# POLYRIBOSOMES OF HAMSTER CELLS: TRANSIT TIME MEASUREMENTS

CLIFFORD P. STANNERS

*From The Department of Medical Biophysics, University of Toronto, and The Ontario Cancer Institute, 500 Sherbourne Street, Toronto 5, Ontario, Canada*

ABSTRACT The amount of radioactivity incorporated into completed polypeptides and into nascent polypeptides bound to polyribosomes was measured for rapidly dividing, cultured hamster embryo cells incubated in the presence of labelled amino acids. Using a mathematical analysis which took into account variations in the specific activity of the intracellular amino acid pool, these measurements yielded a value for the length of time required for the synthesis of a primary polypeptide of average length, the "transit time." Subject to a number of reasonable assumptions in the mathematical analysis which were, however, only approximately verified, the transit time was found to be 23 ± 2 sec in four independent experiments. Regardless of the absolute accuracy of this measurement, the method is considered a useful measure of translation independent of transcription.

## INTRODUCTION

Increasing evidence for the control of protein synthesis at the level of translation has been found recently for microorganisms (Kano-Sueoka and Sueoka, 1966; Bautz et al., 1966) and for animal cells (McAuslan, 1963; Salb and Marcus, 1965; Gross et al., 1964). This evidence has served to focus attention on polyribosomes, on which cellular protein synthesis is carried out. Three ribosomal processes in the polysome may be involved in the control of translation: attachment to, movement along, and detachment from the messenger RNA. This study is concerned with the rate of movement of ribosomes along the messenger RNA of hamster embryo cells grown in culture. The choice of system was partially governed by the ability of these cells to undergo changes of biological significance in vitro, such as malignant transformation (Vogt and Dulbecco, 1960) and contact inhibition (Abercrombie and Heaysman, 1954). A measure of the rate of ribosome movement is the length of time required for a ribosome to translate a messenter RNA "cistron" of average length. This time, termed the "transit time," has been estimated by Goldstein et al. (1964) to be 5 sec in *E. coli*. In animal cells, most estimates for the transit time are of the order of 1 min (Penman et al., 1963) though these are usually maximum estimates.

Recently Conconi et al. (1966) have measured the transit time in rabbit reticulocytes by a relatively precise method and have obtained values of about 40 sec for the synthesis of globin molecules in hemoglobin. The reticulocyte, however, is a somewhat specialized cell, since no messenger or ribosomal RNA is synthesized (Marks et al., 1965) and practically all of the protein synthesized is of one type (Dintzis et al., 1958). The method used by these authors could not be applied with precision to the rapidly growing hamster cell for reasons which will become apparent later. An alternate method of high precision has been developed and has yielded the value of about 25 sec, subject to some assumptions. This method entails a mathematical model of the operation of polysomes and includes a consideration of pool problems which arise when radioactive amino acids are taken up by the cells.

The transit time is a measure of the rate of translation independent of the content of messenger RNA in the polysomes. The method should therefore provide a precise measure of translation independent of transcription.

## METHODS

### *Solutions and Chemicals*

Phosphate buffered saline (PBS) was prepared as described by Dulbecco and Vogt (1954).

Sodium H buffer consisted of 0.1 M NaCl, 0.02 M $MgCl_2$, and 0.01 M Tris, with pH 7.4 at 20°C.

Cells were grown in $\theta$ medium (Reader, 1966)[1] plus 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.). This mixture will be referred to as "growth medium."

Puromycin was obtained from the Nutritional Biochemicals Corporation (Cleveland, Ohio). A single batch of $^{14}$C-valine, of specific activity 107 mc/mM (The Radiochemical Centre, Amersham, England) was used in all experiments.

### *Cells*

Hamster embryo (HA) cells were obtained from 13 day old embryos of noninbred Syrian hamsters. The cells were grown in monolayer culture at 37°C in an atmosphere of 5% $CO_2$ and 95% air in 32 oz. medicine bottles, and were subcultured by trypsinization 4–7 times prior to use in experiments. Cultures were prepared 24–48 hr before use and were in the exponential phase of growth with approximately $1.5 \times 10^7$ cells per bottle at the time of experimentation. The doubling time of the cells under these conditions varied from 11–13 hr.

### *Labelling Techniques*

Cultures were washed and incubated with 9.5 ml growth medium. After exactly 30 min, 0.5 ml of a solution of $^{14}$C-valine was added to the growth medium to give a final concentration of 0.87 $\mu$c/ml in experiment 1, and 2 $\mu$c/ml in the other three experiments. It should be stressed at this point that the absolute valine concentration in the medium (47 $\mu$g/ml) was not reduced, as such treatment has been found to reduce the rate of protein synthesis in these cells rapidly and permanently (Taylor and Stanners, 1967).

The time of addition of the isotope was precisely defined in the following manner. Culture

[1] Reader, R. W. 1966. M. A. Thesis. University of Toronto, Canada.

bottles were turned over so that the medium was not in contact with the cells (which form a monolayer on one side of the bottle only). Isotope was added to the medium, then at a prescribed time the bottles were quickly turned over again, flooding the cells with the radioactive medium. To terminate labelling, 400 ml of ice cold PBS was flushed over the cells and the bottle plunged into ice water. To check the efficacy of the cold treatment in terminating protein synthesis, the rate of amino acid incorporation into acid precipitable material was measured at 4°C, and found to be less than 0.05% of the rate at 37°C.

*Preparation of Cellular Extracts*

Cytoplasmic extracts containing ribosomes and polysomes were prepared as described elsewhere (Reader and Stanners, 1967). Briefly, approximately $3 \times 10^7$ cells were removed from the culture bottles with trypsin[2] at 4°C, washed three times by centrifugation and resuspension in cold PBS, centrifuged and suspended in 1.4 ml of sodium H buffer. After 30 min at 0°C, Brij 58 (Atlas Chemical Industries, Brantford, Ontario, Canada), a non-ionic detergent, was added to a concentration of 0.5% and the suspension subjected to 10 strokes of a tight fitting Dounce glass homogenizer. The nuclei were removed by light centrifugation leaving the desired cytoplasmic extract. In experiment 4, sodium deoxycholate (DOC) was added to the extract to a concentration of 0.5%; DOC was not used in the other experiments.

*Sucrose Density Gradient Technique*

Ribosomes and polysomes were separated from lighter cytoplasmic material by the following technique. The cytoplasmic extracts were layered over 28 ml 15% to 30% (w/w) linear sucrose density gradients in sodium H buffer and centrifuged for 95 min at 24,500 rpm and 25°F in the Spinco Model L ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) using the SW 25.1 rotor. After centrifugation, the gradients were pumped through a recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) to measure the optical density (OD) at 260 mμ. Typical optical density patterns for extracts of normal and puromycin-treated cultures are shown in Fig. 1. The proof that the fast-sedimenting broad peak seen in Fig. 1 for the normal culture represents polysomes, and the narrow more slowly sedimenting peak represents free ribosomes has been given elsewhere (Reader and Stanners, 1967). Two fractions were collected from each gradient, one containing the polysomes and free ribosomes ("polysome fraction") and the other the rest of the lighter cytoplasmic material at the top of the gradient ("soluble fraction"). The soluble fraction included a small number of ribosomal subunits. A longer centrifugation demonstrated that a negligible fraction of incorporated radioactive valine was associated with these subunits.

*Measurement of Radioactivity in Fractions*

The radioactivity incorporated into protein in the polysome and soluble fractions was measured as follows. A series of aliquots of different volumes was withdrawn from each fraction and treated with KOH to a final concentration of 1 N (in order to free any labelled amino acids bound to sRNA molecules). An amount of 40% trichloroacetic acid equal in volume to the KOH-treated sample was added to precipitate the protein, and the precipitate was collected on Millipore filters (pore size 0.45 μ). The filters were glued to planchettes, and

[2] The trypsin treatment was found to have no effect on the amount of acid precipitable radioactivity within cells after incubation of cultures with radioactive amino acids.

were counted using a low background (2 cpm) gas flow counter. Each filter was counted for a time sufficient to accumulate at least 500 counts. The measured activities were plotted against sample volume, and the total activity calculated from the slope of the resultant straight line and the total volume of the fraction. This procedure was designed to minimize errors in the measurement of activity due to sampling and due to self-absorption of the $^{14}C$-beta particles in the precipitate itself. The plots described above deviated from linearity only for points corresponding to large samples taken from the soluble fractions.

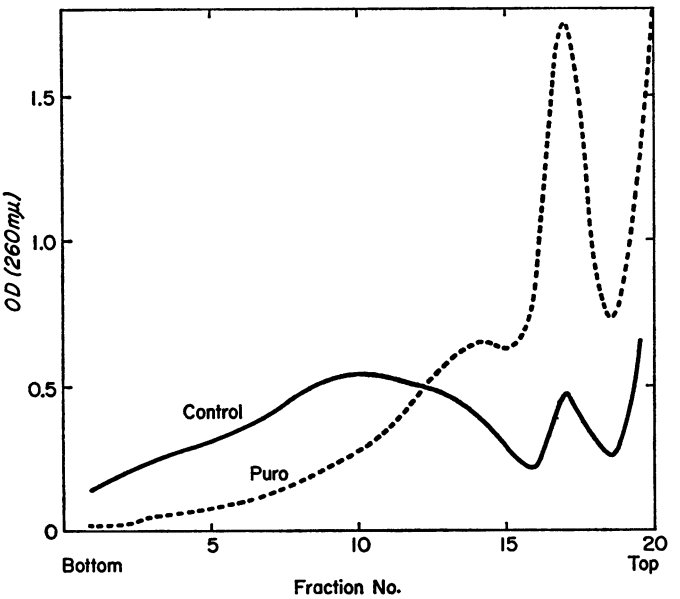


FIGURE 1 Optical density profiles obtained from sucrose density gradient analysis of cytoplasmic extracts from pormal cultures and from cultures treated with puromycin at 100 μg/ml for 5 min. See text for details.

*Experimental Design*

The calculation of the transit time, as defined in the Introduction, required measurements of the rate of synthesis of nascent and completed polypeptides. This was done in the following way. A series of replicate cultures were incubated in medium containing $^{14}C$-valine for various well defined periods ranging from 15 sec to 10 min. Each time point was represented by two identically treated cultures which were pooled in subsequent procedures. Cytoplasmic extracts were prepared and separated into two fractions: the soluble fraction and the polysome fraction as described above. The total optical density at 260 mμ in the polysome fraction and the total alkali-stable, acid-precipitable radioactivity in both the polysome and soluble fractions was measured (see previous section). $P(t)$ and $S(t)$ were calculated for the polysome and soluble fractions respectively as the quotient: total counts per minute (cpm)/total polysome OD. The latter procedure was necessary to normalize variations in cell number or extraction efficiency which inevitably occurred between the replicate cultures; the result should be considered a relative activity rather than a specific activity. The $P(t)$ values obtained were corrected for non-nascent radioactivity bound to the ribosomes as follows. For experiments 1

and 2, a series of replicate cultures was labelled with $^{14}$C-valine in parallel with the first series, but after the various times, puromycin, which has been shown to liberate nascent protein from polysomes quantitatively in other systems (Nathans, 1964; Colombo et al., 1965), was added at 100 $\mu$g/ml and the incubation continued for a further 5 min. The radioactivity still associated with polysomes and ribosomes after puromycin treatment was measured at each time, expressed as total cpm/OD as above, and subtracted from the corresponding $P(t)$ to give the radioactivity in nascent protein only.

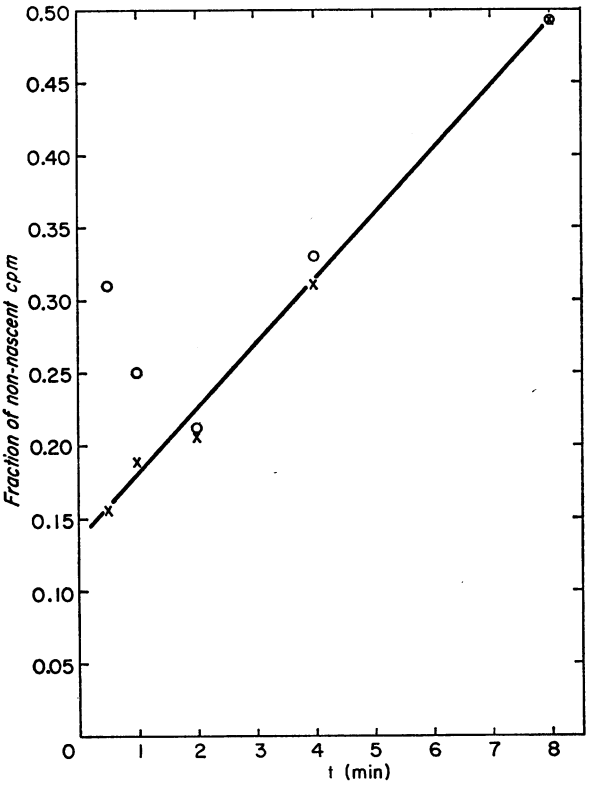


FIGURE 2 The fraction of the total alkali-stable, acid-precipitable radioactivity in the polysome fraction, as a function of time of incubation of cultures in $^{14}$C-valine, which remained associated with the polysome fraction after a 5 min incubation with puromycin administered immediately after the uptake of the labelled valine. Open circles, uncorrected data; crosses, data corrected for 30 sec lag in the action of puromycin, as described in the text.

In previous experiments using HA cells, puromycin at 100 $\mu$g/ml was found to inhibit amino acid incorporation to about 0.1% of the control value, but required about 30 sec to act. This lag was taken into account in the correction of the $P(t)$ values. The fraction of the total ribosome and polysome-bound radioactivity which was *not* released by puromycin is shown in Fig. 2, and can be seen to vary from about 15% at 30 sec to about 50% at 8 min. The line in Fig. 2 has been drawn through points (crosses) corrected for the time lag in the action of puromycin, though the uncorrected values (circles) are also shown. The time lag correction was applied as follows: curves of $P(t) + S(t)$, where $t$ refers to the time of termination of

labelling using ice-cold PBS (as described above), and where $t$ refers to the time of addition of puromycin at 100 $\mu$g/ ml, were superimposed by a shift of coordinates. A near perfect superposition was obtained by moving the "puromycin curve" back about 20 sec and down by a small amount on the ordinate scale. The $P(t)$ values for puromycin-treated cultures were then corrected by multiplying them by the ratio of the values of $P(t) + S(t)$ on the new to the old coordinates, and the values in Fig. 2 are the ratio of this corrected $P(t)$ for puromycin-treated cultures to the $P(t)$ for normal cultures. The time lag correction is obviously greater for

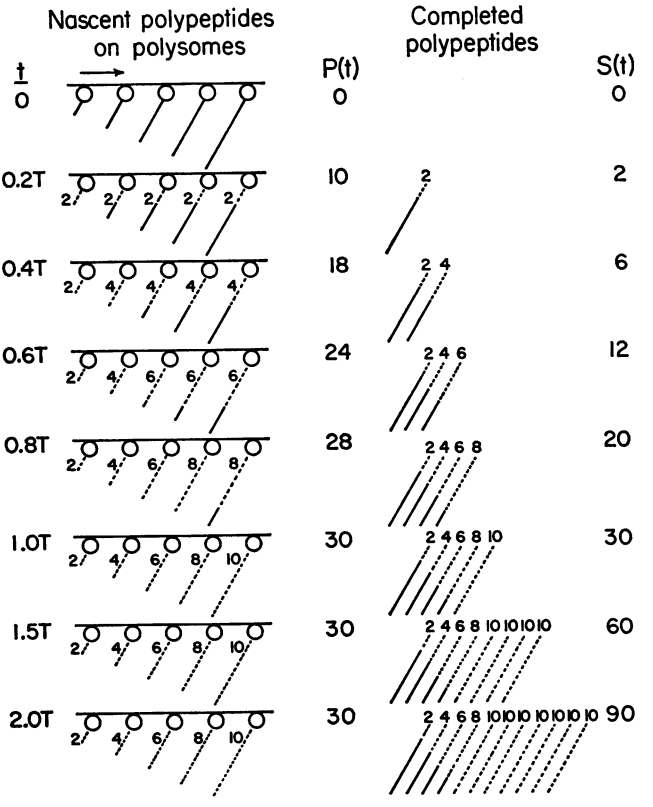


FIGURE 3 A model of polysome function showing the radioactivity (dotted lines) incorporated into nascent polypeptides on a polysome and into released completed polypeptides as a function of time (in units of the transit time, $T$) after administration of a labelled amino acid. $P(t)$ and $S(t)$ have been given values in arbitrary units assuming that $B(t)$ is constant, and that the amino acid administered is uniformly distributed throughout the protein.

small $t$ and virtually disappears at longer times (see Fig. 2). Very similar results could be obtained by simply shifting the time scale on the $P(t)$ curve for puromycin-treated cultures back about 30 sec, then calculating the ratio of these corrected puromycin $P(t)$ values to normal $P(t)$ values at corresponding times.

Since very similar values for the non-nascent fraction of radioactivity in the polysome fraction were obtained in the first two experiments, the puromycin treatment was not applied in the last two experiments. $P(t)$ values for these experiments were corrected for non-nascent radioactivity using the data of Fig. 2. This non-nascent radioactivity was presumably in ribo-

somal structural protein and perhaps in membranous structures attached to the polysomes, though there is no evidence bearing on this point.

## RESULTS

### *The Simplest Case*

The length of time required for the synthesis of a primary polypeptide of average length, the "transit time", $T$, can be obtained from measurements of the incorporation of radioactivity into nascent polypeptides $P(t)$, and into completed free polypeptides $S(t)$, as a function of time, $t$, after incubation of the cells with radioactive amino acids. Operationally, $S(t)$ represents the alkali-stable, acid-precipitable radioactivity which remains at the top of a sucrose density gradient after a short centrifugation of a cytoplasmic extract, while $P(t)$ represents radioactivity which is bound to the polysomes and ribosomes but released after a short incubation of the cells in the presence of puromycin after labelling.

A simple case based on the following assumptions, some of which are obviously unrealistic, will serve as a guide to a more sophisticated treatment to be presented later. The radioactivity which is incorporated into polypeptides will depend on the specific activity of the intracellular amino acid pool, $B(t)$. If $B(t) = b$, constant, $T$ is the same for all polysomes, all radioactivity found in completed polypeptides $S(t)$ is derived from radioactivity originally in nascent polypeptides as measured here, and the currently accepted model of polysome action applies (Warner et al., 1963), then the situation depicted in Fig. 3 pertains. For this case $P(t)$ would be expected to rise from zero, then become constant at $P(T)$ when $t = T$. $S(t)$ should equal $P(t)$ at $t = T$ but continue to rise linearly for $t \geqq T$ (see Fig. 3). Three estimates of the transit time emerge from this model:

$$\frac{P(t)}{S'(t)^*} = \frac{T}{2} \quad \text{for} \quad t \geqq T \tag{1}$$

$$P(t) \text{ becomes constant at } t = T \tag{2}$$

$$P(t) = S(t) \text{ at } tc = T \text{ where } tc \text{ is the time of intersection of } P(t) \text{ and } S(t). \tag{3}$$

* The prime notation in this paper represents the first derivative. The double prime notation represents the secord derivative. That is

$$S'(t) = \frac{dS(t)}{dt} \quad \text{and} \quad S''(t) = \frac{d^2S(t)}{dt^2}.$$

Relationship 1 has been used by others (Conconi et al., 1966) and can be stated: one-half a transit time is required to release one complement of polysome-bound nascent polypeptides for times after all such nascent polypeptides are uniformly labelled. This can be seen to be true from Fig. 3.

In view of the assumptions involved it was not surprising that experimental data did not conform to this model. Fig. 4 shows $P(t)$ and $S(t)$ from a typical experiment. It is evident that the curves intersect at about 30 sec, long before $P(t)$ becomes constant (at about 5 min). Two possible explanations for this contradiction come to mind: either the specific activity of the amino acid pool rises and does not become constant until long after a transit time, or the polypeptides which enter the $S(t)$ fraction are chiefly derived from some other source than the $P(t)$ fraction. If the

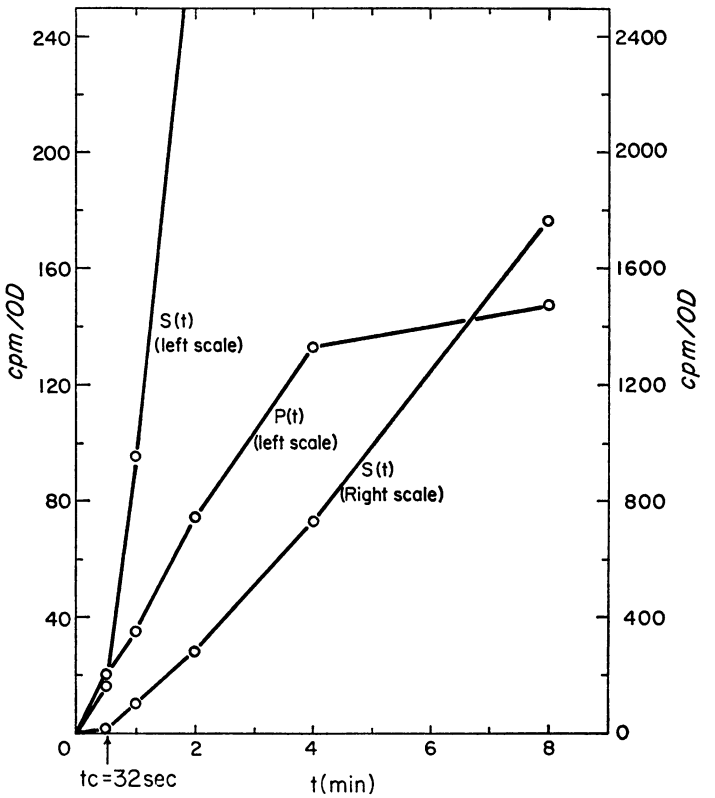


FIGURE 4 Values for $P(t)$ and $S(t)$ from experiment 2 as a function of time after administration of $^{14}$C-valine. The $S(t)$ values have been plotted using two different ordinate scales.

first explanation (the "pool" explanation) held, then $P(t)$ and $S'(t)$ would continue to rise after $t = T$, and the curves would intersect at $tc > T$ since $P(T)$ would be greater than $S(T)$. (A time lag exists between the incorporation of label in nascent polypeptides and the appearance in completed polypeptides; thus the nascent polypolypeptides will have been synthesized from a pool of higher specific activity.) It is intuitively obvious, however, that $tc$ would not be much greater than $T$, since polypeptides cannot accumulate in $P(t)$ as they can in $S(t)$. This explanation is thus

consistent with the observations and would indicate that the transit time is less than 30 sec. The second explanation predicts that $P(t)$ and $S(t)$ would intersect long before $T$ since $S(t)$ is artificially large, being "fed" chiefly from some other source of nascent polypeptides. This explanation is unlikely because an average of only 10% of the total alkali-stable, acid-precipitable radioactivity incorporated by the cells was recovered in fractions (nuclei plus sucrose gradient pellet) not included in $P(t)$ and $S(t)$ for all times up to 20 min. The following consideration will show that the second explanation alone cannot account for the observed behaviour of $P(t)$ and $S(t)$.

The total rate of amino acid incorporation into protein, that is $(P + S)'$, should be proportional to the specific activity of the amino acid pool, $B(t)$. $(P + S)'$ is plotted as a function of time for the data of Fig. 4 in Fig. 5. It is clear that $(P + S)'$, and

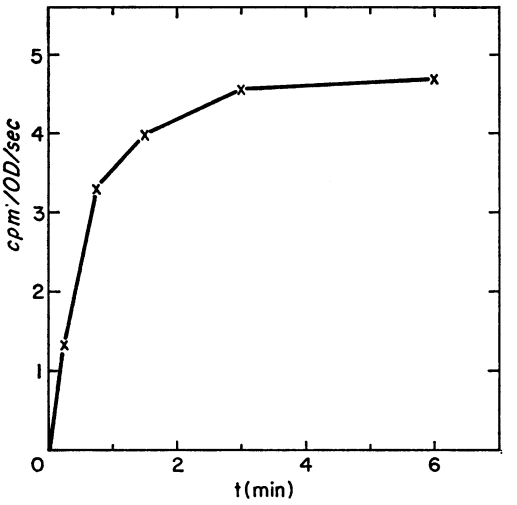


FIGURE 5 The slope of $[P(t) + S(t)]$ plotted as a function of time for the data of Fig. 4.

therefore $B(t)$, rises from zero at time zero and becomes constant only after about 5 min when, from Fig. 4, $P(t)$ also becomes constant. This observation is entirely consistent with the "pool" explanation of Fig. 4. A more quantitative treatment of the data is obviously necessary, however, and in order to do this, mathematical expressions for $P(t)$ and $S(t)$ which include the variation in $B(t)$ must be derived. These expressions could then be applied to the experimental data, and could be used to check assumptions and to yield values for $T$.

*The Derivation of $B(t)$*

$B(t)$ can be obtained empirically from experimental data since it is directly proportional to $(P + S)'$. In order to express $P(t)$ and $S(t)$ mathematically, however, it is necessary to obtain a mathematical expression for $B(t)$. It is important to realize

that the cultures were incubated in medium containing normal concentrations of the amino acids, and that the amount of radioactive valine added represented only $\frac{1}{20}$ or less of the total valine present. The addition of labelled valine should, therefore, have little or no effect on the distribution of total valine between cells and medium. It is seen from Fig. 5 that $(P + S)'$ became constant, and therefore, the valine pool reached its maximum specific activity within 5 min after addition of labelled valine. Since the cells would require about 1 to 2 hr to incorporate all the valine present in the intracellular pool into protein (Taylor, 1967),[3] the valine pool must become labelled by a rapid exchange of valine between cells and medium (Britten and McClure, 1962), rather than by uptake of valine to supply the need for protein synthesis. Also the size of the total cellular valine pool is small compared to the total amount of valine in the medium (Taylor, 1967). If the final specific activity of the cells plus medium is $b$ cpm/mole, the total pool size is constant at $d$ mole, and the total exchange rate is $a$ mole/sec, then the rate of change of $B(t)$ is given by:

$$\frac{dB}{dt} = \frac{ab}{d} - \frac{aB}{d},$$

where the first term, $\frac{ab}{d}$, represents the entry into the cellular pool of valine molecules at specific activity $b$ *from* the medium, and the second term, $-\frac{aB}{d}$, represents the loss (at equal rate) of valine molecules in the cellular pool at specific activity $B$ *to* the medium. This equation has the solution:

$$B(t) = b(1 - e^{-\lambda t}), \qquad \lambda = \frac{a}{d} \tag{4}$$

where $\frac{1}{\lambda}$ is the pool "time constant." Equation 4 can be rearranged:

$$\frac{b - B(t)}{b} = e^{-\lambda t} = \frac{(P + S)'_{max} - (P + S)'}{(P + S)'_{max}}.$$

Thus a semilogarithmic plot of the difference between the maximum slope of $(P + S)$ and the slope of $(P + S)$ at time $t$ divided by the maximum slope of $(P + S)$, against $t$ should give a straight line with a slope of $-\lambda$. Such plots for two of four experiments in which $(P + S)'_{max}$ was determined are shown in Fig. 6. The data give approximate values of 90 and 120 sec for the pool time constant, but show quite large fluctuations. Since replicate, but separate, cultures were used to measure $(P + S)$, and any culture to culture variation would be magnified in taking the first derivative, these fluctuations are perhaps not surprising. Regardless of whether the

[3] Taylor, J. M. 1967. Unpublished data.

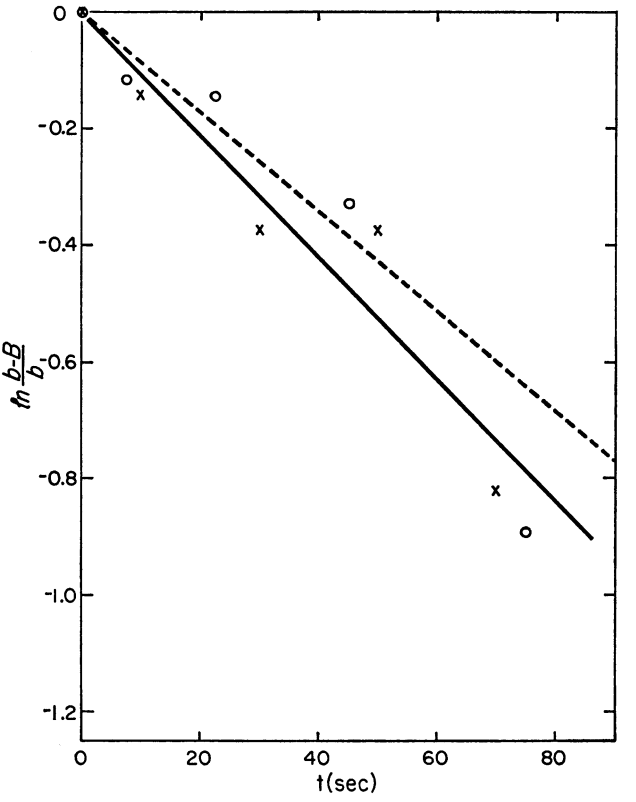


FIGURE 6 The value of $\ln\left\{\frac{b - B(t)}{b}\right\}$ or $\ln\left\{\frac{(P + S)'_{max} - (P + S)'}{(P + S)'_{max}}\right\}$ plotted against time, $t$, for experiment 3 (crosses) and for experiment 4 (circles). The full and broken lines are subjective fits to the data of experiment 3 and experiment 4 respectively.

fluctuations are due to experimental error, or to intrinsic variation in $\lambda$ with time or from culture to culture, it is apparent that $\lambda$ is difficult to determine accurately.

*The Derivation of $P(t)$ and $S(t)$*

Assume that: $T$ is the same for all polysomes, all of the radioactivity in $S(t)$ was originally derived from $P(t)$, and the currently accepted model for the polysome as depicted in Fig. 3 applies. Let $k$ be the number of valine molecules incorporated into polypeptides per sec per length of mRNA by all of the polysome-bound ribosomes in the cells. Assume that $k$ is the same for all such ribosomes. If the length of the mRNA is expressed in time units as the length of time required for a ribosome to translate it, and all mRNA has a single "length" of $T$ sec (first assumption above), then the position of a ribosome on the mRNA can be expressed as $\tau$ seconds, where $0 \leqq$

$\tau \leqq T$. For $t \geqq T$, $P(t)$ can be expressed:

$$P(t) = k \int_0^T \int_{t-\tau}^{t} B(x)\, dx\, d\tau. \tag{5}$$

The integral from $t - \tau$ to $t$ represents the sum of the radioactivity at time $t$ in a nascent chain at position $\tau$ on the mRNA. The integral from 0 to $T$ merely sums the radioactivity in such chains at all positions from 0 to $T$. Similarly, for $t \geqq T$,

$$S(t) = k \int_0^T \int_0^{\tau} B(x)\, dx\, d\tau + k \int_T^t \int_{y-T}^{y} B(x)\, dx\, dy \tag{6}$$

where $y \geqq T$ is the time at which a completed polypeptide was released from the polysome. The first double integral in relationship 6 sums the radioactivity in partially labelled complete polypeptides released from time 0 to $T$. The second double integral sums radioactivity in fully-labelled complete polypeptides released from time $y = T$ up to time $y = t$.

*Expressions for T*

In order to determine $T$, equations 5 and 6 must be expressed in a form in which $T$ is given explicitly in terms of the experimentally measured quantities, $P(t)$, $S(t)$, and $t$. As a guide in this arithmetic, it is useful to recall relationships 1 and 3 which were derived for the simple case, $B(t) = b$. Two of the following expressions for $T$ in which more complex expressions for $B(t)$ are employed will essentially use the concepts embodied in relationships 1 and 3.

*Use of Relationship 1.* Using expression 4 for $B(t)$ in expressions 5 and 6 and integrating one obtains, for $t \geqq T$

$$P(t) = kb\left[\frac{T^2}{2} + \frac{T}{\lambda} e^{-\lambda t} - \frac{e^{-\lambda(t-T)}}{\lambda^2} + \frac{e^{-\lambda t}}{\lambda^2}\right] \tag{7}$$

$$S(t) = kb\left[Tt - \frac{T^2}{2} - \frac{T}{\lambda} + \frac{e^{-\lambda(t-T)}}{\lambda^2} - \frac{e^{-\lambda t}}{\lambda^2}\right]. \tag{8}$$

Taking the first derivative of $P(t)$ and the second derivative of $S(t)$ and dividing:

$$\frac{P'(t)}{S''(t)} = \frac{(e^{\lambda T} - 1)1/\lambda - T}{e^{\lambda T} - 1}, \qquad \text{for } t \geqq T, \text{ or}$$

$$\frac{P'(t)}{S''(t)} \simeq \frac{T}{2}, \text{ for } \lambda T \text{ small.} \tag{9}$$

The assumption that $\lambda T$ is small merely requires that the transit time be small relative to the pool time constant. Thus, if the assumption can be justified experimentally, the ratio of the slope of $P(t)$ and the slope of $S'(t)$ should be constant for experi-

mental data and should yield a value for $T$. When applied to the data obtained here, however, rather large fluctuations were found for this ratio, in spite of the fact that the assumption seemed justified. The rather large variation in values obtained for $T$ was probably due to experimental errors in $P(t)$ and $S(t)$ which were inordinately magnified in taking first and second derivatives. The other possibility, that the assumptions involved in the mathematical analysis are incorrect, will be considered in the Discussion. The method is included here for its possible use in systems where more precise values of $P(t)$ and $S(t)$ can be obtained.

For large $\lambda t$, it can be shown from equations 7 and 8 that

$$\frac{P(t)}{S'(t)} = \frac{T}{2}.$$

This result confirms the fact that for long times, when $B(t) \simeq b$, equations 7 and 8 can be shown to yield relationship 1. Also from equations 7 and 8 it can be seen that under these conditions $P(t)$ and $S'(t)$ become constant, in agreement with the logic presented above. Relationship 1 has been used by Conconi et al. (1966) to calculate transit times for rabbit reticulocytes. It could not be applied successfully here, however, because $P(t)$ was difficult to determine accurately at long times where $B(t)$ was constant. This was due to the fact that the fraction of polysome-bound radioactivity which was not released by puromycin became rather large for such times (see Fig. 2) and to the fact that, at long times, when $S(t)$ was much larger than $P(t)$, small contaminations of the $P(t)$ fraction with material from the $S(t)$ fraction lead to rather large errors in $P(t)$.

*Use of Computer Techniques.* An iterative procedure for determining the coefficients of nonlinear equations as described by Scarborough (1958) was applied to equations 7 and 8 using the data shown in Table I. Three unknown coefficients: $kb$, $\lambda$, and $T$ in the equations were evaluated. The iteration was carried out with the aid of a computer, which, for each experiment, essentially chose the values for the coefficients which gave a minimum deviation by least squares fit between calculated values of $P(t)$ and $S(t)$ using equations 7 and 8 and the experimental values of $P(t)$ and $S(t)$. The "best" values of $kb$, $\lambda$, and $T$ are shown for each experiment in Table I, along with the calculated values of $P(t)$ and $S(t)$. The deviation between calculated and experimental values is often considerable, indicating that the agreement between experiment and theory leaves something to be desired. This matter will be considered further in the Discussion. The mean value for $T$ for the four experiments when evaluated by this technique was 41 $\pm$ 7 sec. I am indebted to Dr. R. G. Baker of this Institute for suggesting this technique and for carrying out the computer calculations.

*Use of Relationship 3.* Relationship 3 states that for $B(t) = b$, the time of intersection of $P(t)$ and $S(t)$, $tc$, is equal to $T$. This relationship does not hold for $B(t)$ variable. In order to obtain the expression relating $T$ and $tc$ using equation 4

TABLE I

$P(t)$, $S(t)$ AND CALCULATED VALUES OF $T$, $\lambda$ AND $kb$ USING ITERATIVE FIT TO EQUATIONS 7 AND 8

| Experiment No. | $t$ | $P(t)$* Experimental | $S(t)$* Experimental | $P(t)$ Calculated | $S(t)$ Calculated | $T$ | $\lambda$ | $kb$ |
|---|---|---|---|---|---|---|---|---|
| | *sec* | *cpm/OD* | *cpm/OD* | *cpm/OD* | *cpm/OD* | *sec* | $sec^{-1}$ | *cpm/OD/sec* |
| 1 | 25 | 9.1 | 4.9 | 6.4 | 2.2 | | | |
| | 40 | 15.5 | 17.6 | 13.2 | 8.1 | | | |
| | 80 | 30.9 | 55.0 | 29.1 | 50.5 | 36 | 1/170 | 0.135 |
| | 150 | 59.3 | 191 | 49.1 | 198 | | | |
| | 300 | 63.8 | 710 | 71.9 | 708 | | | |
| 2 | 30 | 20.1 | 16.5 | 27.7 | 12.5 | | | |
| | 60 | 35.3 | 95.3 | 72.4 | 56.2 | | | |
| | 120 | 74.5 | 281 | 101 | 257 | 51 | 1/37 | 0.082 |
| | 240 | 132.8 | 733 | 107 | 749 | | | |
| | 480 | 147.2 | 1,760 | 107 | 1,759 | | | |
| 3 | 20 | 8.5 | 7.3 | 9.1 | 10.9 | | | |
| | 40 | 26.8 | 26.4 | 48.2 | 22.1 | | | |
| | 60 | 37.8 | 52.8 | 73.6 | 66.7 | 45 | 1/48 | 0.118 |
| | 80 | 53.8 | 106 | 90.2 | 133.1 | | | |
| | 600 | 137 | 2,890 | 122 | 2,841 | | | |
| | 1,200 | 154 | 6,030 | 122 | 6,052 | | | |
| 4 | 15 | 10.2 | 12.4 | 9.6 | 10.5 | | | |
| | 30 | 25.2 | 24.8 | 48.5 | 22.6 | | | |
| | 60 | 64.4 | 85.5 | 90.9 | 136 | 34 | 1/36 | 0.219 |
| | 90 | 97.1 | 210 | 109 | 310 | | | |
| | | | | | | Average 41 ± 7 | | |
| | 600 | | 4,180 | | 4,026 | | | |
| | 1,200 | | 8,370 | | 8,438 | | | |

* Approximately 10 OD units were recovered per fraction. The approximate cpm in $P(t)$ and $S(t)$ can thus be obtained by multiplying these values by 10.

for $B(t)$, equation 7 for $P(t)$ is set equal to equation 8 for $S(t)$. Rearranging terms yields the expression:

$$T - \frac{2}{\lambda^2} e^{-\lambda tc} \frac{e^{\lambda T} - 1}{T} = tc - \frac{1 - e^{-\lambda tc}}{\lambda} \tag{10}$$

which can be solved for $T/tc$ as a function of $\lambda tc$, by setting $tc = 1$, and finding the value of $T$ which satisfies the expression for each value of $\lambda$. The solution can be worked out by iteration and is shown in Fig. 7. It can be seen that $T/tc$ has a minimum value of 0.64 for $\lambda tc = 0.1$ and rises to a maximum of 0.99 for $\lambda tc = 100$.

Thus $T/tc$ is not a strong function of $\lambda$. This is a rather important result since $\lambda$ is difficult to determine accurately. Practically speaking, the value of this treatment can be seen from the close agreement in four separate experiments of the values of $T$ obtained from measured values of $tc$ and $\lambda$, and the application of Fig. 7 (see Table II). The average value of $T$ was found to be 23 sec with a maximum absolute variation of 3 sec. With this treatment, though the values of $\lambda$ do show considerable variation from experiment to experiment, the value of $\lambda$ had very little effect on the calculated value of $T$ (see Fig. 7 and Table II). In fact, if the values of $\lambda$ calculated by the computer technique (Table I), which are quite different from those employed in Table II, are used in this technique, the average value of $T$ changes only from 23 sec

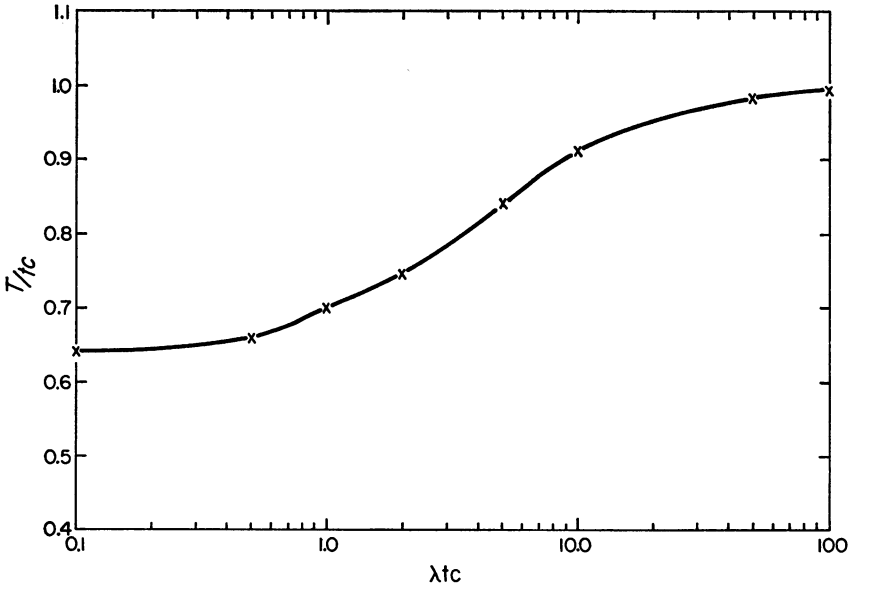


FIGURE 7 A theoretical curve relating $T/tc$ to $\lambda tc$ according to equation 10 of the text.

to 24 sec. The much improved precision obtained by this method over that obtained by the methods outlined above will be considered further in the Discussion.

The measurement of $tc$ would appear to be difficult since $P(t)$ and $S(t)$ intersect at very early times when the activities of the polysome and supernatant fractions are quite low. In practice, however, this measurement is relatively precise because $S(t)$ values increase very rapidly above $P(t)$ values for $T > tc$; $tc$ can thus be estimated as the point of rapid divergence of the two curves.

In view of the good results obtained with this treatment, re-examination of the assumptions involved in the derivation of equation 10 would seem worthwhile. Equation 10 was derived from equations 7 and 8 which, in turn, were derived from expressions 5 and 6. The assumptions used in the derivation of expressions 5 and 6

TABLE II

VALUES OF $T$ CALCULATED FROM TIME OF INTERSECTION OF $P(t)$ AND $S(t)$

| Experiment No. | $tc$ | $\lambda$* | $\lambda tc$ | $T/tc$ from Fig. 7 | $T$ |
|---|---|---|---|---|---|
| | *sec* | $sec^{-1}$ | | | *sec* |
| 1 | 34 | 1/130 | 0.26 | 0.65 | 22 |
| 2 | 32 | 1/110 | 0.29 | 0.65 | 21 |
| 3 | 40 | 1/110 | 0.36 | 0.65 | 26 |
| 4 | 32.5 | 1/200 | 0.16 | 0.64 | 21 |
| Average | | | | | 23 ± 2 |

* Calculated from the initial slope of $(P + S)'$ vs. time.

are given at the beginning of the section devoted to the derivation of $P(t)$ and $S(t)$. The first assumption, that $T$ is constant, seems particularly unrealistic. A myriad of different proteins with different numbers of amino acid residues in their primary sequences are most probably synthesized by these growing cells. It is also possible that variations exist in the rate of assembly of different polypeptides of the same length according to the availability of charged sRNA species corresponding to their messenger RNA's or perhaps to their topography within the cell. Such variation would invalidate the assumption that $k$ in expressions 5 and 6 is constant. Both processes would lead to a distribution of transit time values. In order to determine what relationship the value of $T$ calculated from $tc$ values has to this distribution, the following analysis was developed.

First consider variation in $T$. Since $\lambda tc$ is relatively small experimentally (see Table II), the approximation $B = b\lambda t$ of expression 4 seems valid for $t$ near $tc$. This approximation can also be justified by the fact that both $P(t)$ and $S(t)$ are affected by $B(t)$ and, since the treatment involves equating these functions, the form of $B(t)$ is not critical. If the distribution of transit times is given by $\{Tj\}$, where $ni$ polysomes of a total of $N$ polysomes have transit time $Tj$, the radioactivity in nascent and completed polypeptides, $Pj(t)$ and $Sj(t)$ is given by:

$$Pj(t) = \frac{nj}{N} k \int_0^t \int_{t-\tau}^t B(x)\, dx\, d\tau + \frac{nj}{N} k \int_t^{Tj} \int_0^t B(x)\, dx\, d\tau$$

$$= \frac{nj}{N} \frac{k\lambda b}{6} (3t^2 Tj - t^3) \text{ for } t \leqq Tj; \text{ and}$$

$$Pj(t) = \frac{nj}{N} \frac{k\lambda b}{6} (3tTj^2 - Tj^3) \text{ for } t \geqq Tj \text{ from equation 5.}$$

$$Sj(t) = \frac{nj}{N} k \int_0^t \int_0^\tau B(x)\, dx\, d\tau$$

$$= \frac{nj}{N} \frac{k\lambda b}{6} t^3 \text{ for } t \leqq Tj; \text{ and}$$

$$S_j(t) = \frac{nj}{N} \frac{k\lambda b}{6} (3t^2Tj - 3tTj^2 + Tj^3) \text{ for } t \geqq Tj \text{ from equation 6.}$$

The expressions for $Pj(t)$ and $Sj(t)$ for $t \leqq Tj$ were deduced by application of the principles, described in detail above, used in the derivation of equations 5 and 6. The total radioactivity $P(t)$ and $S(t)$ is given by:

$$P(t) = \sum_{Tj=0}^{t} \frac{nj}{N} \frac{k\lambda b}{6} (3t^2Tj - t^3) + \sum_{Tj=t}^{\infty} \frac{nj}{N} \frac{k\lambda b}{6} (3tTj^2 - Tj^3)$$

$$S(t) = \sum_{Tj=0}^{t} \frac{nj}{N} \frac{k\lambda b}{6} t^3 + \sum_{Tj=t}^{\infty} \frac{nj}{N} \frac{k\lambda b}{6} (3t^2Tj - 3tTj^2 + Tj^3).$$

At $t = tc$, $P(t) = S(t)$, which yields:

$$\sum_{Tj=0}^{tc} \frac{nj}{N} \left(3 \frac{Tj}{tc} - 2\right) + \sum_{Tj=tc}^{\infty} \frac{nj}{N} \left(6 \frac{Tj^2}{tc^2} - 3 \frac{Tj}{tc} - 2 \frac{Tj^3}{tc^3}\right) = 0.$$

More simply,

$$\sum_{\phi j=0}^{1} \frac{nj}{N} (3\phi j - 2) + \sum_{\phi j=1}^{\infty} \frac{nj}{N} (6\phi j^2 - 3\phi j - 2\phi j^3) = 0,$$

where $\phi j = \frac{Tj}{tc}$. This expression can be rearranged to:

$$\sum_{\phi j=0}^{\infty} \frac{nj}{N} (3\phi j - 2) + \sum_{\phi j=1}^{\infty} \frac{nj}{N} (6\phi j^2 - 6\phi j - 2\phi j^3 + 2) = 0. \qquad (11)$$

Since the mean value of $Tj$ is probably less than $tc$ (see argument under The Simplest Case), the distribution of $\phi j$'s probably has its peak at some $\phi j < 1$. Consequently, the second term in equation 11 which starts at $\phi j = 1$ is a sum over just a fraction of the $\phi j$ distribution. The bracketed expression in this term starts at zero for $\phi j = 1$ and remains very small until $\phi j = 2$ when it becomes half (though negative) of the value of the bracketed expression of the first term; as $\phi j$ increases its negative value increases rapidly so that at $\phi j = 5$ it is 10 times as large as the bracketed expression of the first term. Thus, if the distribution of $\phi j$'s is not skewed toward large values, that is, if $\phi j \simeq 5$ or greater is relatively improbable, (or has small $nj$), then the contribution of the second term to equation 11 will be relatively small.

This assumption seems justified since a polypeptide for which $\phi j = 5$ would have to be more than five times longer than the average polypeptide, that is, more than 1000 to 1500 amino acid residues long if the average length is 200 to 300 amino acid residues. To a first approximation, then, equation 11 can be written:

$$\sum_{\phi j=0}^{\infty} \frac{nj}{N} (3\phi j - 2) = 0,$$

or,

$$\sum_{\phi j=0}^{\infty} \frac{nj}{N} \phi j = \frac{2}{3} \sum_{\phi j=0}^{\infty} \frac{nj}{N}$$

or,

$$\bar{\phi} = \frac{2}{3} = \frac{\bar{T}}{tc}. \tag{12}$$

This is very close to the value of $T/tc$ obtained from equation 10 for small $\lambda tc$ (see Fig. 7). Thus, to a first approximation, the treatment for estimating $T$ using values of $tc$ actually yields the mean value of a distribution of $T$ values.

It can be seen from equation 12 that for small $\lambda tc$, $T/tc$ is independent of $\lambda$. Thus, regardless of how slowly the amino acid pool equilibrates with the medium, as long as the increase in specific activity of the pool is linear with time, $\bar{T}/tc$ will be given by equation 12.

Consider next a variation from polysome to polysome in $k$, which is precisely defined in the section entitled "The Derivation of $P(t)$ and $S(t)$." Roughly speaking, $k$ represents the rate at which valine molecules are incorporated into polypeptides. If the distribution of $k$'s is completely independent of the distribution of $T$'s, that is, if polysomes which incorporate valine slowly have the same distribution of messenger lengths as do polysomes which incorporate valine quickly, then equation 12 would apply for each $k$ in the distribution of $k$'s and would therefore hold unchanged. If, however, the distribution of $k$'s depends on the distribution of $T$'s, that is, if for every $Tj$ there corresponds a different average $kj$, then equation 11 would become:

$$\sum_{\phi j=0}^{\infty} \frac{nj}{N} kj(3\phi j - 2) = 0,$$

if the assumptions discussed below equation 11 still held. This equation can be written:

$$\sum_{\phi j=0}^{\infty} \frac{nj}{N} kj\phi j = \frac{2}{3} \sum_{\phi j=0}^{\infty} \frac{njkj}{N}$$

or,

$$\overline{k\phi} = \frac{2}{3}\bar{k}$$

or,

$$tc = \frac{3}{2}\frac{\overline{kT}}{\bar{k}}. \tag{13}$$

Thus, if for every transit time there is a different $k$, this method for estimating transit times would yield a weighted average of the transit time distribution, and polysomes which produce polypeptides at a greater rate would have a greater influence on the transit time estimate in proportion to this rate.

Though the situation leading to equation 13 is considered to be biologically unlikely, it is possible, for example, that ribosomes translate messenger RNA bases more quickly on short polysomes than on long polysomes. This modification of equation 12 is included to cover such an eventuality.

## DISCUSSION

The foregoing treatment provides a means of evaluating the mean transit time ($T$) of ribosomes across messenger RNA molecules in the polysomes of a rapidly proliferating animal cell. In essence the treatment involves a comparison of the amount of radioactivity in nascent polypeptides on polysomes with the amount in completed polypeptides as a function of time after exposure of the cells to a labelled amino acid. A simple treatment of this data, which is similar in some respect to treatments used by others (Conconi et al., 1966) was found to be inadequate because the specific activity of the intracellular amino acid pool took much longer than a transit time to equilibrate with the external medium. Mathematical derivations of the transit time in terms of the measured amino acid incorporations were developed which took into account the variation in the specific activity of the cellular amino acid pool. One derivation yielded a wide range of values for $T$ within a single experiment and between different experiments. A second derivation which involved an iterative fit of experimental data to theoretical equations using a series of values for three unknown parameters including $T$, gave an average value of 41 $\pm$ 7 sec for $T$, but the fit obtained was poor in three out of four experiments. A third derivation, which involved the measurement of the time at which the activity in nascent polypeptides and in completed polypeptides was equal, yielded closely reproducible values for $T$: 23 $\pm$ 2 sec.

The success of the third method relative to the first two methods could be ascribed to the following causes. Since HA cells will not grow in suspension culture using present tissue culture techniques, replicate monolayer cultures were used for each

experimental point. This necessity naturally introduces culture to culture variation which could greatly decrease the precision of the determinations. The first method for determining $T$ required taking first and second derivatives of the data, a procedure which would inordinately magnify variations. The second method actually averaged all the data from the various cultures in any one experiment, but nevertheless required comparison of values obtained between different cultures. The third method, on the other hand, essentially compared activities in two fractions obtained *from the same culture*, which would eliminate culture to culture variation. A second possible reason for the better precision of the third method is that it is quite insensitive to variations in the speed with which the intracellular amino acid pool equilibrates with the medium. This speed, which is characterized by the parameter $\lambda$, is difficult to measure precisely, and could vary considerably from culture to culture. Both of the first two methods are strongly dependent on the value of $\lambda$.

The third method thus emerges as a precise technique for measuring the transit time and could be extremely useful where comparative values for the transit time in cells under different conditions are desired. The values obtained cannot be accepted as absolute, however, because the failure of the first two methods to yield reproducible values for the transit time does not allow a critical evaluation of the assumptions involved in the mathematical development. For example, if some fraction of the radioactivity in completed polypeptides were derived from a source other than polysomes as isolated here, such as nuclei or "lost" polysomes, then, as explained in the Results, the "precise" value of $23 \pm 2$ sec for the transit time could be small by a factor equal to this fraction. The latter fraction is probably not large, however, as less than 10% of the total radioactivity incorporated by the cell was found in cell fractions not included in nascent or completed polypeptides. The assumption that the commonly accepted model of the polysome, as depicted in Fig. 3, applies is a more serious one. No precise information can be invoked to justify it, but the model cannot be grossly wrong as can be seen from the way in which the data roughly coincides with its general precepts. For example, the radioactivity in nascent polypeptides reached a constant value at about the time the specific activity of the amino acid pool reached its final constant level (see Figs. 4 and 5). Also, the values of the transit time calculated by the different methods were not grossly different e.g. $41 \pm 7$ sec by the second method versus $23 \pm 2$ sec by the third.

It is important to realize that the transit time is a measure of translation independent of transcription. Simple measurements of total amino acid incorporation can depend both on translation and on the amount of messenger RNA in the cell. The methods outlined in this paper could therefore be very useful in separating the effects of various agents or of different physiological states on translation and on transcription.

I thank Mr. P. Ottensmeyer, Dr. R. G. Baker, Dr. G. F. Whitmore, and Dr. J. E. Till for useful discussion and criticism during the formulation of the mathematical treatment. Mr. G. McKenzie

and Mr. W. Mehring rendered capable technical asistance in the execution of the experiments. This research was supported by grant MA-1877 from the Medical Research Council of Canada and by the National Cancer Institute of Canada.

*Received for publication 19 July 1967.*

## REFERENCES

ABERCROMBIE, M., and J. E. M. HEAYSMAN. 1954. *Exptl. Cell Res.* **6**:293.
BAUTZ, E. F. K., T. KASAI, E. REILLY, and F. A. BAUTZ. 1966. *Proc. Natl. Acad. Sci. U. S.*, **55**:1081.
BRITTEN, R. J., and F. T. MCCLURE. 1962. *Bacteriol. Rev.* **26**:13.
COLOMBO, B., L. FELICETTI, and C. BAGLIONI. 1965. *Biochem. Biophys. Res. Commun.* **18**:389.
CONCONI, F. M., A. BANK, and P. A. MARKS. 1966. *J. Mol. Biol.* **19**:525.
DINTZIS, H. M., H. BORSOOK, and J. VINOGRAD. 1958. *In* Microsomal Particles and Protein Synthesis. Pergamon Press, Inc., N. Y. 65.
DULBECCO, R., and M. VOGT. 1954. *J. Exptl. Med.* **99**:167.
GOLDSTEIN, A., D. B. GOLDSTEIN, and L. I. LOWNEY. 1964. *J. Mol. Biol.* **9**:213.
GROSS, P. R., L. I. MALKIN, and W. A. MOYER. 1964. *Proc. Natl. Acad. Sci. U. S.* **51**:407.
KANO-SUEOKA, T., and N. SUEOKA. 1966. *J. Mol. Biol.* **20**:183.
MARKS, P. A., E. R. BURKA, F. M. CONCONI, W. PERL, and R. A. RIFKIND. 1965. *Proc. Natl. Acad. Sci. U. S.* **53**:1437.
MCAUSLAN, B. R. 1963. *Virology.* **21**:383.
NATHANS, D. 1964. *Proc. Natl. Acad. Sci. U. S.* **51**:585.
PENMAN, S., K. SCHERRER, Y. BECKER, and J. E. DARNELL. 1963. *Proc. Natl. Acad. Sci. U. S.* **49**:654
READER, R. W., and C. P. STANNERS. 1967. *J. Mol. Biol.* **28**:211.
SALB, J. M., and P. I. MARCUS. 1965. *Proc. Natl. Acad. Sci. U. S.* **54**:1353.
SCARBOROUGH, J. B. 1958. *In* Numerical Mathematical Analysis. 4th edition. The Johns Hopkins Press, Baltimore, Md.
TAYLOR, J. M., and C. P. STANNERS. 1967. *Biochim. Biophys. Acta.* **138**:627.
VOGT, M., and R. DULBECCO. 1960. *Proc. Natl. Acad. Sci. U. S.* **46**:365.
WARNER, J. R., P. M. KNOPF, and A. RICH. 1963. *Proc. Natl. Acad. Sci. U. S.* **49**:122.