dextranase on dextran. Glucose and isomaltose, the main products of the action of other dextranases on dextran, are not produced. The accumulation of tri-, tetra-, penta- and higher oligosaccharides suggests that the shortest isomaltodextrin chain which the enzyme can attack is one composed of six glucose units. Although the action of the dextranase on individual isomaltodextrins is in agreement with the accumulation of tri-, tetra-, penta- and hexa-saccharides, it indicates that the shortest chain readily cleaved by the enzyme is in fact one composed of seven glucose units. The need for a substrate of such long chain length is of interest. All other dextranases are capable of hydrolysing chains of from two to four glucose units in length, as are most α -amylases and cellulases. Members of the oligosaccharide mixture which result from dextranase activity are capable of supporting the growth of *L. bifidus*.

Although three strains of *L. bifidus* which secrete dextranase have been obtained, two other human strains of the organism which did not produce dextranase have been isolated. Strains of *Lactobacillus brevis*, *L. bulgaricus*, *L. acidophilus* and *L. plantarum* (one of each) were also found to be without action on dextran. A more detailed examination of the distribution of this unusual dextranase in strains of lactobacilli appears to be warranted.

SUMMARY

1. Two bovine rumen strains and one human strain of *Lactobacillus bifidus* have been shown to secrete an extracellular dextranase.

2. The dextranase was isolated from cell-free culture fluids by precipitation with ammonium sulphate.

3. Although the enzyme rapidly destroyed the opalescence of a dextran solution, reducing sugars were produced only after prolonged incubation.

4. The products of the action of the enzyme on dextran were a mixture of isomalto-triose, -tetraose, -pentaose and higher oligosaccharides. Glucose and isomaltose were not produced.

5. Isomaltose, isomalto-triose, -tetraose and -pentaose were not attacked by the enzyme. Isomaltohexaose was only slightly hydrolysed, whereas isomalto-heptaose and -octaose were hydrolysed readily.

6. The dextranase had maximum activity in the range pH 5.4–6.5 and the temperature range 40–50°.

REFERENCES

- Bailey, R. W. (1959). *Biochem. J.* **71**, 23.
 Bailey, R. W. & Oxford, A. E. (1958). *J. gen. Microbiol.* **19**, 130.
 Barker, S. A., Bourne, E. J., Bruce, G. T., Neely, W. G. & Stacey, M. (1954). *J. chem. Soc.* p. 2395.
 Bayly, R. J. & Bourne, E. J. (1953). *Nature, Lond.*, **171**, 385.
 Hehre, E. J. & Sery, T. W. (1952). *J. Bact.* **63**, 424.
 Hulten, E. & Nordström, L. (1949). *Acta chem. scand.* **3**, 1045.
 Jeanes, A., Wilham, C. A., Jones, R. W., Tsuchiya, H. M. & Rist, C. E. (1953). *J. Amer. chem. Soc.* **75**, 5911.
 Jermy, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
 Malpress, F. H. & Hytten, F. E. (1958). *Biochem. J.* **68**, 708.
 Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
 Rogosa, M., Wiseman, R. F., Mitchell, J. A., Disrealy, M. N. & Beaman, A. J. (1953). *J. Bact.* **65**, 681.
 Sery, T. W. & Hehre, E. J. (1956). *J. Bact.* **71**, 373.
 Shaffer, P. A. & Hartmann, A. F. (1921). *J. biol. Chem.* **124**, 425.
 Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
 Tsuchiya, H. M., Jeanes, A., Bricker, H. M. & Wilham, C. A. (1952). *J. Bact.* **64**, 513.
 Turvey, J. R. & Whelan, W. J. (1957). *Biochem. J.* **67**, 49.
 Whelan, W. J., Bailey, J. M. & Roberts, R. J. P. (1953). *J. chem. Soc.* p. 1293.
 Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.

The Initiation of Deoxyribonucleic Acid Synthesis in the Connective-Tissue Cell, with some Observations on the Function of the Nucleolus

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The zone of cells surrounding the explant in a conventional tissue culture enlarges by the emigration of cells from the explanted tissue and by the multiplication of cells which have emigrated. It is not clear, however, whether cells multiply within the explant before emigration or whether emigration necessarily precedes multiplication. The experiments described in the present paper had three

aims: to determine when the synthesis of deoxyribonucleic acid in the cells of the emigration zone began; to measure the duration of the phase of synthesis of deoxyribonucleic acid in the individual cell and to establish the relation of this phase to the time of mitosis; and to examine the connexion between the synthesis of deoxyribonucleic acid and the synthesis of protein and ribonucleic acid. The

work involved identifying and counting those cells in the emigration zone which, at a given time, were synthesizing deoxyribonucleic acid. This was done by preparing radioautographs of the emigration zone after the cells had been exposed for a suitable period to tritium-labelled thymidine, it being generally accepted that this compound is incorporated into deoxyribonucleic acid only. The high resolution of radioautographs produced by tritiated thymidine is well known. In the present experiments the resolution was even further enhanced by the fact that connective-tissue cells, when grown on a glass surface, spread out and become very flat. When fixed they rarely exceed 1μ in thickness. The radioautographs obtained from these cells were precise enough to permit certain observations to be made on the nucleoli. These observations indicate the site at which the synthesis of deoxyribonucleic acid is initiated in the cell.

EXPERIMENTAL

The connective-tissue cells were derived from the hearts of 5-day-old rats. Small fragments were cut from the heart and explanted on to the surface of microscope slides. About 50 fragments were deposited on each slide under a thin film of a medium consisting of 20% of bovine serum and 80% of Eagle's solution (Eagle, Oyama, Levy, Horton & Fleischman, 1956). The slides were kept for about 1 hr. at room temperature in moist chambers, by which time the fragments had stuck to the glass. No plasma was used to anchor the explants. The cells therefore emigrated from the tissue directly on to the glass surface and the resulting emigration zones consisted of a single layer of flattened cells. The slides were incubated at 37° in glass flasks containing enough of the same medium to cover the explants completely.

In order to determine which of the cells in the emigration zone were synthesizing deoxyribonucleic acid (DNA) the preparations were incubated for a given time with tritiated thymidine (supplied by Schwarz Laboratories Inc., N.Y., U.S.A.) at a concentration of $0.5\mu\text{Ci/ml.}$ of medium. The slides were then removed from the flasks, washed four times in 0.85% NaCl soln. and then fixed by immersion for 15 min. in methanol. After fixation the slides were tapped gently so that the fragments of explanted tissue were dislodged; the cells of the emigration zones, however, remained adherent to the slide. These cells were extracted with ice-cold 5% (w/v) trichloroacetic acid and washed under running water for an hour or two. Stripping film (Kodak AR 10) was then laid down over the slides and developed after 7 days. Finally the cells were stained through the film with Nuclear-fast red.

In the experiments in which the duration of the phase of DNA synthesis was determined, it was necessary to permit the cells to continue growing after a given period of exposure to the labelled thymidine. In these cases the slides were removed from the flasks after the period of incubation with labelled thymidine, washed several times in 0.85% NaCl soln. containing an excess of unlabelled thymidine and then transferred to other flasks containing thymidine-free medium. In other experiments, which are

described later, the cells of the emigration zones were brought into suspension by treatment with trypsin and then cultivated in small coverslip chambers. (For details see Harris, 1955a, b, 1956.)

In order to determine which of the cells were synthesizing ribonucleic acid (RNA) at any given time the preparations were incubated for 1 hr. in medium containing $[8\text{-}^{14}\text{C}]$ -adenine (supplied by The Radiochemical Centre, Amersham, Bucks) at a concentration of $2\mu\text{Ci/ml.}$ and then fixed. The cells were extracted with ice-cold 5% (w/v) trichloroacetic acid and washed under running water. Some of the preparations were then incubated for 2 hr. at 37° with crystalline pancreatic ribonuclease at a concentration of 0.5 mg./ml. in phosphate buffer, pH 6.7. Radioautographs of the preparations which had been treated with ribonuclease were compared with radioautographs of those which had not. Labelled adenine which was not extracted by ice-cold 5% (w/v) trichloroacetic acid but which was extracted by ribonuclease was considered to have been incorporated into RNA.

To determine which of the cells were synthesizing protein the preparations were incubated for 1 hr. in medium containing $[1\text{-}^{14}\text{C}]\text{DL-valine}$ (supplied by The Radiochemical Centre) at a concentration of $2\mu\text{Ci/ml.}$ and then fixed. The cells were extracted for 30 min. with 5% (w/v) trichloroacetic acid at 90° and for a further 30 min. with hot ethanol-ether (1:1). Radioautographs were then prepared. Labelled valine, which was not extracted by the above procedures, was considered to have been incorporated into protein.

RESULTS AND DISCUSSION

Initiation of synthesis of deoxyribonucleic acid

Connective-tissue cells made their appearance at the edges of the explants after incubation for about 8 hr. At this time and at intervals afterwards each of a series of fourteen preparations was exposed for 1 hr. to tritiated thymidine and then fixed. Radioautographs were prepared and the proportion of cells synthesizing DNA was determined for each slide. The results are illustrated in Fig. 1. It will be seen that only 10–15% of the earliest cells to appear were synthesizing DNA at the time of their emigration from the explant. Between the eighth and twentieth hours of incubation there was no increase in the proportion of cells synthesizing DNA, although by the end of this time the emigration zones contained several hundred cells. After 20 hr. the percentage of cells synthesizing DNA began to rise, reaching a maximum of 50–60% between 40 and 48 hr. Thereafter the percentage of cells synthesizing DNA fell gradually.

The above findings do not exclude the possibility that the cells which were not synthesizing DNA at the time of emigration from the explant might have synthesized DNA in the 8 hr. or so preceding emigration. This possibility was eliminated by the following experiment. The explants were put into medium containing tritiated thymidine from the outset and incubated in this medium until a small

number of cells had emigrated (a period of about 10 hr.). The percentage of labelled cells in the emigration zones was then determined. Again, only 10–15 % of the cells were found to be labelled; the great majority had thus not synthesized any DNA before emigrating. It is possible that even in 10 hr. the labelled thymidine might not have penetrated to the centre of the explants; but there is no reason to suppose that it would not have reached the cells at the edges of the explants, and these are the first to emigrate. It can be concluded then that 85–90 % of the cells emigrate before they begin to synthesize DNA and that there is a lag period of about 20 hr., of which the cells spend 12 hr. outside of the explant, before DNA synthesis begins.

The small proportion of cells which synthesize DNA either before or during emigration are presumably cells which were in the phase of DNA synthesis, or about to enter this phase, when the animal was killed. The heart of a 5-day-old rat is a rapidly growing structure, and one would therefore expect that at any one time a certain proportion of the cells in it would be in the process of synthesizing DNA.

Duration of the phase of synthesis of deoxyribonucleic acid

To determine the duration of the phase of DNA synthesis and its relationship in time to mitosis the following experiment was carried out. Twenty-four slides, each with about 50 explants attached, were incubated in non-radioactive medium for 30 hr., by which time emigration zones containing several hundred cells had been formed around most of the explants. At successive hourly intervals over the

Fig. 1. Initiation of DNA synthesis in the cells of the emigration zone. The cells first make their appearance at the edges of the explants after incubation for 8 hr. Between 85 and 90% of them are not synthesizing DNA at this stage; they begin to do so after a lag period of about 20 hr. Each point represents a count of all the cells in six emigration zones.

next 24 hr. tritiated thymidine was added to each of the flasks in turn. Each slide was incubated in the medium containing the tritiated thymidine for 1 hr., then washed in unlabelled thymidine and transferred to thymidine-free medium. At the end of 24 hr. all the slides were fixed and radioautographs prepared. All the cells in metaphase, anaphase or telophase in each slide (between 50 and 100) were examined and the proportion showing labelled chromosomes determined. The passage from metaphase to telophase takes no more than a few minutes in these cells. By selecting such mitotic figures in the emigration zones for examination groups of cells were obtained which were synchronized in these stages of mitosis at the time of fixation; and each of these cells had been exposed to tritiated thymidine at a known period before mitosis. If the chromosomes in a mitotic figure were labelled then that cell had obviously been in the phase of DNA synthesis at the time of exposure to the labelled thymidine; if the chromosomes were not labelled the cell was presumed not to have been in this phase at the time of exposure. From the proportion of labelled mitotic figures in each of these slides an estimate of the duration and timing of the phase of DNA synthesis was made.

The results of two such experiments are superimposed on one graph in Fig. 2. There is great variation in the duration and time of onset of DNA synthesis in different cells and less variation in the time at which DNA synthesis ceases. A median value derived from several hundred cells puts the beginning of DNA synthesis between 13 and 14 hr.

Fig. 2. Duration of the phase of DNA synthesis and its relation to the time of mitosis. There is wide variation in the time at which the phase of DNA synthesis begins, but less variation in the time at which it ends. A period of at least an hour, but generally longer, elapses between the end of DNA synthesis and mitosis. M, Mitosis; O, expt. 1; ●, expt. 2. Each point represents between 50 and 100 mitotic figures.

before metaphase and the end of DNA synthesis between 3 and 4 hr. before metaphase. Despite the variation from cell to cell it is evident that the phase of DNA synthesis occurs in the latter part of the intermitotic period and that there is an interval of at least an hour, but usually longer, between the end of DNA synthesis and metaphase. These findings are in general agreement with those obtained by Firket & Verly (1958) with chick-embryo connective-tissue cells.

Effect of inhibiting synthesis of normal protein and ribonucleic acid in the lag phase

With the incorporation of [^{14}C]valine and [^{14}C]adenine, under the conditions described, as criteria it was shown by radioautography that all the cells synthesized protein and RNA continuously. It was of interest to determine whether synthesis of protein and RNA was a prerequisite of DNA synthesis. The amino acid analogues DL- β -2-thienylalanine, DL-*p*-fluorophenylalanine, 3-fluoro-DL-tyrosine and DL-canavanine were used to interfere with the synthesis of protein, and 8-azaguanine and 5:6-dichloro-1-(β -D-ribofuranosyl)-benzimidazole with the synthesis of ribonucleic acid.

Each of the amino acid analogues had previously been found to inhibit the multiplication of the connective-tissue cells (H. Harris, unpublished work). Thienylalanine at a concentration of 5 mM, fluorophenylalanine at 2 mM, fluorotyrosine at 2 mM and canavanine at 10 mM arrested multiplication of the cells without killing them within 24 hr. If cells in which multiplication had been arrested by the analogue were transferred to medium free of the analogue multiplication resumed after a lag of 30–40 hr. The effects of thienylalanine and fluorophenylalanine could be reversed by phenylalanine, the effect of fluorotyrosine by tyrosine and that of canavanine by arginine. With thienylalanine, alkaline hydrolysis of the cell protein showed that the analogue had actually been incorporated. Similar analyses were not carried out with the other analogues used, although it seems likely from work with bacteria (Munier & Cohen, 1956) that these analogues are also incorporated into the protein.

Twelve slides, each with about 50 explants, were incubated in the usual medium for 18 hr. Two slides were exposed to labelled thymidine for 1 hr. at this stage and then fixed; radioautographs of these showed that 85–90% of the cells in the emigration zones were still in the lag phase. The remaining slides were washed several times in 0.85% NaCl soln. and divided into two groups. One group was transferred to flasks containing the usual medium, the other to flasks containing medium free from phenylalanine but with thienyl-

alanine at a concentration of 5 mM. Incubation was continued. Every 2 hr. one slide from each group was exposed to labelled thymidine for 1 hr. and then fixed; the percentage of cells synthesizing DNA was determined for each preparation. The results of the experiment are shown in Fig. 3. It will be seen that in the control group the percentage of cells synthesizing DNA rises as usual after the twentieth hour of incubation. In the preparations exposed to thienylalanine this rise is abolished. It thus appears that when normal protein synthesis in the lag phase is prevented by the use of thienylalanine synthesis of DNA does not occur. But the synthesis of DNA is not immediately interrupted by transfer of the cells to the medium containing thienylalanine. The small proportion of cells in which DNA synthesis had already begun at the time of the transfer continued to synthesize DNA for some time; in a few cells synthesis of DNA could still be detected 8 hr. after the transfer. Grain counts showed, however, that although these cells continued to synthesize DNA in the presence of thienylalanine the rate of synthesis fell progressively. As judged by grain counts the amount of labelled thymidine incorporated by DNA-synthesizing cells between the seventh and eighth hour after their transfer to

Fig. 3. Effect of DL- β -2-thienylalanine on the initiation of DNA synthesis. The rise in the percentage of cells synthesizing DNA which ordinarily follows the lag phase is abolished, but cells already synthesizing DNA continue to do so for some hours. Preparations were transferred to fresh media at the point indicated by the arrow. $\circ$, Controls; $\blacksquare$, medium contains DL- β -2-thienylalanine at a concentration of 5 mM, but no phenylalanine. Each point represents a count of all the cells in six emigration zones.

medium containing thienylalanine was less than 25% of the amount incorporated in the corresponding period by the controls.

Essentially the same results were obtained with fluorophenylalanine at a concentration of 2 mM, fluorotyrosine at 2 mM and canavanine at 10 mM in media from which the corresponding natural amino acid had been omitted.

The effects produced by 8-azaguanine and the dichlorobenzimidazole riboside were less clear-cut than those produced by the amino acid analogues. This was perhaps because neither of these compounds, nor any other, suppresses the synthesis of natural RNA completely. Azaguanine forms spurious RNA, but apparently permits some normal RNA synthesis to continue (Mandel & Markham, 1958); and even at concentrations approaching the limit of solubility the dichlorobenzimidazole riboside does not abolish the incorporation of labelled adenosine into RNA completely (Tamm, 1958).

8-Azaguanine, at a concentration of 0.01 mM,

Fig. 4. Effect of 8-azaguanine on the initiation of DNA synthesis. The rise in the percentage of cells synthesizing DNA which ordinarily follows the lag phase is not abolished, but it is more gradual and reaches a much lower level than in the controls. Preparations were transferred to fresh medium at the point indicated by the arrow. O, Controls; ■, medium contains 8-azaguanine at a concentration of 0.5 mM. Each point represents a count of all the cells in six emigration zones.

arrested the multiplication of the connective-tissue cells without killing them within 24 hr. The effect produced at a concentration of 0.1 mM appeared to be similar, but at 1 mM some cells died within 24 hr. The dichlorobenzimidazole riboside at the limit of solubility arrested cell multiplication, but also caused the death of some cells within 24 hr. At one-tenth of this concentration, cell multiplication was not arrested.

The effect of exposing cells in the lag phase to 8-azaguanine is shown in Fig. 4. The preparations were incubated for 18 hr. in the usual medium and then transferred to medium containing 8-azaguanine at a concentration of 0.5 mM. It will be seen that the rise in the percentage of cells synthesizing DNA which ordinarily occurs at the end of the lag phase was not completely abolished, but it was perhaps more gradual than in the controls and it reached a much lower level. Thus although azaguanine introduced in the lag phase did not prevent the onset of DNA synthesis in all cells, it did prevent it in most. Like the amino acid analogues, however, azaguanine did not immediately interrupt the synthesis of DNA once this had begun. Cells which were in the phase of DNA synthesis at the time of transfer to medium containing azaguanine continued to synthesize DNA for many hours, although grain counts showed a progressive fall in the rate of DNA synthesis.

The dichlorobenzimidazole riboside at the limit of solubility gave similar results.

Although the experiments with azaguanine and the dichlorobenzimidazole riboside are less convincing than those with the amino acid analogues, they indicate none the less that interference with normal RNA synthesis in the lag phase prevents the onset of DNA synthesis in most cells. If normal RNA synthesis could be completely suppressed or vitiated without killing the cells, it is possible that the results would more closely resemble those obtained with the amino acid analogues.

EXPLANATION OF PLATE 1

Radioautographs of the nuclei of connective-tissue cells cultivated in medium containing tritiated thymidine. The cells were stained through the film with Nuclear-fast red. Magnification $\times 2600$.

- The nucleus shows two prominent nucleoli and one smaller one. There is no labelling over any part of the nucleus.
- The labelling is over the nucleoli or in close association with them; the rest of the nucleus has no more activity than the background.
- There is quite heavy labelling over the nucleoli and some labelling over the rest of the nucleus.
- There is no labelling over the nucleoli whereas the rest of the nucleus is heavily labelled.

H. HARRIS—THE INITIATION OF DEOXYRIBONUCLEIC ACID SYNTHESIS IN THE CONNECTIVE-TISSUE CELL,
WITH SOME OBSERVATIONS ON THE FUNCTION OF THE NUCLEOLUS

(Facing p. 58)

Role of the nucleolus in the initiation of synthesis of deoxyribonucleic acid

The nucleoli in the connective-tissue cell are very prominent structures (Plate 1a). There are usually one, two or three nucleoli per cell, but more than three are occasionally found. During the course of the present experiments it was noticed that the grains in radioautographs of cells which had been exposed to tritiated thymidine were not randomly distributed over the nucleus. Three patterns of labelling emerged. They are shown in Plate 1 (b, c, d). In cells which had been exposed to the labelled thymidine for an hour these patterns were often obscured by the density of the radioautographs; but if the time of incubation with tritiated thymidine was reduced to 5 or 10 min. the three types of labelling became very clear. In Plate 1b the labelling is over the nucleoli or in close association with them; the rest of the nucleus has no more activity than the background. In Plate 1c there is quite heavy labelling over the nucleoli and some labelling over the rest of the nucleus. When both the nucleolus and the rest of the nucleus are labelled the relative intensity of labelling may vary: either one may be more heavily labelled than the other. In Plate 1d there is no labelling over the nucleoli whereas the rest of the nucleus is heavily labelled.

Some indication of the duration of the three phases represented by these three types of radioautograph could be obtained from their relative frequencies in a population of cells multiplying at random. Although strict randomness is difficult to achieve, satisfactory conditions were obtained in populations of dissociated connective-tissue cells grown from standard inocula in coverslip chambers. The technique has been previously described (Harris, 1955a). The multiplication of cells grown in this way shows no detectable synchronization after the first 48 hr. Incubation of such cells with tritiated thymidine for 5 min. periods gave radioautographs in which about 55% of the labelled cells showed no labelling over the nucleoli, about 40% labelling over nucleoli and the rest of the nucleus and 5% or less labelling over the nucleoli only. It can be inferred then that in a phase of DNA synthesis lasting 10 hr. the stage represented by Plate 1b probably lasts half an hour or less, that represented by Plate 1c about 4 hr. and that represented by Plate 1d 5-6 hr.

Unless either nucleolar labelling or labelling over the rest of the nucleus is initiated, suspended for a period of time and then resumed, which seems very unlikely, the stage represented by Plate 1c must be intermediate in time between that represented by Plate 1b and that represented by Plate 1d. But none of the data so far presented permits one to decide whether the stages showing nucleolar

labelling precede or succeed the stage showing no nucleolar labelling. This problem was resolved by studying a population of cells in which some measure of synchronization had been produced. If most of the cells in a population are synchronized in the phase preceding DNA synthesis and then permitted to pass into and out of the phase of DNA synthesis, the incidence of any event occurring in the early part of the phase of DNA synthesis will rise as the cells pass into this phase and fall as the cells pass out of it; conversely, the incidence of any event occurring in the latter part of the phase of DNA synthesis will fall as the cells pass into this phase and rise as the cells pass out of it. Such conditions are to be found in the emigration zone when the cells pass from the lag phase into the phase of DNA synthesis, but more marked synchronization can be produced when the cells of the emigration zone are brought into suspension by treatment with trypsin and then cultivated as

Fig. 5. Variation in the proportion of labelled cells showing nucleolar labelling during the period when most of the cells in a partially synchronized population pass into and out of the phase of DNA synthesis. As increasing numbers of cells pass into the phase of DNA synthesis, the percentage of labelled cells showing nucleolar labelling rises; as increasing numbers pass out of this phase the percentage of labelled cells showing nucleolar labelling falls. O, Percentage of cells synthesizing DNA; ■, percentage of labelled cells showing either form of nucleolar labelling (Plate 1b or c); ●, percentage showing labelling in association with the nucleolus only (Plate 1b). Each point represents a count of 600 cells.

dissociated cells (Harris, 1955*a*). It was found that after the cells had been dissociated and had again become adherent to the glass surface on which they were to be grown, only 10 % of them or less were in the phase of DNA synthesis. During the next 36 hr. the percentage of cells synthesizing DNA rose rapidly and later fell rapidly. The fluctuations in the proportion of labelled cells showing nucleolar labelling during this period are set out in Fig. 5. The cells were exposed to tritiated thymidine for 5 min. periods at the times shown. It will be seen that as increasing numbers of cells move into the phase of DNA synthesis the percentage of labelled cells showing nucleolar labelling rises; and as increasing numbers move out of the phase of DNA synthesis the percentage showing nucleolar labelling falls. This is the case both for the stage represented by Plate 1*b* and that represented by Plate 1*c*. Conversely, the percentage of labelled cells showing no nucleolar labelling (Plate 1*d*) falls as increasing numbers of cells move into the phase of DNA synthesis and rises as increasing numbers move out of it. It follows then that the stages showing nucleolar labelling precede the stage showing no nucleolar labelling and that the probable sequence of events is represented by Plate 1*b*, *c* and *d*, in that order. There are therefore good grounds for concluding that the process of DNA synthesis is initiated at the nucleolus. Too little is known about the nucleolus to permit useful speculation at this stage about the possible biochemical role which it might play in this connexion; this problem is under current investigation.

SUMMARY

1. The observations described in the present paper were made on connective-tissue cells derived from explants of rat-heart tissue.

2. Between 85 and 90 % of these cells emigrate from the explants before they begin to synthesize

deoxyribonucleic acid. The lag period preceding the onset of synthesis of deoxyribonucleic acid lasts about 20 hr.; for 12 hr. of this phase, the cells are outside the explant.

3. During the lag period there is continuous synthesis of both protein and ribonucleic acid. These two processes are prerequisites for the synthesis of deoxyribonucleic acid: if either is interfered with this latter synthesis does not take place.

4. The duration of the phase of synthesis of deoxyribonucleic acid varies widely. A median figure, obtained from several hundred cells, puts the beginning of this phase between 13 and 14 hr. before metaphase and its end between 3 and 4 hr. before metaphase; mitosis is thus immediately preceded by a period in which no synthesis of deoxyribonucleic acid occurs.

5. An analysis of the patterns of nuclear and nucleolar labelling in radioautographs of cells exposed to tritiated thymidine indicates that the synthesis of deoxyribonucleic acid is initiated at the nucleolus.

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REFERENCES

- Eagle, H., Oyama, V. I., Levy, M., Horton, C. L. & Fleischman, R. (1956). *J. biol. Chem.* **218**, 607.
 Firket, H. & Verly, W. G. (1958). *Nature, Lond.*, **181**, 274.
 Harris, H. (1955*a*). *Brit. J. exp. Path.* **36**, 115.
 Harris, H. (1955*b*). *Brit. J. exp. Path.* **36**, 454.
 Harris, H. (1956). *Brit. J. exp. Path.* **38**, 512.
 Mandel, H. G. & Markham, R. (1958). *Biochem. J.* **69**, 297.
 Munier, R. L. & Cohen, G. N. (1956). *Biochim. biophys. Acta*, **21**, 592.
 Tamm, I. (1958). In *The Strategy of Chemotherapy*, p. 190. Ed. by Cowan, S. T. & Rowatt, R. Cambridge University Press.

A Substance from Animal Tissues which Induces Ketonuria

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Before insulin was generally available Petrén (1924) found that the addition of meat to the diet of diabetic patients could exacerbate or induce ketosis, whereas a diet containing a high proportion of fat reduced ketosis. Marks & Young (1939) made

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similar observations with dogs in which metahypophyseal diabetes had been induced. A reduction of ketonuria has also been observed when a high-fat diet is fed to alloxan-diabetic rats (Burn, Lewis & Kelsey, 1944; Bornstein & Nelson, 1949), and the exacerbating effect of dietary meat on the ketosis of alloxan-diabetic rats has been observed by Lazaris & Brzezinskaya (1946).