CELL AND TISSUE KINETICS

Edited by R. J. Berry

Volume 11, 1978

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GROWTH FRACTION AND CYCLE DURATION OF HEPATOCYTES IN THE THREE-WEEK-OLD RAT

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(*Received* 7 *March* 1977; *revision received* 17 *August* 1977)

ABSTRACT

The proliferation of hepatocytes in the liver of 3-week-old rats has been investigated by autoradiographic methods. This investigation is a continuation of earlier work on the same topic (Schultze & Maurer, 1972; 1973). 21 days after birth, 102 rats received a single injection of ^{3}H-TdR. The percentage of labelled mitoses was then determined 1 hr later and at various times throughout the interval up to 12 days after application of ^{3}H-TdR. In agreement with earlier work, a first peak of labelled mitoses was found 7 hr after ^{3}H-TdR injection. The area under the peak indicates an S phase duration of 8 hr. In addition a second very broad peak of labelled mitoses was found between 2 and 12 days after pulse labelling. The analysis of the results leads to the conclusion that the hepatocytes of the 3-week-old rat have a growth fraction close to 1 and a doubling time of 6–7 days. This is at variance with earlier results of Post, Huang & Hoffman (1963) and Grisham (1969) who had derived a value of 21·5 hr for the duration of the cell cycle and a value of only 0·1–0·2 for the growth fraction of the hepatocytes.

Post *et al.* (1963) used the method of labelled mitoses to investigate the proliferation of hepatocytes in the 3-week-old rat. They found a first peak of labelled mitoses 7 hr after ^{3}H-TdR injection and a second peak of labelled prophases about 30 hr after ^{3}H-TdR injection. The striking fact that the second peak contained no labelled meta-, ana- or telophases remained unaccounted for, and the authors proceeded to derive from their data a cell cycle time of 21·5 hr. This was considerably shorter than the cycle time of 225 hr which would result from the assumption of a growth fraction of unity, the measured labelling index of 4%, and the S phase duration of 9 hr. It was, accordingly, assumed that the growth fraction is small, namely of the order of 0·1.

Grisham (1969) found for the hepatocytes in rats of age 4–5 weeks (60 g body weight) two peaks of labelled metaphases, 7 and 30 hr after ^{3}H-TdR injection. In agreement with Post *et al.*, he inferred a growth fraction of 0·1–0·2.

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0008–8730/78/0500–0241 $02.00 © 1978 Blackwell Scientific Publications Ltd

These experiments were repeated by Schultze & Maurer (1972, 1973). The existence of a first peak of labelled mitoses 7 hr after ^{3}H-TdR application was confirmed for the liver of the 3-week-old rat; however, there was no evidence of a second peak at 30 hr. Furthermore, the grain count over the interphase nucleus was reduced, during the first peak at 7 hr, by a factor of 2; after that it remained constant for the duration of the experiment (54 hr). In addition the number of labelled interphase cells increased by a factor of 2 following the first mitotic peak, thereafter it remained constant. These findings were additional evidence against the existence of a second maximum of labelled mitoses at 30 hr. As the experiment was not continued beyond 54 hr, it was merely possible to conclude that the cycle time had to be larger than 54 hr. An experiment with continuous ^{3}H-TdR infusion over 7 days did not lead to further conclusions.

For these reasons an FLM experiment over 13 days has now been carried out on the hepatocytes of 3-week-old rats. In this experiment an extremely broad (2–12 days) second peak of labelled mitoses is found with a maximum at 6–7 days. As will be shown, this implies that in the liver of the 3-week-old rat, the growth fraction is close to 1.

MATERIALS AND METHODS

Animals. 3-week-old Wistar rats of weight 41·3 ± 5·3 g were used. The animals came without exception from litters of six offspring. Larger litters were reduced to six animals and smaller litters were not used. During the experiment the animals were left with their mothers for suckling. At the same time they had access to standard food (Altromin R 10) and water *ad libitum*. Before and during the experiment the animals were kept under constant conditions in an air-conditioned room at a temperature of 23°C and with 12 hr light/dark regime.

Labelled thymidine. Thymidine-methyl-^{3}H (^{3}H-TdR; 6·7 Ci/mmol) from New England Nuclear Chemicals was used. ^{3}H-TdR was checked for radiochemical purity by thin layer chromatography.

Autoradiographic technique. After decapitation of lightly ether-anaesthetized animals, the ventral edge of the left liver lobe was removed, fixed in formalin and embedded in paraplast. Sections 3 μm thick were Feulgen stained and were dipped in Ilford K2 emulsion. The autoradiographs were then exposed at 4°C for 21 days.

Evaluation of the autoradiographs. All mitoses from early prophase to anaphase were counted. The background on the autoradiographs was less than one grain per nucleus. The labelled mitoses had a mean of twenty-four grains. To ensure that only genuinely labelled mitoses were included, no mitoses with less than four grains were counted as labelled.

EXPERIMENTAL PROCEDURES AND RESULTS

Experiments. Ninety-five rats received, on the twenty-first day after birth, a single injection of 1 μCi ^{3}H-TdR per g body weight. The animals were killed at various times between half an hour and 12·5 days after injection. Autoradiographs were prepared from the liver as described.

Results. Fig. 1 shows the observed fractions of labelled mitoses. Up to 3 days after the beginning of the experiment, each point represents two animals and at least 600 mitoses per animal. At later times (3–12 days) the points are mean values for groups of four to nine animals; on average 700 mitoses were counted per animal.

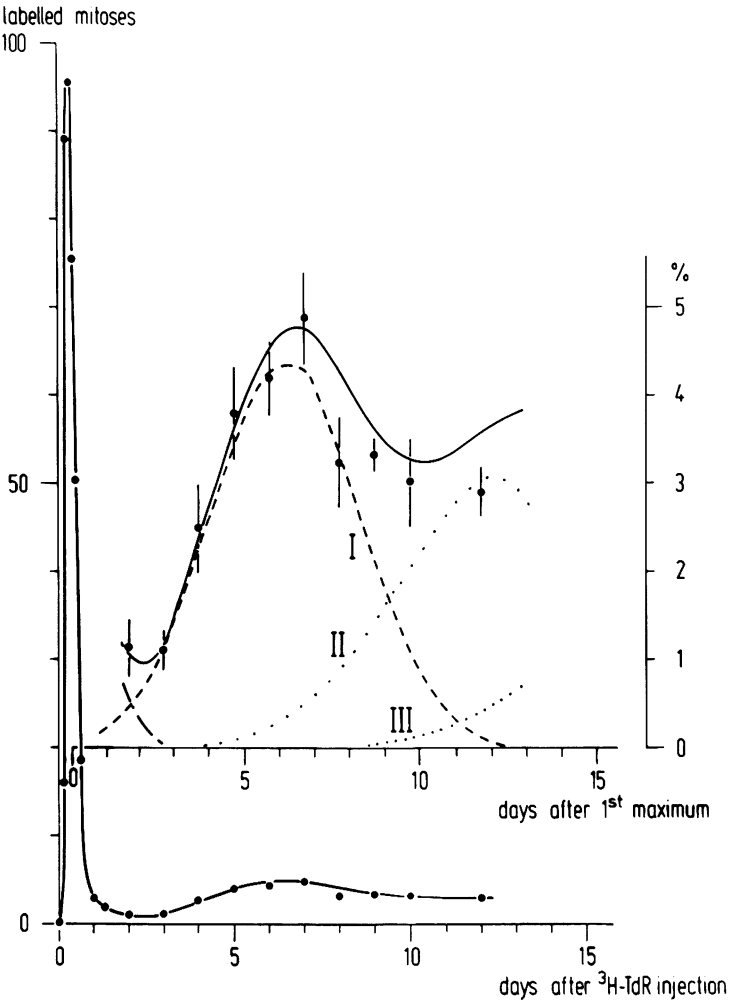

FIG. 1. Per cent labelled mitoses in hepatocytes of 3-week-old rats after a single injection of ^{3}H-thymidine. The points are observed values. The curve shows a first narrow peak at 7 hr and a second broad and flat peak at 6–7 days. The second peak is given in enlarged scale in the insert. The bars represent standard errors. The solid curve results from the superposition of three successive waves I, II, and III of labelled mitoses (see equation 14). The contributions of the three waves of labelled mitoses are represented by the broken line and the two dotted lines.

Estimation of standard errors. The standard errors of the mean in Fig. 1 are calculated in such a way that each animal carried the same statistical weight regardless of the number of mitoses counted. This corresponds to the assumption that the statistical fluctuations are predominantly due to differences between animals and not to the counting of only a limited number of cells.

The validity of this assumption was checked by comparing the standard errors in Fig. 1 with the theoretical standard errors which result from the limited cell count according to the Poisson distribution. The resulting standard errors were substantially smaller than the ones in Fig. 1. This confirms that the statistical error results mainly from the variation between animals.

First peak of the FLM-curve. The first peak in Fig. 1 occurs 7 hr after ^{3}H-TdR injection. This is in agreement with the results previously obtained (Schultze & Maurer, 1972, 1973) and also with those reported by other authors. Both from the width of the first peak at the 50% level and from the area under the peak, a value of 8 hr is obtained for the duration of the S-phase.

Second peak of the FLM-curve. The second peak is spread over a remarkably broad range from about 2 to 12 days after the application of ^{3}H-TdR, but its maximum is only 5%. This low maximum is to be expected, since it leads to approximate equality of the areas under the first and the second wave of labelled mitoses. In fact the area under the second wave, i.e. the area under the FLM-wave from 2 to 12 days is approximately equal to 8 hr. One concludes from the standard errors given in Fig. 1 that, in spite of its low maximum, the existence of the second wave is established with high statistical significance.

Labelling index. The labelling index has been determined for nine rats 21 days after birth either 30 or 60 min after ^{3}H-TdR injection. A value of $3{\cdot}22 \pm 0{\cdot}26\%$ has been obtained.

Radiation effects. It is commonly assumed that radiation effects can be disregarded in FLM-experiments if an activity of ^{3}H-TdR of 1 μCi per g body weight is given and if the duration of the experiment is 2–3 days. In the present experiment the labelled nuclei received an absorbed dose of approximately 10 rad per day, i.e. the dose up to the time of the second peak was close to 50 rad. A dose of this size, spread over several days, is unlikely to have a substantial effect on the FLM curve.

DISCUSSION

Mode of proliferation of hepatocytes in the 3-week-old rat

Before a discussion of the FLM-curve in Fig. 1, the proliferation processes in the liver of the 3-week-old rat must be considered.

Stöcker & Butter (1968) and also Grisham (1969) have found a maximum labelling index of hepatocytes (5·5% and 9%, respectively) a few days after birth. Thereafter the labelling index decreases steadily until after several months it reaches the very low value of 0·1%, which is well established for the adult rat. A decrease of about 50% occurs between the age of 21 and 45 days. The mitotic index is subject to a similar change, it is 0·6% in the 3-week-old rat and decreases to 0·04% in the adult rat. However, the S phase duration of 7–9 hr remains constant from the day of birth up to 4–6 months of age (Grisham, 1969; Schönung, 1973). One must conclude that there is in this age interval a steady decrease in the growth fraction and/or a steady increase of the cycle duration of the hepatocytes.

Various authors have reported that during the first 3 weeks of life, the liver contains only mononuclear diploid cells. At about the third week of life a small number of binuclear diploid cells appear; these cells result from mitotic divisions of mononuclear diploid cells without cytokinesis. Later these binuclear cells give rise to mono- and binuclear cells of higher ploidy (See Carrière, 1969).

First peak of the FLM-curve. The liver of the 3-week-old rat consists almost exclusively of mononuclear diploid cells. As would therefore be expected, the first peak of labelled mitoses 7 hr after ^{3}H-TdR application consists almost entirely of labelled mononuclear mitoses of diploid cells.

Second peak of the FLM-curve. The second peak of labelled mitoses represents the second mitotic division of the ^{3}H-TdR labelled mononuclear, diploid cells. This second mitotic

division gives rise to a mixture of mono- and binuclear, diploid, labelled cells. It is essential that all mitoses in this second peak are those of mononuclear diploid cells. The character of the labelled cells resulting from these divisions has no influence on the peak.

From the curve in Fig. 1 one concludes, even without detailed analysis, that at age 3 weeks the diploid hepatocytes have a mean cycle duration of about 6–7 days. According to earlier calculations (Schultze & Maurer, 1972, 1973) this corresponds to a growth fraction of 1·0.

The conclusion is consistent with the fact that the weight of the 3-week-old rat liver doubles in about 6–8 days (Schultze & Maurer, 1972, 1973). At this age interval the hepatocytes represent 88% of the liver volume (Greengard, Federman & Knox, 1972). The increased weight results therefore essentially from proliferation of the hepatocytes; their doubling time must accordingly be 6–8 days.

Determination of the growth fraction and the labelling index LI_p of hepatocytes

In this section a mathematical treatment will be given of the data represented in Fig. 1. A formal treatment is required because one cannot simply assume that the usual formulae for growth fraction and labelling index remain valid if, as in the present case, one deals with large variations in cell cycle duration. It could then be that the doubling time of the population is substantially shorter than the mean cycle duration.

There is an additional reason to present a formal analysis: it is of general interest to obtain quantitative relations for a case which is in two aspects characteristically different from the normal situation. The first aspect is the large fluctuation of cycle duration; the second aspect is that the cycle duration is very long compared with the S phase duration of 8 hr.

The growth fraction, GF, of the hepatocytes is equal to the ratio of the observed labelling index, LI, and the labelling index, LI_p, for the proliferating hepatocytes alone:

$$GF = LI/LI_p \tag{1}$$

Accordingly one determines GF from a comparison of the experimentally determined labelling index, LI, with LI_p. The value of LI_p must be derived from the data in Fig. 1.

From Fig. 1, one concludes that the durations of the S phase and of the G_2 phase are very small compared to the duration of the cycle. Accordingly the number of cells in S phase at a given time, t, is very nearly equal to the cell flow, $u(t)$, into mitosis multiplied by the duration, S, of the S phase. LI_p must therefore be equal to the quantity, $u(t)$ S, divided by the total number, $N(t)$, of proliferating cells:

$$LI_p = u(t)\,S/N(t) \tag{2}$$

Since it is assumed that the growth parameter of the proliferating cells is constant in time, one obtains the relation:

$$N(t) = N_0 e^{bt} \tag{3}$$

One may note that this relation, depending on the value of b, applies to a growing, stationary, or decreasing pool of proliferating cells.

If r is the mean number of cells which result from a mitosis and continue to proliferate, then the cell flow out of mitosis is equal to $r\,u(t)$. The rate of increase of the pool of proliferating cells is equal to the flow of proliferating cells *out of* mitosis, $r\,u(t)$, minus the flow of cells *into* mitosis, $u(t)$. One obtains therefore:

$$\frac{dN(t)}{dt} = bN_0 e^{bt} = (r-1)\,u(t) \qquad (2 \geq r > 0) \tag{4}$$

This provides a relation between the cell flow into mitosis and the total number of proliferating cells:

$$u(t) = \frac{b}{(r-1)} \quad N(t) = \frac{b}{(r-1)} N_0 e^{bt} \tag{5}$$

One can insert this result into equation (2) and obtain thus a formula for the labelling index of the proliferating cells:

$$\mathrm{LI_p} = \frac{b\mathrm{S}}{(r-1)} \tag{6}$$

In the present case it is probably justified to assume $r = 2$. Nevertheless it is desirable to obtain the rigorous formulation which applies to any value of r. To this purpose one can rewrite equation (3):

$$N(t) = N_0 r^{t/T_r} \qquad \text{with} \qquad T_r = \frac{\ln r}{b} \tag{7}$$

T_r is the time in which the proliferative pool increases by a factor r. This quantity T_r is a generalization of the concept of *doubling time*. Inserting this into equation (6) one obtains:

$$\mathrm{LI_p} = \frac{\ln r\, \mathrm{S}}{(r-1)T_r} \qquad \text{or for } r = 2: \qquad \mathrm{LI_p} = \frac{\ln 2\, \mathrm{S}}{T_2} \tag{8}$$

One could now make the simplifying assumption that T_r or T_2 is equal to the mean cycle duration which according to Fig. 1 is about 6·5 days. Then, with $r = 2$, the value $\mathrm{LI_p} = 3{\cdot}35\%$ results. From the observed labelling index LI = 3·2% one obtains, according to equation (1), a growth fraction GF = 0·9. A similar value results if r is somewhat smaller than 2. This is the essential result of the present study.

However, in view of the possible discrepancy between T_r and the observed mean cycle duration, a more rigorous treatment is desirable. This will be presented in the next section.

Determination of the doubling time from the distribution of cycle durations

For the purpose of the formal analysis the usual assumption will be made that the durations of successive cycles are uncorrelated. The distribution of cycle durations will be designated by $\mathrm{f}(T)$. Accordingly $\mathrm{f}(T)\mathrm{d}T$ is the fraction of cells which, being at the beginning of $\mathrm{G_1}$, will need a time between T and $T + \mathrm{d}T$ to reach the beginning of $\mathrm{G_1}$ again.

It has earlier been shown that the doubling time differs from the mean value of the distribution $\mathrm{f}(T)$ (Gilbert, 1972), and it is therefore necessary to derive the formula which permits T_r to be calculated from the distribution $\mathrm{f}(T)$.

By analogy to equation (3) one can give the time dependence for the cell flow $u(t)$ into mitosis:

$$u(t) = u_0 e^{bt} \tag{9}$$

This cell flow consists of cells which had come out of the previous mitosis at various times. The contribution of those cells which had come out of the previous mitosis at time $t - T$ is equal to the cell flow out of mitosis at this earlier time, $ru_0 e^{b(t-T)}$, multiplied by the probability density $\mathrm{f}(T)$, and one obtains therefore for the sum of all contributions to $u(t)$:

$$u(t) = \int_0^{T_{\max}} ru_0 e^{b(t-T)} \mathrm{f}(T)\mathrm{d}T \tag{10}$$

with equations (9) and (7) this leads to

$$r \int_0^{T_{max}} e^{-bt} f(T)\, dT = \int_0^{T_{max}} (1 - T/T_r) f(T)\, dT = 1 \qquad (11)$$

If $f(T)$ is known, one can, by numerical integration, determine the value T_r which satisfies this equation. This method corresponds to the procedure earlier used by Hartmann & Pedersen (1970).

Numerical integration is however not always necessary for the resolution of equation (11); in the next section it will be seen that the experimental data in Fig. 1 are consistent with the assumption that $f(T)$ is a normal distribution with mean cycle time T and variance σ^2. In this particular case one obtains the following solution of equation (11) which is here given without proof:

$$T_r = \left[\frac{1}{2} + \sqrt{\frac{1}{4} - \frac{\ln r}{2} \frac{\sigma^2}{\overline{T}^2}} \right] \overline{T} \qquad (12)$$

One can see that T_r is smaller than $\overline{T}$, and that the difference increases with increasing variance, σ^2.

Determination of the distribution of cycle times from the FLM-curve

Fig. 1 represents the observed fraction of labelled mitoses, FLM(t), at time t after pulse labelling with ^{3}H-TdR. Since the duration of the S phase is only 8 hr and is therefore small compared to the duration of the cycle, one can disregard the details of the first narrow wave of labelled mitoses which occurs at $t = 7$ hr. One can simply assume that all labelled cells pass the first mitosis and enter into G_1 7 hr after labelling. In order to simplify notation this time will be taken as $t = 0$. With this assumption there are S $r\, u(0)$ labelled proliferating cells which leave mitosis at time $t = 0$. Accordingly one obtains the following fraction of labelled mitoses at time t:

$$\text{FLM}\ (t) = S\, r\, u(0)\, f(t)/u(t) = S\, r\, e^{-bt} f(t) \qquad (13)$$

This however applies to the case that the observed FLM-curve represents only mitoses of those cells which have gone through one cycle after $t = 0$. In view of the width of the FLM-curve in Fig. 1, one must accept the possibility that some of the observed labelled mitoses belong to cells which have gone through 2 or perhaps more cycles after $t = 0$. If one take these labelled mitoses into account, one obtains the general formula:

$$\text{FLM}(t) = S\, e^{-bt} \sum_{\nu=1}^{\nu_{max}} r^{\nu} f_{\nu}(t) \qquad (14)$$

Each term in the sum is the contribution of a mitotic wave. Thus $f_\nu(t)$ is the distribution of the time which is needed for a cell to pass through ν successive cycles; $f_\nu(t)$ is the so-called ν-fold convolution of the distribution $f(t)$.

Equation (14) is based on the assumption that the distribution of cycle durations remains the same for the successive waves of labelled mitoses. It is, however, well established that after the age of about 3–4 weeks cells with higher ploidy and with one and two nuclei are formed. Virtually nothing is known about the kinetic parameters of these cells. Therefore, equation (14) can only be considered as an approximation. In view of this uncertainty waves II and III are indicated merely as dotted lines in Fig. 1.

There are well-established computer techniques (see Hartmann *et al.*, 1975; Takahashi, Hogg & Mendelsohn, 1971; Brockwell, Trucco & Fry, 1972; Ashihara, 1973) to derive from experimentally observed FLM-curves the distribution of cycle times and of the durations of various parts of the cycle. Any of these techniques, for example the method of Steel & Hanes (1971), could be applied to the present data. However, equation (14) represents a much simpler problem than the general case. It has therefore been straight-forward to obtain on the computer the mean value and the variance of that Gaussian distribution which leads to the best agreement with the experimental data; for $t = 2$ the best fit was obtained with the mean value $\overline{T} = 6{\cdot}7$ days and the standard deviation $\sigma = 2{\cdot}1$ days. One then obtains from equation (14) the solid curve in Fig. 1. The broken line I, and the dotted lines II and III represent the contributions of the individual mitotic waves, and one can see that there is indeed an overlap between successive waves. According to equation (12) one obtains the value $T_2 = 6{\cdot}5$ days; one may note, at this point, that the long cycle durations of $16{\cdot}7 \pm 2{\cdot}1$ days reflect a prolongation merely of the first part of the cycle, i.e. that they are related only to the G_1- and possibly to a G_0 phase.

The assumption of a value $r = 2$ is not very critical. If one assumes that after a mitosis only $r = 1{\cdot}8$ daughter cells continue to proliferate, one obtains the best fit with a normal distribution of mean value $T = 6{\cdot}6$ days and standard deviation $\sigma = 2{\cdot}1$ days. The resulting FLM curve is very nearly identical to the one obtained in the first case and represented in Fig. 1. The corresponding value of $T_{1{\cdot}8}$ is equal to 6·4 days. A cell loss of 0·2 daughter cells per mitosis is therefore of little influence on the result.

From the value $T_2 = 6{\cdot}5$ days one obtains according to equation (8) a labelling index for the proliferating cells of $LI_p = 3{\cdot}56\%$ and with $LI = 3{\cdot}2\%$, equation (1) leads to $GF = 0{\cdot}9$. From $T_{1{\cdot}8} = 6{\cdot}4$ days one obtains $LI_p = 3{\cdot}8\%$ and $GF = 0{\cdot}84$. The growth fraction is therefore close to 1·0.

The theoretical curve is in fair agreement with the data in Fig. 1. There is, however, a discrepancy insofar as, according to equations (11) and (14), the area under each of the mitotic waves should be equal to the duration of the S phase (8 hr). The area under the broken curve which represents the first broad maximum (wave I) is however only 5·5 hr. Partly, this must be due to the fact that mitoses were counted as labelled only if they had at least four grains in the autoradiographs. The number of grains per labelled mitosis varied broadly with a mean of about twenty-four. The counting limit must therefore have lead to a certain loss of labelled mitoses, with a resulting general decrease of the FLM-curve. This is, however, without influence on the calculated value of GF.

The preceding analysis has not taken into account that the labelling index, LI, of the hepatocytes decreases with increasing age as Stöcker & Butter (1968) have shown. This may influence the calculated value of GF in two ways.

(1) The calculated value of GF refers to age 3 weeks while the observed FLM curve covers an age interval of 3–5 weeks. During this period the labelling index decreases by about 15% per week. If the calculation of GF was based not on the value $LI = 0{\cdot}032$ for 3 weeks but on the LI value for 4 weeks which is 15% less, one would obtain a value for GF which is correspondingly decreased.

(2) Beginning roughly with age 3–4 weeks one finds an increasing number of tetraploid mono- and binuclear cells in the liver in addition to the binuclear diploid cells. Little is known about the kinetics of the tetraploid cells, and the experimental data at later times can therefore not be interpreted clearly. It is however not unlikely that the decrease in the labelling index,

LI, is due at least partly to an increase in cycle duration. This would mean that the theoretical curves in Fig. 1 would be stretched progressively at later times. It also implies that the contribution of waves II and III in Fig. 1 are reduced, and that the area under wave I will be correspondingly increased. Moreover this would lead to closer agreement of the theoretical curves in Fig. 1 with the observations at later times. With this change one obtains a larger value T_r and, therefore, according to equations (8) and (1) a decreased labelling index, LI_p, and an increased value for GF.

The two factors, (1) and (2), have opposite effects on GF, and they may partially compensate each other. The conclusion, therefore, remains that the growth fraction in the liver of the rat of age 3–4 weeks is between 0·8 and 1·0. The earlier assumption (Post *et al.*, 1963; Grisham, 1969) of a growth fraction between 0·1 and 0·2 has not been confirmed.

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