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AN AUTORADIOGRAPHICAL STUDY OF AMINO ACID AND NUCLEOSIDE INCORPORATION DURING THE CELL CYCLE OF *AMOEBA PROTEUS*

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SUMMARY

The incorporation of tritiated thymidine, uridine and leucine, into the acid-precipitable material of DNA, RNA and proteins, respectively, was studied by autoradiography throughout the cell cycle of *Amoeba proteus*. DNA synthesis occupied the first 17 h of the cycle (57 h long) and 2 peaks between 0·5–7 and 9–13 h accounted for the majority of the thymidine incorporation. RNA synthesis was represented by a series of peak uridine grain counts, the 3 major peaks occurring at 10, 26–27 and 47–48 h. The incorporation of leucine also followed a pattern of peaks and dips, the main peaks occurring 1–2 h after the major increases in uridine incorporation. The fraction of label present over the nucleus decreased during the cell cycle, and this was probably due to a lowered incorporation of the leucine label by proteins synthesized in the cytoplasm and destined to become nuclear proteins. The incorporation patterns of 6 amino acids (arginine, aspartic acid, leucine, lysine, serine and valine) were studied individually during 3 periods of the cell cycle: 0–10 h (*S* phase); 20–30 h (early G_2); and 40–50 h (mid–late G_2). Variations in the intensity and timings of the incorporation maxima of the amino acids were observed, although the timings of increased grain counts of some of the amino acids frequently coincided. 'Unique' incorporation peaks (i.e. only observed in one of the amino acids studied) possibly indicate the synthesis of phase-specific proteins. The amino acid and nucleoside incorporation profiles presented in this paper will enable the results obtained from future studies on amoebae to be related to the macromolecular synthesis patterns.

INTRODUCTION

It is a characteristic of eukaryotic cells that different species or sub-species of macromolecules (DNA, RNA and proteins) are synthesized during specific periods of the cell cycle. DNA replication mainly occurs during one period of the cell cycle – the *S* phase (Howard & Pelc, 1953); whilst RNA and proteins are synthesized continuously throughout the cycle, with particular types of RNA or proteins synthesized during periods characteristic for the molecule (Gurdon & Woodland, 1969; Kolodny & Gross, 1969; Mitchison, 1971; Ord, 1973). The results reported here present the patterns of synthesis of DNA, RNA and proteins during the cell cycle of *Amoeba proteus*, as measured by autoradiography of the incorporation of tritiated precursors. From the complete cell cycle, 3 periods were selected for

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incorporation experiments using 6 amino acids (arginine, aspartic acid, leucine, lysine, serine and valine) individually. These experiments will aid the interpretation of nuclear-protein movement studies performed during these periods (Mills, 1981).

MATERIALS AND METHODS

Culture methods

Amoeba proteus (strain $P_{da}X_{69}$) was cultured in a modified Chalkley's medium (Chalkley, 1930; Ord, 1970) at pH 6·0 ± 0·1. The amoebae were maintained at 20 ± 1 deg.C and fed with *Tetrahymena pyriformis* (Prescott & James, 1955). Under these conditions the cell cycle time was 57 ± 2 h.

Labelling procedures

Mitotic amoebae, selected from mass cultures, were monitored for division prior to being maintained in a solid watch glass until the required age. The culture medium, free of food organisms, was replaced with Chalkley's medium containing one of the tritiated amino acids or nucleosides used and, labelling was performed over a period of ½ or 1 h. For protein labelling, one of the following amino acids was used (the specific activity is shown in parenthesis): L-[5-^{3}H]arginine monohydrochloride (2·1 Ci/mmol); L-[2,3-^{3}H]aspartic acid (3·0 Ci/mmol); L-[4,5-^{3}H]leucine (12·0 Ci/mmol); L-[4,5-^{3}H]lysine monohydrochloride (10·0 Ci/mmol); L-[3-^{3}H]serine (4·8 Ci/mmol) or L-[3,4(*n*)-^{3}H]valine (9·2 Ci/mmol.). DNA and RNA labelling was achieved using [*methyl*-^{3}H]thymidine (7·0 Ci/mmol) and [5-^{3}H]uridine (7·5 Ci/mmol), respectively. All radiochemicals were obtained from Radiochemicals Ltd, Amersham. Following the incubation period, the amoebae were washed in cold Chalkley's medium, placed on a clean slide and squashed with a coverslip holding a drop of 20% acetic acid. The coverslip was flicked off after the slide had been completely frozen on dry ice. The cells were postfixed in acetic acid/alcohol (1:3), passed through 2 changes each of absolute and 95% alcohol, and air-dried. The amino acid labelled cells were washed with 10% trichloroacetic acid to remove any unincorporated label.

Autoradiography

The slides were prepared for autoradiography by dipping into liquid emulsion (Ilford L4, diluted 1:1 with distilled water) and exposed for a period of 21 days. After the period of exposure, the slides were developed in D 19 (diluted 1:1) and fixed in 20% sodium thiosulphate. The amoebae were stained in 0·4% light green and made permanent. The number of grains over the nucleus and cytoplasm was counted under oil at a magnification of × 1000 with the aid of an eyepiece grid graticule. Ten areas were counted over the cytoplasm; these counts were corrected for background, and the average number of grains in 100 μm^2 calculated. All the grains over the nucleus were counted and the size of the nucleus noted. The nuclear count was then corrected for background and to an area of 100 μm^2. The nuclear counts were also corrected for any grains contributed by the cytoplasm overlaying the nucleus, by subtracting the cytoplasmic count from the nuclear count to give a true nuclear count. This correction will occasionally result in an over-correction, depending on the degree of squashing of the cell, but some type of standardization was thought to be necessary.

RESULTS

Incorporation during the cell cycle

Analysis of the autoradiograms revealed that the nucleus had incorporated thymidine mainly during the first 16–17 h of the cell cycle – the *S* phase (Fig. 1A), indicating that DNA synthesis was initiated immediately following mitosis. Two major peaks of thymidine incorporation occurred during *S* phase: the first between 0·5 and 7 h

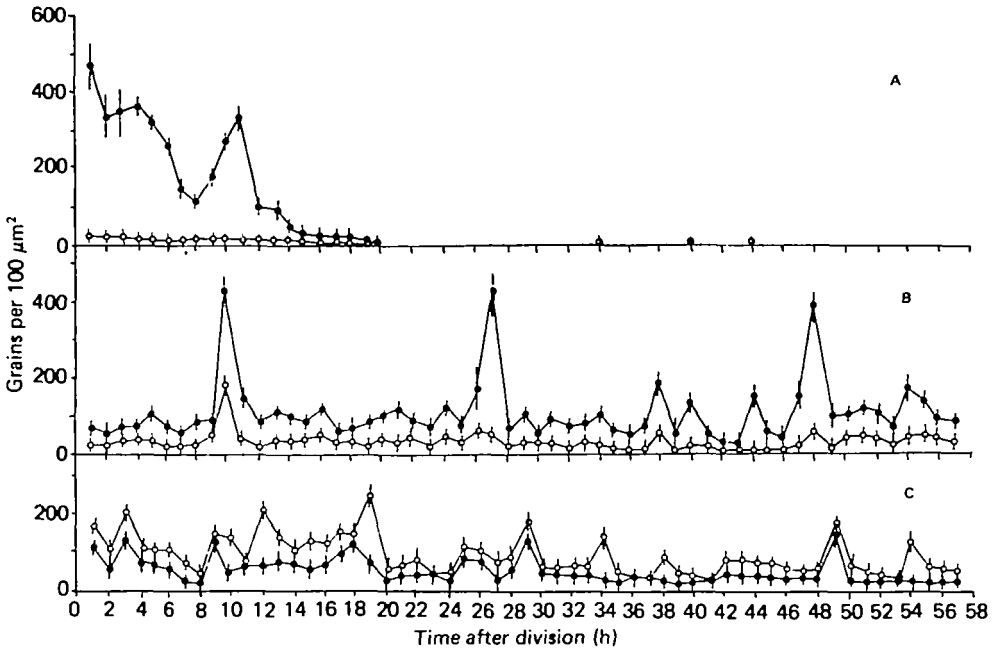

Fig. 1. Pattern of thymidine (A), uridine (B) and leucine (C) incorporation during the cell cycle of *A. proteus*. Each point represents the mean of at least 30 amoebae. The vertical lines indicate standard error with 95% confidence limits. Cells were incubated for 1 h in one of the tritiated precursors before fixation and autoradiography. (●) Nuclear grains; (○) cytoplasmic grains.

and the second smaller peak between 9 and 13 h. During *S* phase, approximately 9% of the grains were present over the cytoplasm. The pattern of uridine incorporation showed that RNA was synthesized continuously throughout the cell cycle; the major peaks of uptake accounted for approximately 25% of the total nuclear incorporation and occurred at 10, 26–27 and 47–48 h after division (Fig. 1B). Minor peaks were observed at 38, 40, 44 and 54–55 h. The ratio of nuclear uridine grains to cytoplasmic grains showed that as the cycle proceeds, the nucleus retains an increasing amount of the labelled RNA during the incubation period (Fig. 2). The extent of leucine incorporation, taken as an indicator of protein synthesis, was also variable (Fig. 1C), and was higher during the first 19 h of the cycle than for the remainder of the generation time. Increased incorporation of the leucine occurred 1–2 h after the RNA maxima at 26–27 and 47–48 h, and for approximately 7 h following the 10 h RNA peak. The extent to which leucine-labelled proteins are present in the nucleus was calculated from the ratio of grains over the nucleus to those present in the cytoplasm, and this showed that less nuclear proteins were labelled as the cell cycle proceeded: 0·44 in the first 4 h (early *S* phase); approximately 0·37 throughout the majority of the cycle; and 0·22 in the period prior to mitosis. The times of the peak incorporations of thymidine, uridine and leucine during the cell cycle are summarized in Table 1.

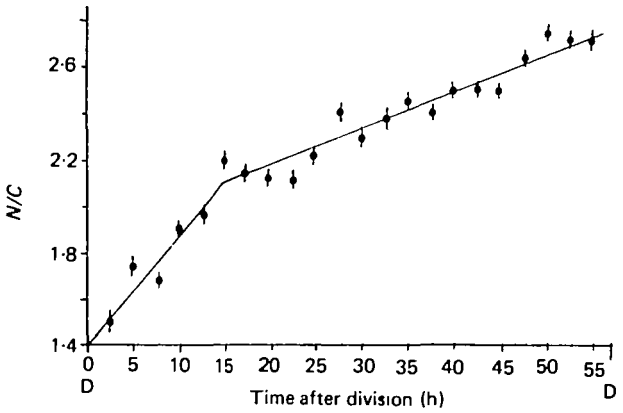

Fig. 2. The ratio of nuclear grains (*N*) to cytoplasmic grains (*C*) during the cell cycle of *A. proteus* following uridine labelling. The ratios were calculated from the data presented in Fig. 1. D, division.

Table 1. *Timings of the main thymidine, uridine and leucine incorporation peaks during the cell cycle of* Amoeba proteus

Thymidine:	0·5→7	9→13									
Uridine:		10		26/27		38	40	44	48	51	54
Leucine:	3	9	12→19	25	29	34			49		54

Hours after division ----→

Main peaks are underlined.

Incorporation during selected periods of the cycle

From the complete cell cycle (Fig. 1), 3 periods were selected for incorporation studies using, individually, 6 amino acids (arginine, aspartic acid, leucine, lysine, serine and valine) and uridine. These periods were 0–10 h (*S* phase); 20–30 h (early G_2); and 40–50 h (mid–late G_2). Incubation periods of ½ h were used in all the following experiments, and this minimal labelling period resulted in reducing the height of the peaks observed compared to those in the complete cell cycle study.

During the first period (0–10 h) the pattern of RNA synthesis, as measured by uridine incorporation (Fig. 3A) was similar to that observed previously (Fig. 1B), with a major peak at 9·5–10 h and a smaller increase in grain count at 4·5 h. The individual amino acids exhibited different variable incorporation profiles (Fig. 3B–G), although the increased grain counts observed at 4–4·5 and 7·5 h were present in the majority of the patterns. However, on comparing closely related amino acids greater similarities were observed, i.e. leucine with valine (Fig. 3B, C) and lysine with arginine (Fig. 3E, F). The nuclear incorporation of arginine, aspartic acid and lysine exceeded that of the cytoplasmic grains during the majority of the first 9–9·5 h, indicating that the majority of newly synthesized proteins labelled with these amino acids during this period were probably nuclear proteins. In the early G_2 period studied (20–30 h), major peaks of uridine incorporation occurred at 20 and 23·5 h (Fig. 4A). Differing

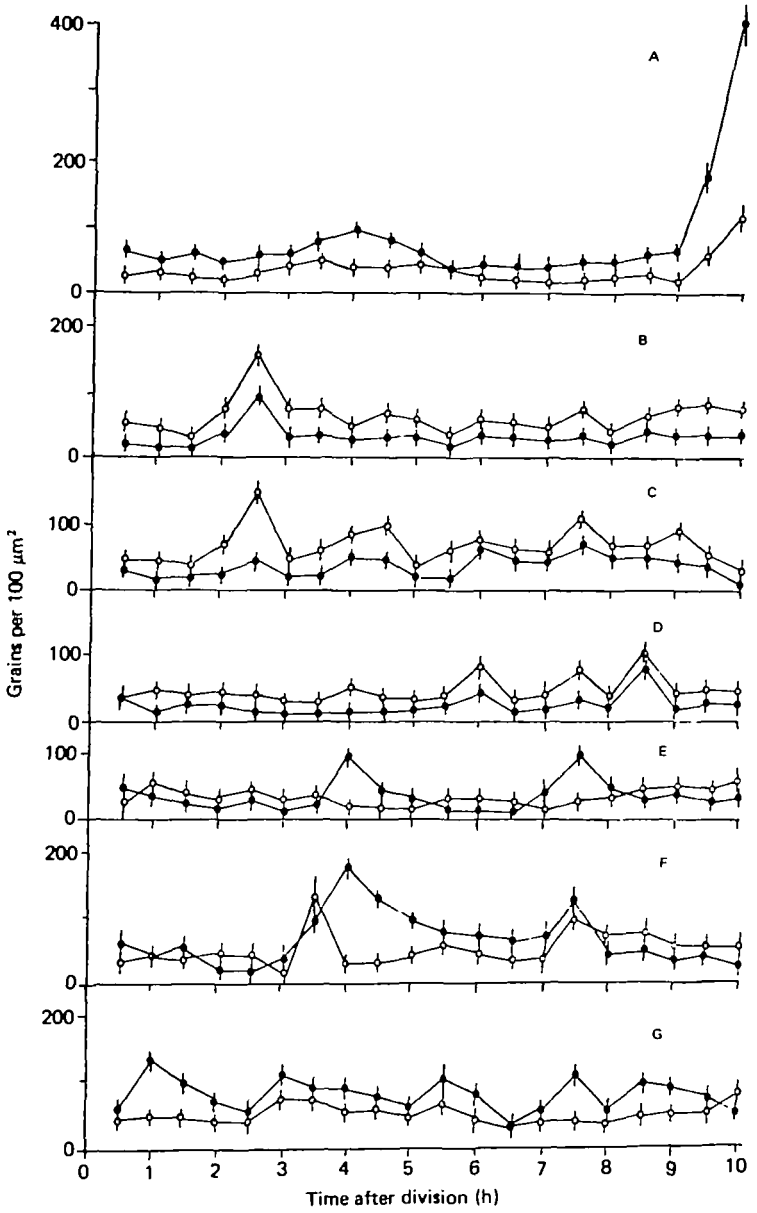

Fig. 3. The pattern of grain counts obtained following autoradiography of amoebae labelled with uridine (A), leucine (B), valine (C), serine (D), lysine (E), arginine (F) and aspartic acid (G) during the period 0–10 h after division. Each point represents the mean of at least 30 amoebae, and the vertical lines indicate standard errors with 95 % confidence limits. Cells were incubated for $\frac{1}{2}$ h in one of the tritiated precursors prior to fixation and autoradiography. (●) Nuclear grains; (○) cytoplasmic grains.

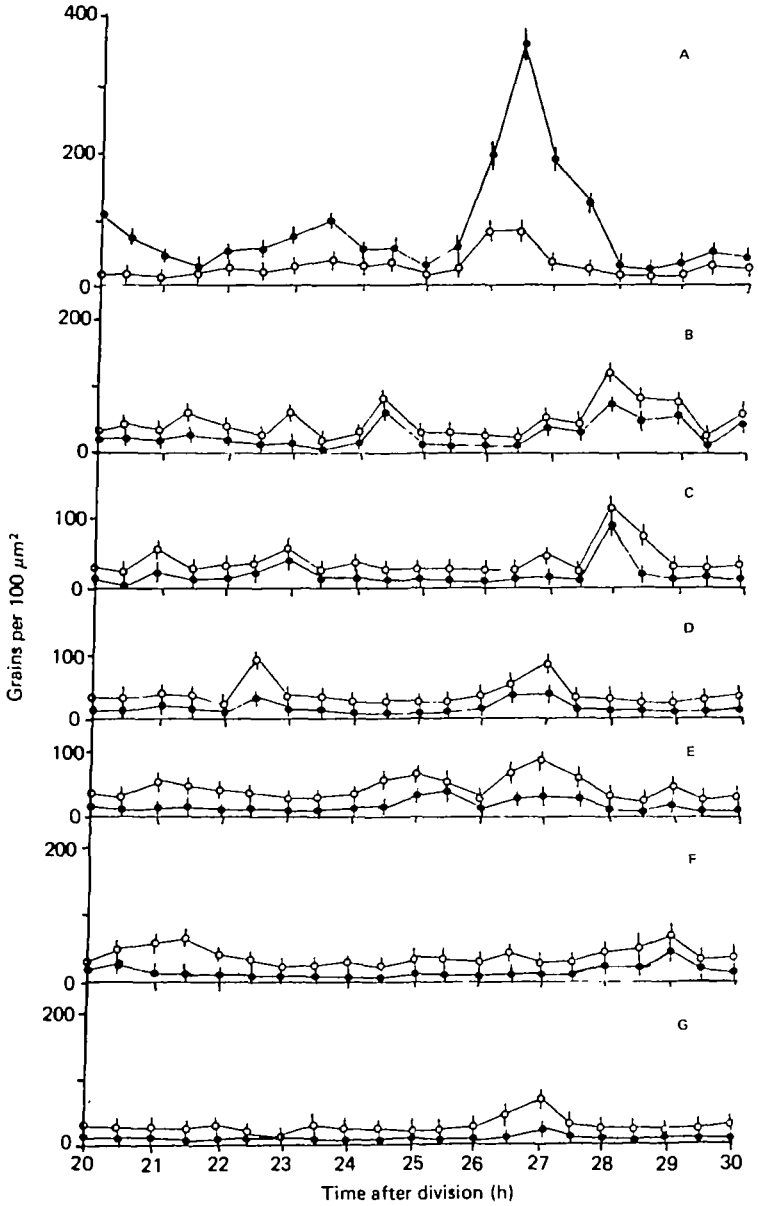

Fig. 4. Pattern of uridine (A), leucine (B), valine (C), serine (D), lysine (E), arginine (F) and aspartic acid (G) incorporation during the early G_2 period studied (20–30 h). Other details as in Fig. 3.

patterns of amino acid incorporation were found, although some common peaks could be distinguished (Fig. 4B–G). The increased amino acid grain counts at 27–28 h lagged 1–2 h behind the major uridine incorporation peak (RNA) at 26–27·5 h, suggesting that the delay represents the time required for the RNA to migrate into the cytoplasm and then be available for translation. During the mid–late G_2 period (40–50 h) studied, increases in uridine incorporation occurred at similar times to those found in the complete cell cycle study, with peaks at 40·5, 43–45 and 47·5–48·5 h, the latter being the major peak (Fig. 5A). The amino acid incorporation patterns for this period were again variable and depended on which amino acid was used (Fig. 5B–G). Increased amino acid incorporation again lagged behind the main RNA peak by 1–2 h. Following the minor RNA peaks, increased incorporation of one or two of the amino acids was particularly noticeable. During this period, the cytoplasm accumulated a greater proportion of the amino acid label than in the previous periods, suggesting that the majority of labelled newly synthesized proteins were cytoplasmic proteins.

DISCUSSION

The experiments of Chatterjee & Bell (1976) on leucine and lysine incorporation during the post-*S* period in amoebae suggested a cyclic variation in protein synthetic activity. The present study has shown that the incorporation of labelled precursors into macromolecules does follow a variable, but reproducible, pattern throughout the cell cycle. Peak incorporation of thymidine, uridine and leucine into DNA, RNA and protein, respectively, occurs during specific periods of the cell cycle, and from this autoradiographic study some of the interactions between the synthetic patterns are observed.

The cell cycle of amoeba lacks a G_1 phase, thus entering *S* phase directly after mitosis. The length of the *S* period compares favourably with previous reports on amoebae with a shorter cycling time: 17 h in a cell cycle of 57 h compared to 12–13 h in a 48 h cycle (Ord, 1968). However, the lengths of these *S* periods are longer than those reported for other amoebae strains (Prescott & Goldstein, 1968; Ron & Prescott 1969). The presence of cytoplasmic grains during *S* phase was possibly due to incorporation of the thymidine by mitochondria or 'DNA-containing bodies', as this level of incorporation continued throughout the cell cycle. However, the strain of amoebae used in this study appears to contain very few 'DNA-bodies' (Smith, 1978). The pattern of RNA synthesis observed (as measured by uridine incorporation) closely resembled the series of peaks and dips reported by Ord (1973) for uridine incorporation during the first 60% of the amoeba cell cycle. Variable patterns of RNA synthesis have been reported for HeLa cells (Kim & Perez, 1965; Pfeiffer & Tolmach, 1968), and *Physarum* (Mittermayer, Braun & Rusch, 1964; Cummins, Weisfield & Rusch, 1966). The peak of uridine incorporation at 10 h occurs at a time close to the decrease in thymidine incorporation, thus suggesting that large amounts of DNA and RNA cannot be synthesized simultaneously. An increasing ratio of nuclear to cytoplasmic uridine grains was found as the cycle proceeded, possibly indicating either nuclear retention of a higher proportion of the newly synthesized

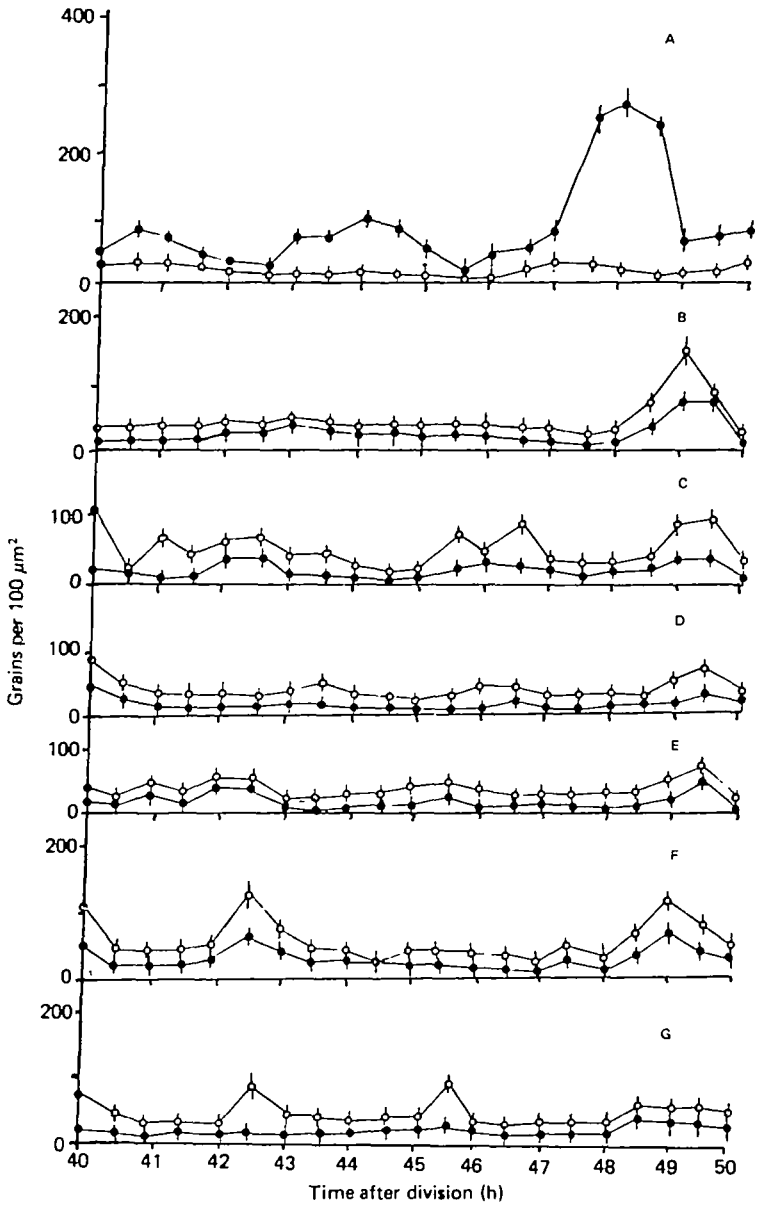

Fig. 5. Pattern of grain counts obtained following labelling with uridine (A), leucine (B), valine (C), serine (D), lysine (E), arginine (F) and aspartic acid (G) during the period 40–50 h (mid–late G_2). Other details as in Fig. 3.

RNA; rapid turnover of the RNA and re-utilization of the degradation products; or an increased movement of RNA into the nucleus, for example in the form of the shuttling RNA described by Goldstein & Ko (1975).

The incorporation of leucine indicated a variable pattern of protein synthesis throughout the cell cycle; this was consistent with the initial findings of Ord (1973), who reported that incorporation of leucine followed a series of peaks and dips. Electrophoretic studies by Kolodny & Gross (1969) have shown that individual proteins in HeLa cells were synthesized only during specific periods of the cycle. A recent report, however, has suggested that all proteins were synthesized throughout the HeLa cell cycle, and when differences did appear they were due to variation in the relative intensity rather than in the appearance of new polypeptides (Bravo & Celis, 1980). The periodic synthesis of RNA (as measured by the incorporation of uridine) is possibly due to the sequential transcription of DNA, as observed in *Saccharomyces cerevisiae* by Tauro, Halverson & Epstein (1968). This process would result in the peak-and-dip pattern of amino acid incorporation observed in this study on amoebae, i.e. the synthesis or increased synthesis of phase-specific proteins, particularly as the extent of amino acid incorporation was seen to increase 1–2 h after the major peaks of RNA synthesis. The delay may reflect the time of the nucleocytoplasmic translocation of the RNA, as approximately 1 h is required for 50% of the newly synthesized RNA to migrate into the cytoplasm (Mills, 1981). Following some of the minor RNA peaks, only one or two amino acids showed increased grain counts, possibly indicating the synthesis or increased synthesis of one specific type of protein. The higher nuclear incorporation of aspartic acid during early *S* period probably represents an increased synthesis of non-histone proteins, whilst the lysine and arginine incorporation peaks at 4 and 7·5 h are probably due to the synthesis of nuclear histones, which has been shown to be tightly coupled to DNA synthesis (Spalding, Kagiwara & Mueller, 1966; Wilkes, Birnie & Old, 1978). Recently, histone synthesis was shown to occur throughout the G_1 and *S* phases of S49 mouse lymphoma and Chinese hamster ovary cells (Groppi & Coffino, 1980); however, due to the lack of a G_1 phase in amoebae, histone synthesis presumably occurs mainly during the *S* period. The fraction of leucine label present in the nucleus during the cell cycle gave an indication of the changing protein requirements of the cell. A higher proportion of the proteins labelled during early *S* phase were destined to become nuclear proteins (e.g. DNA replication proteins, histones, nuclear envelope proteins); whilst the majority of labelled proteins during the period prior to mitosis were cytoplasmic proteins, e.g. division-specific proteins.

The ease of synchronization of amoebae, with a minimum of disturbance, allows a clearly defined pattern of macromolecular synthesis to be obtained, which occurs as a consequence of the cell cycle and not as a result of any distortion caused by chemically or physically induced synchrony. The variable patterns of synthetic activity reported in this paper form a basis for future experiments designed to provide a more complete picture of cellular synthesis and which can exploit the temporal resolution ($\frac{1}{2}$ h labelling in a 57 h cycle) of the present technique.

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