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Uptake of Amino Acids and Thymidine During the First Cell Cycle of Synchronized Hamster Cells

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Summary. The net total uptake of four amino acids (valine, leucine, lysine and methionine) used at concentrations required for growth, and of thymidine at tracer concentrations, has been studied during the first cell cycle of an asparagine-dependent strain of transformed BHK cells synchronized by asparagine starvation. The rate of the total uptake of the amino acids, the free pool of the amino acids taken up, and the rate of their incorporation into protein at the end of the first cell cycle were, on an average, 12-fold that at the beginning of the cell cycle. The increase in these parameters during the cell cycle was not linear. The uptake of thymidine started before the onset of DNA synthesis and proceeded linearly beyond the peak of the S phase. The rate of accumulation of thymidine into the acid-soluble fraction also increased during the S phase, apart from a tendency to plateau off at the peak of this phase. It reached a second plateau towards the end of the cell cycle, and then declined slightly. Evidence is presented which suggests that the total quantity of protein synthesized during the cell cycle is more than the newly synthesized protein present in the cells at the end of the cell cycle; this indicates degradation and/or secretion of a substantial proportion of the newly synthesized protein. The total protein synthesized at different time points in the cell cycle appeared to contain different proportions of the amino acids used.

A majority of cells in tissues of adult organisms spend a large proportion of their life span in the resting stage. Yet it is often possible to trigger many of these cells (e.g. hepatic parenchymal cells, following partial hepatectomy) into the division cycle. Available evidence strongly suggests that the primary event which initiates a resting cell into the division cycle, occurs at the cell membrane (for references, *see* Everhart & Rubin, 1974). It would, therefore, be of interest to look closely at changes in a well-defined membrane function, such as the uptake of nutrients, during the *first* cell cycle of resting cells triggered into synchronous cell division.

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In the present investigation, we have studied the net total uptake of valine, leucine, lysine and methionine by an asparagine-dependent strain of BHK cells during their first cell cycle after initiation of synchronous growth following a "resting period" of no growth. The four amino acids were chosen because they are not made by the cells and do not represent common pools. The cells were synchronized by asparagine deprivation over a period of 24 hr, followed by a shift to an asparagine-containing medium. The uptake was studied at high external concentrations of the amino acids, which were used for growth. We have also concurrently studied the net total uptake of thymidine in these cells. The uptake of several nutrients including amino acids and thymidine, during a mammalian cell cycle, has been studied earlier (*inter alia,* Mitchison, 1971; Sander & Pardee, 1972; Everhart & Rubin, 1974). However, most of the studies on changes in the rate of uptake during the *first cell cycle* following triggering of resting cells into division, have been confined to density-inhibited cells (for references, *see* Bhargava & Vigier, 1976). Sander and Pardee (1972) studied the changes in the transport of a nonmetabolizable amino acid analogue, α-aminoisobutyric acid, during the first cell cycle of CHO and L cells synchronized by isoleucine deprivation.

Materials and Methods

Cells, Synchronization and Incubation

An asparagine-dependent strain of BHK/21/13 cells transformed by hamster sarcoma virus, called HS5 (Montagnier, Gruest & Boccara, 1971), was used. The cells were grown in Eagle's minimal essential medium as modified by Macpherson and Stoker (1962), containing twice the prescribed concentration of amino acids, 10% calf serum and 10% bactotryptosephosphate (BTP, which contains asparagine); the pH was maintained at 7.2 by aerating with 90% air + 10% CO_2. For synchronization, the cells were first grown in Roux bottles in the above medium (the "growth medium"). Towards the end of the logarithmic phase, the cells were detached by trypsin [0.08% in Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS) containing 0.014% EDTA], washed once with the growth medium and then suspended in the same medium without BTP. After 24 hr at 37 °C, the cells were released by trypsin, washed and resuspended in the BTP-free medium to which asparagine (15 μg/ml) and serine (10 μg/ml) were added (the "complete medium"). Five-ml aliquots of the cell suspension were pipetted into petri dishes (60 mm diameter) at room temperature, and incubated at 37 °C. Zero hour is considered to be the time at which the cells were transferred to 37 °C, which was done within 10–20 min of suspension of the cells in the complete medium. A separate dish was used for each amino acid and for each time point.

At the specified time (unless the time was less than 3 hr), the medium was decanted off the required number of dishes, and the cells were washed twice, as rapidly as possible,

by decantation, with the "incubation medium" (growth medium without serum, BTP, asparagine or serine). After the last wash, 5 ml of the incubation medium containing ^{14}C-thymidine (12 Ci/mmole) and the ^{3}H-labeled amino acid (5–30 Ci/mmole) was added, and the dishes incubated at 37 °C for 30 min; in the trace quantities used, the labeled amino acids did not significantly alter the concentration (0.2–0.8 μmoles/ml) of the amino acid already present. The labeled compounds were obtained from C.E.A., Saclay, France.

Since the cells required 2 hr to attach to petri dishes, the 0, 1 and 2-hr samples could not be worked as above; they were transferred to 10 ml conical centrifuge tubes and washed twice by centrifugation, each time with 5 ml of the incubation medium. They were then suspended in the ^{3}H-amino acid and ^{14}C-thymidine-containing incubation medium (5 ml), transferred quantitatively to a petri dish and incubated for 30 min.

Estimation of Uptake

After incubation with the labeled precursors, the petri dishes were put on ice, the incubation medium decanted off and the cell monolayer washed 5 times, by decantation, with ice-cold PBS containing unlabeled amino acids used as precursors ($\sim 10^{-4}$ M) and sodium azide (10^{-3} M). The entire washing was completed in less than 5 min. Radioactivity was often measured in all the washings; the total radioactivity in the fifth wash was always less than 1% of that in the cells at the end of this wash.

After the fifth wash, 1.5 ml of the trypsin solution was added and the cells incubated for 4 min at 37 °C. The detached cells were transferred quantitatively with a pasteur pipette to 6-ml tubes; the dish was rinsed with 1.0 ml of ice-cold PBS and 2 ml of 12% trichloroacetic acid (TCA) added to the cell suspension. The mixture was allowed to stand in the cold for at least 1 hr and then centrifuged. Radioactivity was determined in 1.5 ml of the supernatant (the acid-soluble fraction; total volume 4.5 ml). The acid-insoluble residue was washed twice with 5% TCA. In most experiments, the residue was dissolved in 1–4 ml of 0.6–1.2 N NaOH, and 0.1–0.5 ml aliquots used for estimation of radioactivity [the acid-insoluble radioactivity, on account of protein (^{3}H) and DNA (^{14}C)]. Protein was estimated in the alkali solution by Folin's reagent. In other experiments, the TCA precipitate was heated at 98 °C for 30 min with 1 ml of 5% TCA and centrifuged; the supernatant was removed and the precipitate dissolved in alkali as above. Radioactivity was determined in 0.1–0.5 aliquots of the supernatant, as well as of the alkali solution of the precipitate. Protein was estimated in the alkali solution either by Folin's reagent or by measurement of $E_{280\ nm}$ (an optical density of 1.0, using a light path of 1 cm, was taken to correspond to 1 mg protein/ml). The radioactivity in the precipitate was entirely in protein; no measurable ^{14}C was present. In the supernatant, containing hydrolyzed ^{14}C-thymidine-labeled DNA, some tritium counts were also present which accounted for 6% of the total radioactivity due to ^{3}H in the TCA-insoluble fraction. The origin of these counts was not investigated; they did not represent acid-soluble material left adhering to the cells, as they were much larger than the radioactivity in the second wash of the cold TCA precipitate.

When the cold TCA precipitate was not dissolved directly in alkali but first treated with hot TCA, only the ^{14}C counts in the hot TCA supernatant were taken to be derived from DNA; the sum of the ^{3}H counts in the hot TCA supernatant and in the alkali solution of the hot TCA residue, that is, the total acid-insoluble radioactivity, was taken to represent incorporation of the ^{3}H-amino acid into protein.

All the total uptake values (that is, the sum of radioactivity in the acid-soluble and the acid-insoluble fractions) given represent the *net* uptake as the back-flux, if any, was not estimated.

Measurement of Radioactivity

Up to 0.5 ml of the solution in water, TCA or alkali was added to 12 ml of Bray's fluid, and the radioactivity measured in a liquid scintillation counter (counting efficiency: 40% for ^{3}H, and 80% for ^{14}C). Appropriate corrections were made for quenching.

Results

Pattern of Synthesis of DNA and of Increase in Protein

Fig. 1 shows the results of incorporation, in a typical experiment, of ^{14}C-thymidine into DNA during a 30-min pulse given to cells at various times during the first cell cycle, following asparagine starvation for 24 hr. The incorporation started at 6–8 hr, reached a peak at 12–14 hr and then fell, reaching a minimum, in every experiment, at 17 hr; it rose again between 18–20 hr after staying at the minimum level for 1–3 hr. Synthesis

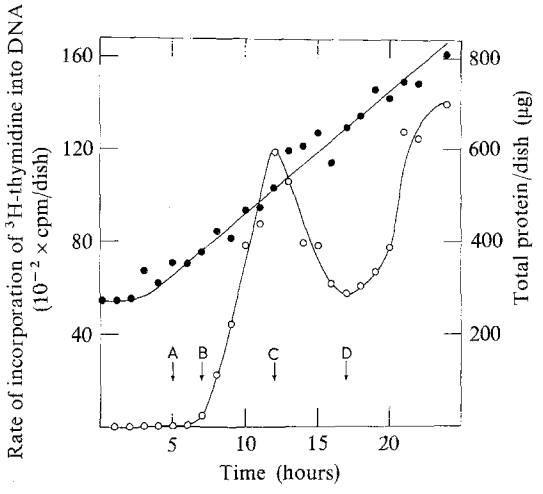

Fig. 1. Changes in the total protein content and in the rate of incorporation of ^{14}C-thymidine into DNA during the first cell cycle of synchronously grown BHK cells. Cells were synchronized as described in the text and grown in a set of petri dishes (1.2×10^{6} cells in 5 ml of the growth medium per dish). At the specified time, a set of dishes was taken out, the cells washed as described in the text, and incubated for 30 min at 37 °C in the serum-free incubation medium containing ^{14}C-thymidine (300,000 cpm). The cells were then washed and processed for estimation of total protein and of radioactivity in DNA as described in the text; protein was estimated by Lowry's method. The values given are the average of four dishes. ○, rate of DNA synthesis; ●, protein content. *A, B, C* and *D* refer to the time points at which, respectively, the uptake of thymidine began (*see* Fig. 2), the incorporation of thymidine into DNA began, this incorporation reached the peak, and this incorporation reached the lowest value after the peak

of DNA, as determined by absolute measurement of DNA, followed the same pattern as the incorporation of thymidine into DNA (E.P. Allin, *unpublished*), and the number of cells at 20 hr was approximately twice that at 0 hr. We have, therefore, taken the first cell cycle to last from 0–17 hr and the second cell cycle to begin at 17 hr.

Unlike the pattern of DNA synthesis, the pattern of increase in the amount of total cellular protein during the first cell cycle showed significant variation among experiments, especially in regard to (a) the initial lag (*c.f.* Mitchison, 1971), and (b) the subsequent increase which was linear in most cases (e.g. Fig. 1) but occasionally nonlinear. The protein content of the cells at 17 hr was, however, always about twofold that at 0 hr (Fig. 1).

Changes in the Rate of the Total Uptake of Thymidine and of Its Accumulation in the Acid-soluble Pool

No detectable uptake of thymidine was observed in the first 4 hr; the uptake commenced between 4 and 7 hr (Fig. 2). After a slight initial lag, the rate of the total uptake of thymidine increased linearly until

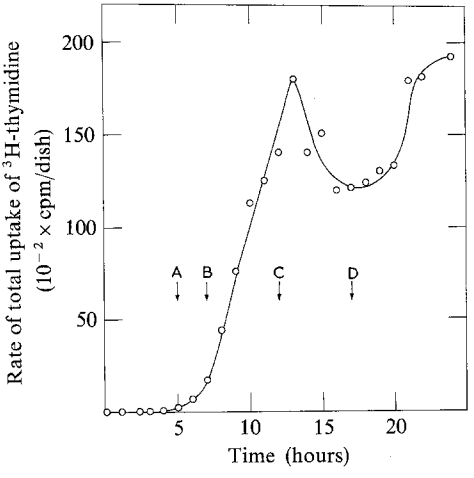

Fig. 2. Changes in the rate of the net total uptake of ^{14}C-thymidine during the first cell cycle of BHK cells. The cells were incubated at the stated period with ^{14}C-thymidine for 30 min, as in Fig. 1. The cells were then washed and the radioactivity in the acid-soluble and the acid-insoluble fractions determined as described in the text. The uptake values given are the total of the values for these two fractions and are averages of four dishes. For explanation of *A, B, C* and *D*, *see* Fig. 1

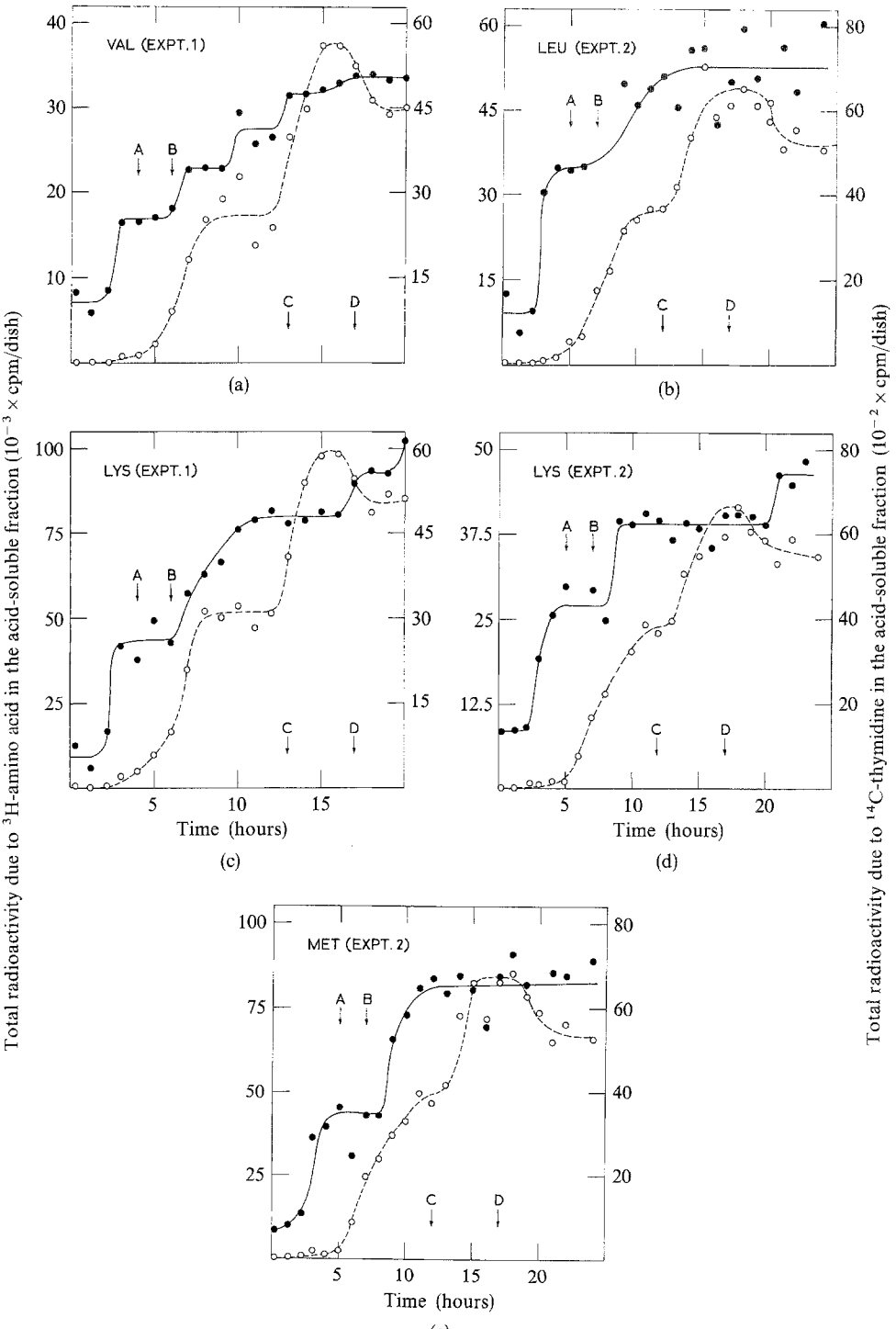
VAL (EXPT. 1)
LEU (EXPT. 2)
LYS (EXPT. 1)
LYS (EXPT. 2)
MET (EXPT. 2)
A
B
C
D
(a)
(b)
(c)
(d)
(e)
Time (hours)
Total radioactivity due to ^{3}H-amino acid in the acid-soluble fraction (10^{-3} × cpm/dish)
Total radioactivity due to ^{14}C-thymidine in the acid-soluble fraction (10^{-2} × cpm/dish)

Fig. 3

it reached a peak at 13–15 hr; that is, *after* the peak of DNA synthesis in the S phase; the rate then fell abruptly, reaching a minimum value at the same time as the rate of DNA synthesis towards the end of the S phase. The uptake was maintained at the reduced level for several hours and then rose again, presumably as the cells entered the second S phase.

It has been reported earlier that the uptake of thymidine in mammalian cells does not begin until the beginning of the S phase (*cf.* Mitchison, 1971; Everhart & Rubin, 1974). In this investigation, the uptake of thymidine appeared to start just *before* the onset of the S phase (Fig. 2).

Labeled thymidine (and derivatives) began to appear in the acid-soluble pool between 4 and 7 hr (Fig. 3). The rate of accumulation of thymidine in the free pool gradually increased until about the time of maximal DNA synthesis, when it had a tendency to plateau off for 1–2 hr; this rate then rose again, and continued to increase even when the rate of DNA synthesis fell. The above rate reached a second plateau before cell division, then fell a little and plateaued off again at the time of cell division. The drop in the rate of accumulation of thymidine in the free pool from the peak value after the S phase to the low value at the time of cell division, was much less than the corresponding drop in the rate of its incorporation into DNA from the peak value in the S phase.

Uptake of Amino Acids

Fig. 4 shows the changes in the rates of total (acid-soluble + acid-insoluble) uptake of labeled lysine, leucine, valine and methionine at various time points during the first cell cycle in a typical set of experiments. The corresponding data for the acid-soluble and the acid-insoluble

Fig. 3. Changes in the rate of accumulation of four ^{3}H-labeled amino acids and of ^{14}C-thymidine in the acid-soluble fraction during the first cell cycle of BHK cells. The cells were incubated at the stated period, for 30 min, with ^{14}C-thymidine (372,000 cpm in Expt. 1, 300,000 cpm in Expt. 2) and the ^{3}H-labeled amino acid [valine (14.3×10^6 cpm) or lysine (24.9×10^6 cpm) in Expt. 1; and leucine (24.5×10^6 cpm), lysine (27.8×10^6 cpm) or methionine (18.0×10^6 cpm) in Expt. 2]. The cells were then washed and the radioactivity in the total acid-soluble fraction determined as described in the text. In this and the following two figures, the data of two experiments is presented for lysine to illustrate the extent of reproducibility in these studies. For explanation of *A, B, C* and *D, see* Fig. 1. ○, ^{14}C-thymidine; ●, ^{3}H-amino acids

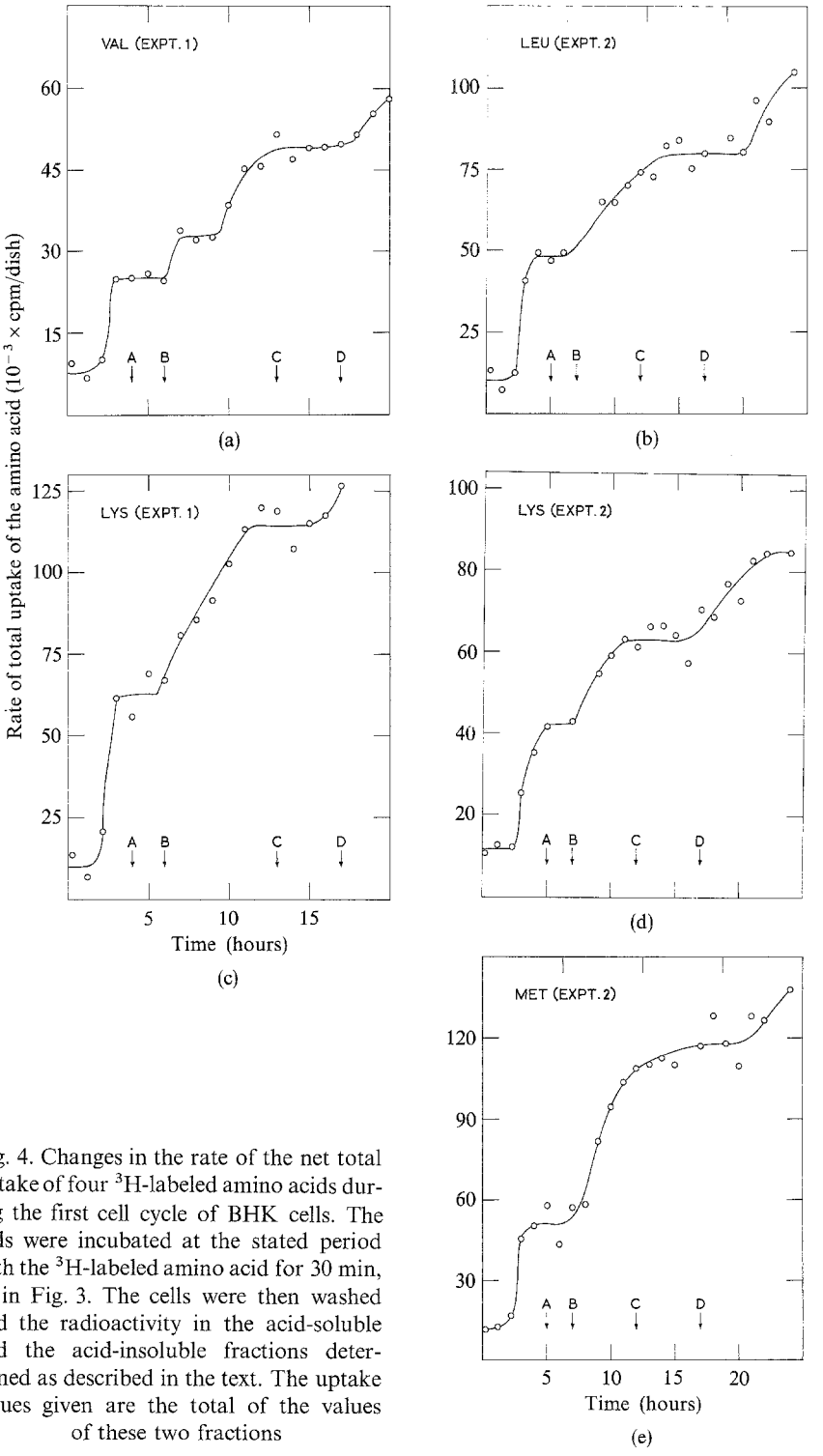

Fig. 4. Changes in the rate of the net total uptake of four ^{3}H-labeled amino acids during the first cell cycle of BHK cells. The cells were incubated at the stated period with the ^{3}H-labeled amino acid for 30 min, as in Fig. 3. The cells were then washed and the radioactivity in the acid-soluble and the acid-insoluble fractions determined as described in the text. The uptake values given are the total of the values of these two fractions

fractions taken separately in these experiments, are given in Figs. 3 and 5, respectively. The experimental conditions used for the uptake studies were the same as those used for growth of the cells except that serum, BTP, serine and asparagine were not present. In a separate series of experiments using ^{3}H-arginine and ^{3}H-leucine, it was shown that the total uptake of amino acids into the cells *in 30 min* in the absence of serum, BTP, serine and asparagine, was the same as in their presence; the absence of serum affected amino acid uptake in these cells only after 45 min.

The following conclusions can be drawn in regard to the total uptake of lysine, leucine, valine and methionine (Fig. 4)[1]:

(a) The uptake of these amino acids by the 0 hr cells was significant.

(b) After a lag of about 2 hr, the rate of total uptake rose rapidly (the lag may be related to the fact that about 2 hr were required for attachment of the cells to the petri dish).

(c) The rise in the rate of uptake was not linear; periods of rapid rise were interspersed with periods of slower or no rise (the plateaus).

(d) The patterns of uptake for the different amino acids were reproducibly and significantly different.

(e) The uptake at 17 hr, that is at approximately the time when the cells divided, was 6–13 times (average 9 times) that at the beginning of the cell cycle.

The total uptake of the ^{3}H-amino acids at different time points in the cell cycle, expressed as per milligram of protein contained in the cells at that time point, reached a peak value at 6–7 hr, that is, close to the period of commencement of DNA synthesis. In no case was the total uptake of amino acid per milligram of cellular protein constant during the cell cycle; the uptake therefore was not determined by, nor related to, the amount of protein present in the cell.

Although qualitatively, the pattern of change in the rate of accumulation of the amino acids in the acid-soluble fraction (Fig. 3), or of change

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1 The curves we have presented for the total uptake of amino acids (Fig. 4) and for the radioactivity in the acid-soluble and the acid-insoluble fractions taken separately (Figs. 3 and 5), are based on the following two considerations.

(a) Comparison of data from several experiments; to illustrate the reproducibility obtained, we have given the data of two experiments with lysine in Figs. 3–5.

(b) Analysis of the data (using an IBM 1620 computer) for fit of a variety of functions such as linear, quadratic and cubic polynomials. The fit was considered satisfactory if the mean percentage relative error was less than 10. In 14 out of the 15 cases, none of the functions tried fitted the data satisfactorily, and in no case was the fit as good as the manual fit for a step function, the one we have used.

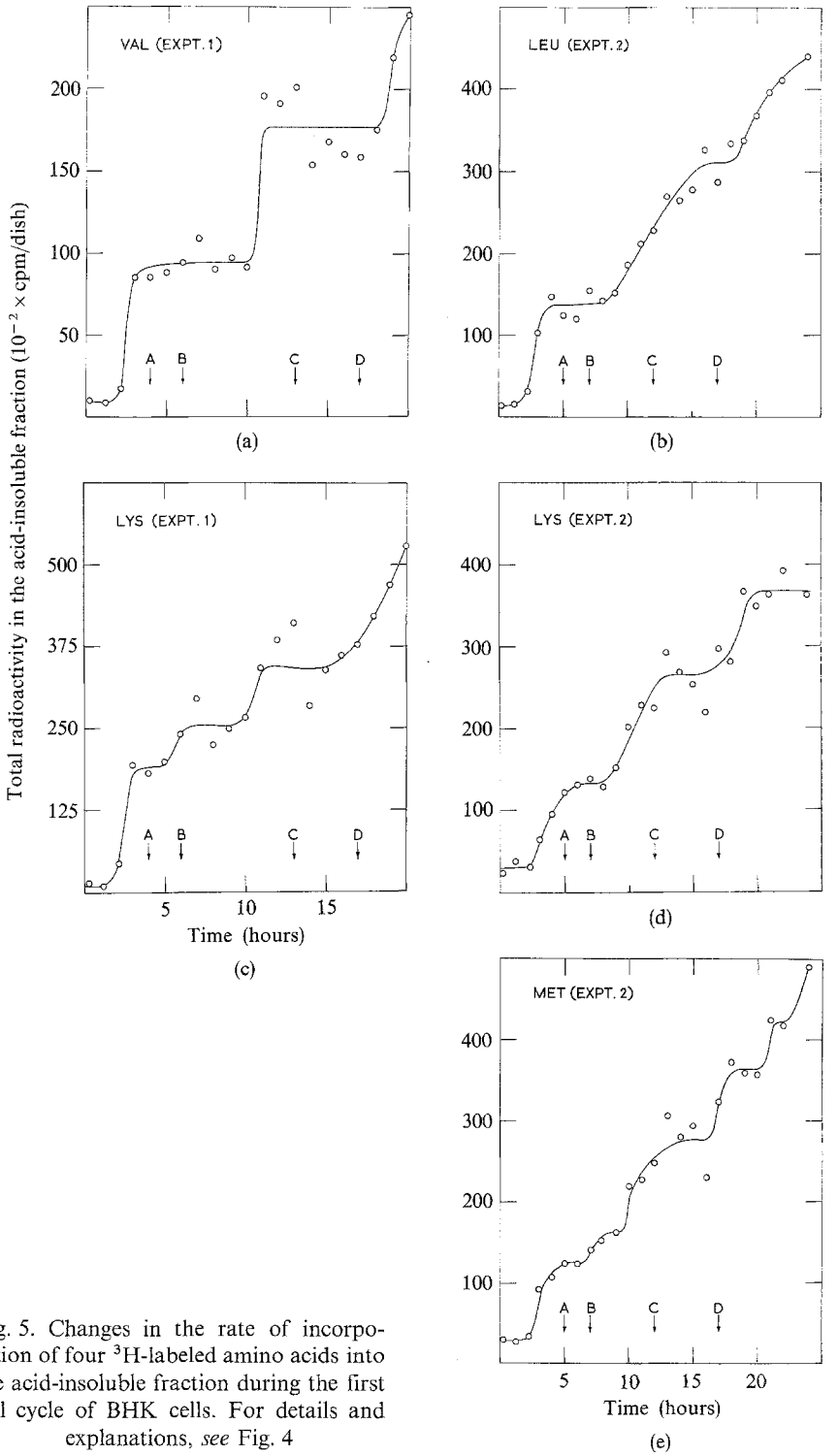

Fig. 5. Changes in the rate of incorporation of four ^{3}H-labeled amino acids into the acid-insoluble fraction during the first cell cycle of BHK cells. For details and explanations, *see* Fig. 4

in the rate of their incorporation into protein (Fig. 5), was similar to the pattern of change in the rate of the total uptake (Fig. 4), there were significant differences between the three patterns in the case of each amino acid, in the timing and duration of the periods of rapid rise in the rate and of the periods during which there was no, or relatively little, rise in the rate. Perhaps the most significant difference was that the rate of incorporation of the amino acid into protein generally continued to increase (except in the case of valine) beyond the peak of the S phase, when the rate of accumulation of all the amino acids in the acid-soluble fraction reached a peak value (*cf.* Figs. 3 and 5). Therefore, the peak of the S phase would appear to represent the attainment of an equilibrium situation, at or beyond which the amount of amino acid taken up by the cell equalled the amount of amino acid incorporated into protein, none contributing to expansion of the free pool.

As equilibration of the internal pool with the external amino acids is likely to have occurred in 30 min (Stanners, 1968; Wiebel & Baserga, 1969; Stanners & Becker, 1971), it would appear from Fig. 4 that the size of their intracellular pool increased during the cell cycle, reaching the maximum value (5- to 10-fold of the 0 hr value) at the time of the peak of the S phase. This conclusion is in agreement with earlier observations (Mitchison, 1971).

It would appear that at least in the early part of the cell cycle (up to the peak of DNA synthesis), the rate of incorporation of an amino acid into protein was related in a general way to the expansion of the pool of the amino acid (*cf.* Figs. 3 and 5). The rate of incorporation of the amino acids into protein increased 10- to 37-fold (average 20-fold) during the cell cycle (Fig. 5). This is in agreement with earlier work on several cell systems showing that the rate of protein synthesis increases through the cell cycle (for references, *see* Mitchison, 1971).

Change in the Specific Activity of Protein

Table 1 gives the specific activity of the total cellular protein following a 30-min label with the four ^{3}H-amino acids at various time points during the cell cycle. The specific activity varied by a factor of 1.3–2.7 during the cell cycle. This variation could be due to (a) a change in the specific activity of the free amino acid pool, or (b) a change in the rate of total protein synthesis, or (c) a change in the rate of synthesis of proteins rich in that particular amino acid. The first two possibilities appear

Table 1. Specific activity of the total cellular protein following uptake by BHK cells of labeled amino acids for 30 min at various time points during their first cell cycle[a]

Time (hr)	Specific activity of protein (cpm $\times 10^{-2}$/mg)				
	Expt. 1		Expt. 2		
	Val	Lys	Lys	Met	Leu
0	30	37	66	–	51
1	33	69	–	95	73
2	67	316	90	140	123
3	*447*	*958*	201	280	292
4	378	864	261	*441*	*462*
5	383	904	*414*	330	323
6	*616*	*1,415*	366	322	390
7	441	999	–	362	*395*
8	214	–	305	362	337
9	*227*	531	386	371	–
10	177	523	406	*517*	385
11	339	580	*463*	450	*501*
12	*350*	636	427	447	441
13	309	*649*	*465*	*493*	*504*
14	263	506	446	457	440
15	*267*	*535*	425	449	429
16	219	520	379	394	*591*
17	215	497	423	525	467
18	223	524	431	*525*	*502*
19	268	541	*516*	476	460
20	269	*574*	488	514	507
21	–	–	509	549	*526*
22	–	–	*518*	*589*	411
24	–	–	401	572	*660*

[a] The specific activities are calculated from the data of Fig. 5 and the corresponding values for total protein obtained as in Fig. 1. Peak values are italicized.

unlikely as the change in the specific activity of total cellular protein during the cell cycle was not correlated either with the change in the rate of the total uptake of the amino acid, or with the change in the rate of its uptake into the acid-soluble or the acid-insoluble fraction.

Distribution of ^{3}H-Amino Acids in the Acid-soluble and the Acid-insoluble Fractions

We also calculated the ratio of radioactivity in the acid-insoluble fraction to that in the acid-soluble fraction following a 30-min uptake

of the four ^{3}H-amino acids by the cells at various time points in the first cell cycle. For all the amino acids, this ratio was 0.10–0.35 at 0 hr, and 0.5–0.9 towards the end of the cell cycle; at no time did it exceed 1.0. Therefore, throughout the cell cycle, less than half of the amino acid taken up in 30 min found its way into protein.

Discussion

Definition and Limitations of the Cell System Used

The cells used in this study were a mutant strain which requires asparagine for growth. They were synchronized by deprivation of asparagine for 24 hr when the cells appeared to accumulate in early G_1. It has been shown that protein and RNA synthesis continues in these cells at a low level throughout the period of asparagine starvation (Montagnier *et al.*, 1971). There was no significant cell death during this period and most of the cells divided when the growth medium was replenished with asparagine. Other workers have found that cells deprived of a few essential nutrients stay viable for long periods, in a state of suspended growth analogous to the stationary phase (Eagle, Piez, Fleischmann & Oyama, 1959; Tobey & Ley, 1971; Vaughan, Pawlowski & Forchammer, 1971; Everhart & Prescott, 1972). In several of these studies too, protein synthesis was found to continue in the deprived cells at a rate much lower than that obtained in exponentially growing cells. We have therefore considered the cells used by us in the asparagine-deprivation stage, to be analogous to resting mammalian cells.

Complete cell synchrony was not achieved in this study even in the first cell cycle (Fig. 1); we were therefore unable to continue these studies into the second cycle. Our observations for the first cell cycle may not hold true in their entirety for the subsequent cell cycles in which the G_1 phase would be expected to be shorter than in the first cell cycle (*cf.* Sander & Pardee, 1972; Everhart & Rubin, 1974). Further, in the first cell cycle such as the one we have studied, it is difficult to distinguish the true "cell cycle effects" from the "deprivation effects" caused by the absence of a nutrient for a substantial period, and from the "transition effects" due to a shift from the resting to the dividing state. Our observations and conclusions must be viewed in the light of the above limitations.

A significant aspect of this investigation is that the uptake of the amino acids was studied at the same external concentration of amino acid as was obtained in the growth medium (0.8 μmoles/ml for lysine, leucine and valine, and 0.2 μmoles/ml for methionine). It has already been reported that the difference between rates of incorporation of precursors when used in trace or at substrate levels, can be as much as 100-fold (Clark & Schmidt, 1967).

The Pattern of Uptake of Amino Acids and Its Relation to Amino Acid Pool and Protein Synthesis

Our observations suggest that the increase in the total uptake of an amino acid during the first cell cycle is a consequence of a series of discrete rather than continuous events. It is also shown that the free amino acid pool expands during the cell cycle, reaching a maximum value, presumably due to saturation of the pool, by 12 hr in the case of all the amino acids (Fig. 3).

It is pertinent to ask if enhancement of the rate of protein synthesis during the cell cycle is a consequence or cause of the increase in size of the intracellular pool of free amino acids. Although this question cannot be answered unequivocally, the fact that in no case did enhancement of the rate of incorporation into protein *precede* the increase in the size of the free amino acid pool, would tend to favor the former possibility.

It appears intriguing that the rate of addition to *total* cellular protein did not change very much during most of the cell cycle (Fig. 1), although the rate of incorporation of each of the ^{3}H-amino acids into protein, measured over a 30-min period, increased enormously (Fig. 4). The former would be a measure of *net* increase in total cellular protein, while the latter a measure of total cellular protein made in 30 min. Our results, therefore, show that these two were not equivalent in our experiments. This could be due to (a) an increase during the cell cycle in the rate of degradation of proteins, and/or (b) an increase in the rate of release of labeled protein into the medium. It has already been shown that a significant proportion of the total protein synthesized by cells grown in tissue culture may appear in the medium (Eidam & Merchant, 1965; Kruse, Miedema & Carter, 1967).

From the data of Table 1, it would appear that valine-rich proteins were synthesized at 3–6 hr, lysine-rich at 5–6 and 13 hr, methionine-rich

at 10–13 hr, and leucine-rich at 4, 11, 13 and 16 hr. There is already considerable evidence suggesting that different proteins are synthesized at different times of the cell cycle (Mitchison, 1971; Thomas & Pardee, 1971; *cf.* Roti, Okada & Eberle, 1973).

It is unlikely that the increase we observed in the rates of the total uptake of amino acids during the first cell cycle was merely a reflection of an increase in cell-surface area, as it is improbable that the surface area increased over the cell cycle by the same large factor by which the rates of uptake increased. Our observations do not throw further light on the mechanism of the substantial increase we observed in the rates of uptake of amino acids during the cell cycle.

Relationship Between Amino Acid Uptake and DNA Synthesis

The rates of total uptake of the amino acids and of their incorporation into protein, at the time of commencement of DNA synthesis, were many-fold higher than those at the beginning of the cell cycle (0 hr), and 33–66% of those at the end of the cell cycle (17 hr). This observation is in accord with the generally held view that prior protein synthesis is essential for DNA synthesis to occur during the cell cycle (*inter alia,* Schneiderman, Dewey & Highfield, 1971; Yamada & Hanaoka, 1973).

At the time of commencement of DNA synthesis, the free pool of each amino acid reached about half the saturation level. At the peak of DNA synthesis the pool was virtually saturated. Although by no means proved, it is an interesting possibility that the elevation of the intracellular free amino acid pool to the maximum permissible level could be an important determinant of the completion of cell division.

Relationship Between Thymidine Uptake and Other Events

The following observations suggest that the correlation between thymidine uptake and DNA synthesis, if any, is not strict: (a) there was a significant lag between the commencement of thymidine uptake and that of its incorporation into DNA; (b) the rate of the total uptake of thymidine continued to increase even after the rate of DNA synthesis decreased towards the end of the S phase; (c) the rate of the total uptake of thymidine fell much less steeply than that of DNA synthesis towards the end of the cell cycle. Therefore, as is suggested by a recent

study of Everhart and Rubin (1974), thymidine uptake is likely to be controlled by an event other than the commencement of DNA synthesis.

Sequence of Events

This investigation suggests that when resting BHK cells are triggered into division, the following events occur sequentially during the first cell cycle, after attachment of the cells to the substratum: (a) rapid rise in the net total uptake of amino acids, in their incorporation into protein and in the size of the free amino acid pool; (b) commencement of the uptake of thymidine; (c) half-saturation of the free amino acid pool and commencement of DNA synthesis; (d) full saturation of the free amino acid pool at the time of the peak of the 'S' phase; (e) decline in the rate of DNA synthesis; and (f) decline in the rate of the total uptake of thymidine. The rates of the total uptake of amino acids and of their incorporation into protein continue to rise till the end of the cell cycle.

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