvage pathway could be taken as a measure of the final flow into RNA if endogenous flow along the *de novo* pathway were minimal. There are reasons for believing such conditions hold with the process of ribonucleoside incorporation into lymphocytes. (i) Incorporation of intermediates of the *de novo* pathway is poor compared with the incorporation of nucleosides (Lucus, 1967; Forsdyke, 1968). (ii) Feed-back inhibition of the early enzymes of the *de novo* pathway would occur if a sufficient intracellular concentration of nucleotides could be maintained by the salvage pathway (Blair & Potter, 1961; Tatibana & Ito, 1969; Lan, Sallach & Cohen, 1969; Ito & Uchino, 1971). Thus irrespective of the site of the rate-limiting step in the system it seems likely that the maximum rate determined from the slopes of isotope-dilution plots would be of the same order as the true rate of RNA synthesis. This would be in keeping with previous work with phytohaemagglutinin-stimulated leukocyte cultures (see the appendix) in which the rate of quantitative RNA increase could be correlated with the rate of nucleoside incorporation, assuming such incorporation to be mainly into stable RNA species. In the latter system, the activity of the *de novo* pathway rises late in the culture period at a time when the pool of salvage precursors is decreasing (Forsdyke, 1968; Ito & Uchino, 1971).

Validity of methods of expressing stimulations of radioactive labelling. The effects of stimulating agents (i.e. hormones, antigens) on tissues are often assayed by measuring changes in the labelling of a given quantity of tissue by some radioactive compound. The degree of stimulation is usually either presented as the percentage change in labelling, or as the additive change in labelling, over that of the corresponding control tissue without the stimulant. To compare different experiments correctly, one should if possible first determine which method of presenting the experimental results is appropriate for comparative purposes. The isotope-dilution approach may assist this.

For example, the present work indicates that plasma stimulates the rate of cell labelling with [^{3}H]uridine, when compared with serum. It can be seen from Fig. 8 that, except at low plasma concentrations, there is a constant ratio between the reciprocal of the radioactivity at a given plasma concentration, and the reciprocal of the radioactivity at the same concentration of serum. If the ratios of the reciprocals are constant, then the ratios of the radioactivities are constant. Hence it would appear valid to compare the percentage stimulation by plasma in an experiment with say 30% plasma and serum, with the percentage stimulation in an experiment with 40% plasma and serum, and the stimulations might be averaged for statistical purposes.

In previous work (Forsdyke, 1968), a comparison was made between the degrees of lymphocyte stimulation by phytohaemagglutinin at different

serum concentrations. It was found, (i) that changing the serum concentration altered the labelling in control cultures, and (ii) that the desired experimental result could be obtained empirically by plotting the stimulation of labelling at each serum concentration as a percentage of the labelling of the corresponding control cultures without phytohaemagglutinin. The present work suggests that the difference in the labelling of control cultures at different serum concentrations was due to a competitive nucleoside pool. Such a pool would be expected to affect both control and phytohaemagglutinin-stimulated cultures to a proportionate extent. Hence it would appear valid to compare percentage stimulations at different serum concentrations.

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APPENDIX

Some worked examples

Calculation of maximum rate of uptake of nucleoside. The slope of the plot at a cell concentration of 0.36×10^6/ml is approx. 8μM/15×10^{-4} reciprocal units (Fig. 3*c*). The 1ml cultures contained 1μCi of [^{3}H]uridine (22.8Ci/mmol), which equals 1/22.8μM-[^{3}H]uridine. Thus 8μM-uridine corresponds to 8×22.8 dilution units, and $n=8\times22.8/15\times10^{-4}=121600$ c.p.m. The maximum rate of nucleoside incorporation

$= 100\cdot n/(D.E.R.C.T.)$
$= 100\times121600/(2.2\times10^{12}\times34\times22.8\times0.36\times3)$
$= 6.6$pmol/h per 10^6 cells
$= 1100$ molecules/s per cell
(Avagadro's number $= 6\times10^{23}$).

Calculation of the competitive abilities of the pools in serum and medium 199. The value of B in Fig. 2 is approx. 36% (Fig. 4). Hence $A=100\cdot B/(100+B)\simeq25\%$, which means that medium 199 has a competitive ability towards [^{3}H]uridine equivalent to 25% of the competitive ability of an equal volume of serum. If the competitive ability in serum is equivalent to Z μM-uridine, then the competitive ability in medium 199 is equivalent to $Z/4\mu$M-uridine. The intrinsic pool (p) is contributed both by serum (0.2ml of a 1ml culture) and by medium 199 (0.8ml of a 1ml culture) and is equivalent to 3μM-uridine (Fig. 3*c*). Thus $0.2Z+0.8Z/4=3\mu$M, and hence the competitive ability of serum is 7.5μM whereas the competitive ability of medium 199 is 1.9μM.

Correlation between the rate of increase of total RNA and the rate of incorporation of [^{3}H]*uridine in pig leukocyte cultures.* (*a*) Quantitative RNA increase.

For 5×10^6 cells in a 5ml culture there is a total RNA increase over a 2-day period of approx. $0.5E_{260}^{1cm}$ units (Fig. 1 of Forsdyke, 1967), which is equal to 14.7 μg of RNA if the $E_{1cm}^{0.1\%}$ for RNA at 260nm is 34 (Forsdyke, 1967). Assuming the RNA to be synthesized from precursors in the culture medium (5ml), then 1ml of culture medium would contribute precursors equivalent to 2.9 μg of RNA.

(*b*) Rate of incorporation of [^{3}H]uridine. The average value of n over a 2-day culture period is approx. 125000 c.p.m./30min per 10^6 cells in a 1ml culture (Fig. 5 of Forsdyke, 1968). The maximum rate of nucleoside incorporation

$$= 100 \cdot n/(D.E.R.C.T.)$$
$$= 100 \times 125000/(2.2 \times 10^{12} \times 10 \times 31 \times 1 \times 0.5)$$

= 36.7 pmol of uridine/h per 10^6 cells
= 0.43 μg of uridine/48h per 10^6 cells
= 2.7 μg of RNA if [^{3}H]uridine is incorporated solely into uridylate residues (mol.wt. 306), which comprise 20% of the RNA bases (Forsdyke, 1968).

(*c*) Decrease in pool size. The value of p falls from 7 μM-uridine to zero over 2 days of culture (Fig. 5 of Forsdyke, 1968).

p = 0.007 μmol of uridine/ml of culture
= 1.7 μg of uridine/ml of culture
= 10.7 μg of RNA (from the above premises).

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