SUMMARY

The level and properties of DNA polymerase activity assayable in extracts of avian erythroid cells was studied. The enzyme was detectable in the dividing cells (erythroblasts) of the erythropoietic series and also the immature non-dividing erythrocytes. It could not be assayed in mature erythrocytes. Investigations showed that activity began to decline at the time of the last cell division of the erythroid series. Properties of the enzyme did change in different cell types; however, the changes did not correlate with cessation of DNA synthesis. Some preliminary results on DNA synthesis by isolated nuclei are also reported and these showed that only nuclei from erythroblasts could synthesize DNA in vitro in the absence of primer.

INTRODUCTION

In previous papers (Williams, 1972a-c; Appels, Wells & Williams, 1972) the various cell stages occurring in the avian erythropoietic series have been described and some attempts to elucidate the nature of the control mechanisms regulating the orderly sequence of events reported. Particular attention has been focused on the steps involved in cessation of DNA synthesis as this point marks a change from a dividing cell series involved in many synthetic functions to a terminal stage characterized by the accumulation of haemoglobin and loss of all macromolecular synthetic functions. It has been shown that the terminal cell fails to enter a new S phase (Williams, 1972a); that this probably occurs at the same cell stage in the normal and anaemic animal (Williams, 1972b); and that the production of the unique avian histone is not likely to be a controlling step in the cessation of cell division (Appels et al. 1972). However, a reduction in the level of one enzymic activity involved in DNA synthesis, i.e. deoxythymidine kinase, did correlate with the cessation of DNA synthesis (Williams, 1972c). Assays of DNA polymerase levels in cell extracts were also undertaken and are reported in this paper. The level of activity assayed was much lower in non-dividing than dividing erythroid cells.

On the basis of these results along with the earlier ones and the work of other authors some suggestions are advanced concerning the mechanism of the control of avian erythropoiesis.

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Materials and methods pertaining to the preparation of avian erythroid cells, their separation on the basis of density and labelling with [3H]deoxythymidine have all been previously reported (Williams, 1972a).

Deoxynucleotide triphosphates. Unlabelled triphosphates were from Sigma as the sodium salt. For use they were dissolved in 10^{-4} \text{M} \text{EDTA} and neutralized to pH 6-8 with 1 \text{M} \text{Tris}.

\( \alpha^{32} \text{P-} \text{dTTP} \) was prepared by the method of Symons (1969) in which \( \alpha^{32} \text{P-} \text{dTMP} \) is synthesized chemically from 3-O-acetyl deoxythymidine or deoxythymidine and phosphorylated to dTTP by an \textit{E. coli} kinase preparation.

\textit{Salmon DNA} (highly polymerized) from Sigma - was at 2.0 mg/ml in 0.01 \text{M} \text{KCl}, 4 \text{mM} \text{EDTA}, 0.01 \text{M} \text{Tris HCl} pH 7.5, for use. Denatured DNA was prepared by heating the solution at 100 °C for 10 min then cooling rapidly on ice. Hyperchromicity was 25-30%.

Activated DNA was prepared by incubating DNA with pancreatic deoxyribonuclease (Sigma No. DN-C) at 37 °C in the presence of 6 \text{mM} \text{MgCl}_2. \text{DNase} was removed with carboxymethyl cellulose (Keir, 1962) and the activated DNA dialysed against the above buffer. Tests showed that optimum priming activity was obtained when DNA at 2.0 mg/ml was incubated with \( 2\times10^{-4} \text{mCl} \text{m} \text{ml} \) of DNase for 1 h; no release of acid-soluble nucleotide was detected; hyperchromicity fell from 25 to 21%. Denatured DNA for deoxyribonuclease assays was prepared by incubating Salmon DNA at 2.0 mg/ml in 0.5 \text{M} \text{KOH} for 4 h then dialysing against the above buffer. This removed any RNA and then the solution was additionally heat denatured.

Preparation of cell extracts

For the standard extracts 1 vol. of cells was suspended in 15 vol. of polymerase buffer (0.15 \text{M} \text{KCl}, 0.01 \text{M} \text{Tris HCl} pH 7.5; 5 \text{mM} \text{mercaptoethanol and 1 mM} \text{EDTA}), (approx. \( 2 \times 10^8 \text{cells per ml of buffer} \), and lysed by twice freezing and thawing. The supernatant extract was then obtained after centrifugation at 35,000 \text{g} for 60 min.

Extracts could be stored at −15 °C but were always assayed within 1 week without loss of activity.

Preparation of nuclei

Cells were suspended in cold sucrose buffer (0.44 \text{M} \text{sucrose, 0.01 M} \text{Tris HCl} pH 7.5; 3 \text{mM} \text{MgCl}_2; 5 \text{mM} \text{mercaptoethanol; 1 mM} \text{EDTA}) (Dounce & Ickowicz, 1969). Saponin was added to 0.25%. After standing on ice for 10 min nuclei were recovered and washed 3 times in the above buffer minus saponin by centrifugation at 1500 \text{g} for 5 min. Electron microscopy showed that the preparation consisted of nuclei plus membranes – there was no contamination by soluble cytoplasmic proteins for when nuclear preparations were solubilized by sonication and examined in a spectrophotometer no absorption characteristic of haemoglobin was observed.

Assay of DNA polymerase activity

Assays were similar to those of Keir (1965) measuring the incorporation of \( \alpha^{32} \text{P-} \text{dTTP} \) (supplied as \( \alpha^{32} \text{P-} \text{dATP} \)) into acid-insoluble material.

The standard assay was in a final volume of 0.25 ml and contained 0.05 ml cell extract. Per assay there was 15 \text{nmol} (with native DNA primer) or 3 \text{nmol} (with denatured DNA) \text{MgCl}_2; 15 \text{nmol} \text{KCl}; 5 \text{nmol} \text{Tris HCl} pH 7.5; 0.1 \text{mM} \text{EDTA}; 50 \text{nmol} \text{dATP}, \text{dTTP}, \text{dGTP}, \text{dCTP} plus 5 \times 10^5 \text{cpm} \alpha^{32} \text{P-} \text{dTTP} (0.5 \text{Ci/mole}); 200 \text{µg} \text{DNA primer (native or denatured)}; 15 \text{µmol} \text{mercaptoethanol}. When activated DNA primer was used 150 \text{µg} were added.

Incubations were for 1 h at 37 °C and were terminated by the addition of 3 volumes of cold water plus carrier BSA and DNA followed immediately by 12 ml of 7% trichloroacetic acid and 1% pyrophosphate. The precipitate was obtained by centrifugation, redissolved in 0.5 ml of cold 0.2 \text{M} \text{NaOH} and precipitated twice more. It was finally collected on fibre filters (Whatman GF/C) to allow determination of radioactivity as previously reported (Williams, 1972a).
DNA polymerase in avian erythroid cells

DNA synthesis by isolated nuclei

The assay was similar to the above except that primer DNA was omitted.

The incubation mixture contained in 0.25 ml: 0.1 ml nuclear preparation (3 x 10^7 nuclei); 1.5 μmol MgCl₂; 50 nmol dATP, dCTP, dGTP; 7.5 x 10⁶ cpm α³²P-dTTP (0.5 mCi/μmol). In some experiments cytoplasmic extracts were also added.

After incubation at 37 °C the reaction was stopped by adding 15 ml of cold sucrose buffer. The nuclei were pelleted and resuspended to be precipitated and processed for counting as described for DNA polymerase assays on supernatant extracts.

Assay of deoxyribonuclease activity

This was assayed by determining the release of acid-soluble 260 nm-absorbing material from DNA (Keir, 1962).

Incubations were as for the DNA polymerase assays except that deoxynucleotide triphosphates were omitted. The incubation was terminated by the addition of 3 ml of 5% perchloric acid. After holding the tubes for 1 h at 4 °C the precipitate was removed by centrifugation and the supernatants filtered through oxoid membranes prior to reading O.D. 260 nm. Time course incubations were always performed and controls lacking DNA included in all assays.

It was found that the avian deoxyribonuclease hydrolysed denatured DNA 7 times more rapidly than native DNA and thus the former substrate was used in the standard assay.

RESULTS

Assay requirements

The assay requirements for avian erythroid polymerase were similar to those found for other eukaryotic DNA polymerases (Mantsavinos & Munson, 1966; Keir, 1965; Loeb, Mazia & Ruby, 1967). The incorporation of α³²P-dTTP into acid-insoluble material was more than 95% dependent on addition of DNA primer and magnesium ions. It was also dependent on the presence of 4 deoxynucleotide triphosphates to the extent that omission of any one of dATP, dCTP or dGTP reduced activity by 60% while omission of all 3 caused a 90% loss of incorporation.

The product of the reaction was hydrolysed to the extent of 93% by deoxyribonuclease, but not ribonuclease under the same conditions.

In the assay conditions used incorporation was always proportional to time and amount of extract.

Levels of DNA polymerase activity in extracts from different cell preparations

Prior to studying avian erythroid cells fractionated on density gradients the levels of DNA polymerase in extracts from mature blood, anaemic blood and anaemic bone marrow cells were determined. The DNA polymerase levels were assayed with native and denatured DNA primer.

Deoxyribonuclease-activated DNA was also used as a primer as it is well known (Keir, 1962, 1965) that this enzyme can affect the assayed activity of DNA polymerase by improving the priming potential of the DNA.

The results are shown in Table 1. Significant DNA polymerase activities were found in all extracts except those prepared from normal blood cells which were predominantly mature erythrocytes. Anaemic blood cells more than 99% of which were non-dividing cells, retained quite a high level of DNA polymerase activity, with the
A. F. Williams

Table 1. Levels of DNA polymerase in extracts of erythroid cells

<table>
<thead>
<tr>
<th>Source of extract and no. of samples</th>
<th>DNA polymerase activity (nmol/h/0.05 ml extract)</th>
<th>N/D ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native DNA</td>
<td>Denatured DNA</td>
</tr>
<tr>
<td>Normal blood (3)</td>
<td>0.004 ± 0.001</td>
<td>0.002 ± 0.002</td>
</tr>
<tr>
<td>Anaemic blood (7)</td>
<td>0.323 ± 0.05</td>
<td>0.160 ± 0.045</td>
</tr>
<tr>
<td>Anaemic bone marrow (6)</td>
<td>0.72 ± 0.23</td>
<td>1.05 ± 0.21</td>
</tr>
<tr>
<td>Ehrlich Ascites (1)</td>
<td>0.22</td>
<td>0.50</td>
</tr>
</tbody>
</table>

With activated DNA primer

<table>
<thead>
<tr>
<th></th>
<th>Unheated</th>
<th>Heated</th>
<th>U/H ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal blood (1)</td>
<td>0.002</td>
<td>0.001</td>
<td>—</td>
</tr>
<tr>
<td>Anaemic blood (3)</td>
<td>0.82 ± 0.26</td>
<td>0.44 ± 0.18</td>
<td>1.85</td>
</tr>
<tr>
<td>Anaemic bone marrow (2)</td>
<td>2.25 ± 0.12</td>
<td>1.63 ± 0.07</td>
<td>1.38</td>
</tr>
</tbody>
</table>

DNA polymerase activity of cell extracts was assayed in the standard assay with the exception that for assays of Ehrlich Ascites extract 6 mM MgCl₂ was used with native as well as denatured DNA.

The values shown for erythroid extracts are mean ± 2 S.E. of mean for the number of determinations shown in brackets.

The different extracts were made equivalent in terms of packed cell volume with 3.3 x 10⁻³ ml of cells for each assay. This was equivalent to 2.75, 1.95 and 1.45 x 10⁷ cells from normal blood, anaemic blood and anaemic bone marrow cells, respectively.

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extracts from anaemic bone marrow cells (a large number of which are erythroblasts) showing the highest activity of all. (Full details of cellular composition of the various cell sources can be found in Williams, 1972a). If the data had been expressed on the basis of cell number rather than cell volume the difference between anaemic blood and bone marrow extracts would be greater still (see legend to Table 1).

When deoxyribonuclease-activated DNA was used as primer the picture was the same with the exception that the levels of activity in extracts containing DNA polymerase were higher. As the differential between anaemic blood and anaemic bone marrow extracts was maintained it seems unlikely that the higher levels in the bone marrow extracts could be explained by higher deoxyribonuclease activity effecting the nature of the primer DNA.

From the results in Table 1 one can tentatively suggest that DNA polymerase activities are higher in erythroblasts than in polychromatic erythrocytes, but are finally lost on maturation of the polychromatic to the mature erythrocyte.

**DNA priming and Mg²⁺ optima of the DNA polymerase in erythroid cell extracts**

In assaying DNA polymerase in erythroid cell extracts a full study was made of the enzymic properties with respect to DNA priming requirements and Mg²⁺ optima. This was because some authors (Keir, 1965; Loeb et al. 1967) have postulated that the properties of the DNA polymerase in this regard may reflect its intracellular role.
DNA polymerase in avian erythroid cells

Fig. 1. Effect of primer DNA and Mg^{2+} concentration on erythroid DNA polymerase activity. A, B, effect of level of DNA primer and [Mg^{2+}] on activity of extract from anaemic blood: ■, native DNA primer; ○, denatured DNA primer. C, D, effect of level of DNA primer and [Mg^{2+}] on activity of extract from anaemic bone marrow: □, native DNA primer; ○, denatured DNA primer. All reagents except the one being varied were as in the standard assay. The addition of primer DNA is shown as µg assay (0.25 ml). DNA polymerase activity is given as nmol α³²P-dTMP incorporated into acid-insoluble material/0.05 ml extract/h. The number of cells contributing to each assay was as in Table 1.
Table 2. DNase activities in anaemic blood and bone marrow cell extracts

<table>
<thead>
<tr>
<th>Deoxyribonuclease activity (ΔO.D. per h/0.1 ml extract)</th>
<th>Anaemic bone marrow cells</th>
<th>Anaemic blood cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity</td>
<td>0.389 ± 0.022 (5)</td>
<td>0.068 ± 0.008 (4)</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.148 ± 0.044 (4)</td>
<td>0.008 ± 0.008 (4)</td>
</tr>
<tr>
<td>Nuclear</td>
<td>0.248 ± 0.012 (2)</td>
<td>—</td>
</tr>
</tbody>
</table>

Cells were lysed in the presence of DNA polymerase buffer by freezing and thawing and supernatant and nuclear extracts prepared. The figures shown are mean values ± 2 S.E. – in brackets are the number of separate extracts assayed. The number of cells contributing to each assay was twice that given in Table 1. If total hydrolysis of DNA substrate had occurred ΔO.D. would be 1.6.

Specifically it has been suggested that the replicative DNA polymerase may be primed preferentially by native DNA and have high Mg\(^{2+}\) optima.

As can be seen from Table 1 and Fig. 1 (A and c) DNA polymerase from different avian erythroid cell extracts did show different properties with respect to DNA priming requirements with the enzyme from anaemic bone marrow cell extracts favouring denatured DNA, while that from anaemic blood cells showed higher activity with native DNA. However, this result was the opposite to that predicted if a replicative polymerase required native DNA as in the anaemic bone marrow extracts about 60% of the cells are in S phase while in anaemic blood only 0.2% are synthesizing DNA (Williams, 1972a).

Mg\(^{2+}\) ion optima (Fig. 1B and D) differed in both extracts with different DNA primers; a higher level of Mg\(^{2+}\) being needed for maximal activity with denatured DNA than with the native form. This difference has not been reported with DNA polymerases from other cell extracts (Loeb et al. 1967). However, one can be fairly sure that the results are not due to trivial artifacts since DNA polymerase extracts of Ascites tumour cells were found to have the properties expected (Shepherd & Keir, 1966) when assayed under exactly the same conditions as used for the erythroid extracts. That is, with Ascites extracts denatured DNA was the better primer (Table 1), and the enzyme with both this and native DNA had its Mg\(^{2+}\) optima at 6 mM (author's unpublished observation).

Despite the description of some unusual properties for erythroid DNA polymerase assayed in cell extracts there were no correlations of interest to be found between the enzyme's properties and the DNA-synthetic capabilities of the cell population from which the extract was derived.

Deoxyribonuclease activities and assays for other activators and inhibitors

The main point of interest outlined thus far is the difference in levels of DNA polymerase in various erythroid cell extracts. As assays of DNA polymerase activity are notably prone to differences due to effects other than the level of the enzyme.
Table 3. Mixing experiments to test for activators and inhibitors of DNA polymerase

<table>
<thead>
<tr>
<th>Extract</th>
<th>DNA polymerase activity (nmol/h/0.05 ml extract) assayed with</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native DNA</td>
<td>Denatured DNA</td>
<td></td>
</tr>
<tr>
<td>Normal blood (NB)</td>
<td>0.01</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Anaemic blood (AB)</td>
<td>0.32</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Anaemic bone marrow (ABM)</td>
<td>0.81</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>NB + AB</td>
<td>0.31 (-6%)</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>NB + ABM</td>
<td>0.88 (+7%)</td>
<td>1.11 (-5%)</td>
<td></td>
</tr>
<tr>
<td>AB + ABM</td>
<td>1.11 (-3%)</td>
<td>1.04 (-20%)</td>
<td></td>
</tr>
</tbody>
</table>

Extracts from the various erythroid cell preparations prepared as in Table 1 were assayed alone and in mixtures in the standard assay. The values shown are means of assays done in triplicate. The figures in brackets show the percentage deviations of the observed value when extracts were mixed from that calculated on the basis of the extracts assayed independently.

Changes in DNA polymerase levels and properties in erythroid cells fractionated on BSA density gradients

Having characterized the DNA polymerase activities in crude extracts from mixed erythroid cell preparations, those of the more homogeneous preparations obtainable after density gradient centrifugation (Williams, 1972a) were studied. Experiments with these purified preparations allowed a closer identification of the cell types in which the changes in levels and properties of avian erythroid DNA polymerase occurs.

Results of an experiment with fractionated anaemic bone marrow cells are shown in Fig. 2. A typical separation of cell types was obtained (compare with Williams, 1972a) and is characterized in Fig. 2A by the data on median cell volume and percentage cells labelled with [3H]deoxythymidine. At densities less than 1.068 g cm⁻³ most cells were dividing erythroblasts while after this density most were non-dividing.
Fig. 2. DNA polymerase activities from anaemic bone marrow cells of different densities. 1 ml of packed cells of anaemic bone marrow was incubated for 15 min in 10 ml of EBMS with 12.5 μCi of [3H]deoxythymidine at 37 °C. After the incubation the cells were centrifuged on a BSA density gradient. On the cell fractions obtained the following parameters were determined. A, •, median cell volume (μm³); •, [3H]-deoxythymidine incorporation as cpm/2 x 10⁷ cells; ▼, % cells labelled. B, DNA polymerase activities (nmol α3²P-dTMP incorporated/2 x 10⁷ cells/h assayed in the standard assay with native and denatured DNA primer): ■, native DNA; ●, denatured DNA. C, DNA polymerase activities as for B but assayed with deoxyribonuclease-activated DNA: □, unheated primer; ○, heated primer.
DNA polymerase in avian erythroid cells

Fig. 2B shows DNA polymerase activities assayed with native and denatured DNA. In erythroblast fractions the activity was relatively constant per cell number even though the cell volume fell by about 50%. The activity began to decline as one progressed from fractions containing erythroblasts to others containing polychromatic erythrocytes. However, DNA polymerase activity was not proportional to the number of labelled cells (at a density of 1.075 g cm⁻³ 5% of the cells were labelled compared with 55% in erythroblast fractions, but DNA polymerase activity was still at 25% of its maximum value). This confirms the results from unfractiuated cell preparations showing that non-dividing cells still contain DNA polymerase.

The change in priming ratio away from a preference for denatured DNA was observed to a limited extent. However, this change appeared to occur in cells more mature than those which had just ceased DNA synthesis and this confirmed the view that any such correlations are unlikely to be an important factor as far as control of replication is concerned.

Fig. 2C shows DNA polymerase activities assayed with deoxyribonuclease-activated DNA. The results bear out the conclusions from Fig. 2B.

Histological staining of cells suggested it was unlikely that the DNA polymerase activities observed are due to any significant extent to non-erythroid cells. In all fractions shown in Fig. 2 at least 87% of the cells were of the erythroid series. Of the non-erythroid cells lymphocytes accounted for 1-4% of cells throughout the gradient. Lymphocytes are small differentiated cells which do not incorporate [³H]deoxythymidine and have low DNA polymerase levels (Loeb, Agarwal & Woodside, 1969). Mature heterophils constituted 8-10% of cells at densities greater than 1.075 g cm⁻³, but these obviously do not markedly contribute to DNA polymerase activities. In the erythroblast fractions there were other blast cells. However, it is unlikely that these had larger amounts of DNA polymerase than erythroblasts for the non-erythroid blast cells tended to concentrate in the lowest-density fractions and extracts from these cells did not have higher DNA polymerase activities than the more homogeneous small erythroblast fractions toward the middle of the gradient.

In experiments with fractionated anaemic bone marrow cells one examines only the cell range to the level of early to mid-polychromatic erythrocytes. By fractionating anaemic blood one can observe the continued loss of DNA polymerase activity in more mature cells. When experiments with these cells fractionated on BSA density gradients were done a continuation of the pattern set in the anaemic bone marrow studies was observed. With increasingly denser cells of greater maturity DNA polymerase levels declined markedly to barely detectable levels. This was expected from the fact that DNA polymerase activity cannot be assayed in extracts from mature erythrocytes.

Nuclear DNA polymerase

If cell extracts were prepared in the standard way outlined in Methods, 97.5% of the total DNA polymerase activity was to be found in the supernatant fraction. Other extraction methods were investigated, notably lysis without freezing (by the addition
Table 4. Distribution of avian erythroid DNA polymerase in anaemic bone marrow and blood cells lysed by various methods

<table>
<thead>
<tr>
<th>Cell preparation and buffer</th>
<th>Ratio nuclear/total DNA polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freeze-thaw</td>
</tr>
<tr>
<td>Anaemic bone marrow</td>
<td></td>
</tr>
<tr>
<td>Polymerase buffer</td>
<td>0.025 (1)</td>
</tr>
<tr>
<td>Sucrose buffer</td>
<td>—</td>
</tr>
<tr>
<td>Anaemic blood</td>
<td></td>
</tr>
<tr>
<td>Polymerase buffer</td>
<td>—</td>
</tr>
<tr>
<td>Sucrose buffer</td>
<td>—</td>
</tr>
</tbody>
</table>

Cells were lysed by the methods shown and nuclear and supernatant extracts prepared. These were assayed in the standard assay with the exception that unheated activated DNA was used as primer.

The figures are the means of the number of observations shown in brackets. Total polymerase activities were 2.7 nmol/h/0.05 ml extract for anaemic bone marrow extracts and 0.58 nmol/h/0.05 ml extract for those from anaemic blood. The amounts of cells contributing to each assay are as for Table 1.

Of saponin to 0.25 %) and use of a sucrose buffer instead of polymerase buffer (see Methods for buffer compositions).

While the method of lysis did not affect the distribution of DNA polymerase between nuclei and cytoplasm, the buffer used did. As can be seen from Table 4 when erythroblast cell fractions were assayed 25 % of the enzyme was retained in the nucleus when preparations were in sucrose buffer.

This result is consistent with those of Keir (1965), who found that the presence of 0.15 M KCl extracted DNA polymerase from cell nuclei.

Teng, Block & Roychoudhury (1970) have isolated a polymerase from nuclei of Ehrlich Ascites cells which prefers native DNA in comparison to the total cell activity which is more active with denatured DNA. The question of priming preference was investigated with polymerase from erythroblast nuclei. These were initially isolated in sucrose buffer and then extracted with polymerase buffer. The priming ratio was then determined on this extract and found to be 0.77:1.0 with native compared with denatured DNA. This was the same as determined for the whole cell extract (see Table 1 and Fig. 1c).

A further point of interest was the observation that under all conditions the percentage of DNA polymerase in the nuclei was low in anaemic blood cells (Table 4). It seemed possible that the movement of DNA polymerase from the nuclei correlated with the cessation of DNA synthesis. However, when this was examined with cells from anaemic bone marrow separated on BSA density gradients clear-cut correlations were not obtained. In one experiment the dividing cells had 30 % of the polymerase assayed in their nuclei compared with 15 % for the most immature non-dividing cells. In 2 other experiments no differences were found.
DNA polymerase in avian erythroid cells

Fig. 3. DNA synthesis in vitro by isolated nuclei compared with that in corresponding whole cells. Cells were obtained from normal and anaemic blood and from anaemic bone marrow fractionated into light and heavy fractions by centrifugation in BSA of density 1.069 g cm⁻³. Cells from each preparation were suspended at 4 × 10⁷ cells/ml in EBMS and incubated with 5 μCi of [³H]deoxythymidine/ml. After 20 min incubation triplicate samples containing 2 × 10⁶ cells were taken and the amount of isotope in acid-insoluble material estimated. The results are shown as cpm of ³H incorporated/2 × 10⁷ cells/20 min. From the rest of the cells of each preparation, nuclei were isolated and assayed for the incorporation of ³²P-dTTP into DNA; 3 × 10⁷ nuclei were added per assay. Incorporation in vitro is plotted against time. ■, mainly erythroblasts; ●, mainly early polychromatic erythrocytes; ○, mid- and late polychromatic erythrocytes; □, mature erythrocytes.

Thus, it seemed that the low levels of nuclear polymerase in the mid- and late polychromatic erythrocytes of anaemic blood were due to changes occurring after the cessation of DNA synthesis.

DNA synthesis by nuclei in the absence of primer DNA

The fact that nuclei from erythroblasts contained significant amounts of DNA polymerase suggested that they may be capable of DNA synthesis in the absence of primer DNA. Some preliminary experiments examining this question have been carried out.
It was found that nuclei prepared in sucrose buffer from erythroblasts could incorporate $^{32}\text{P-dTTP}$ into acid-insoluble material without added DNA primer. This incorporation was dependent on the presence of dATP, dCTP and dGTP to the extent that their omission caused a 90% reduction in counts incorporated. The product of the assay was totally resistant to incubation in 0.25 N KOH at 37 °C for 1 h, but hydrolysed by deoxyribonuclease. The activity was destroyed if the cells or nuclei were at any stage frozen and thawed.

Having to some extent characterized the assay, nuclei were prepared from low-density anaemic bone marrow cells (mainly erythroblasts); higher-density bone marrow cells (non-dividing early polychromatic erythrocytes); anaemic blood cells (mid- and late polychromatic erythrocytes) and mature erythrocytes.

These were assayed for their ability to incorporate $^{32}\text{P-dTTP}$ into DNA. As shown in Fig. 3 this activity correlated strikingly with the ability of the whole cells from which the nuclei were prepared to incorporate $[\text{H}]\text{deoxythymidine}$ into DNA.

The capacity to synthesize DNA seemed inherent in the nuclei as cytoplasmic extracts from erythroblasts could not stimulate the inactive nuclei to DNA synthesis (author's unpublished observations).

Friedman & Mueller (1968) found a similar effect in studies on nuclei from synchronized HeLa cells. Nuclei from cells incapable of DNA synthesis were not stimulated by cytoplasmic extracts from S-phase cells.

**DISCUSSION**

To summarize, the experiments reported in this paper suggest that DNA polymerase activity is constant in erythroblast fractions of the erythropoietic series (although cell volume falls), and begins to decline only when DNA synthesis ceases. From this stage on the activity declined gradually to undetectable levels in the mature erythrocyte.

Although the properties of the DNA polymerase, particularly its preference for native or denatured DNA, were not the same at all cell stages, the differences observed did not seem to be significant with regard to control of DNA synthesis. Similarly, simple correlations between an exclusively nuclear localization of the enzyme and DNA synthesis were not found.

Preliminary observations on DNA synthesis by isolated nuclei suggested that only nuclei from dividing cells of the erythroid series would support DNA synthesis in vitro.

When the results on DNA polymerase activities are compared with those reported for deoxythymidine kinase levels in avian erythropoiesis (Williams, 1972b) similarities are apparent. The only difference being the rate of loss of activity beginning at the last cell division. It seems possible that at this stage of erythropoiesis many synthetic activities begin to be lost from the cell. Results in this paper suggest that deoxyribonuclease, and some nuclear factor involved in DNA synthesis may be in this category as well as DNA polymerase and deoxythymidine kinase. It is also possible that ribosomal RNA synthesis ceases at this time as this occurs to a small extent only in anaemic blood cells (Attardi, Parnas, Hwang & Attardi, 1966; Scherrer & Marcuard, 1968).

One major problem with regard to control of erythropoiesis is the definition of the
point at which transcriptional control of cell function ceases. Studies on tissue culture of erythropoietic cells from embryonic sources have emphasized the importance of early events in erythropoiesis, particularly the recruitment of stem cells into the differentiating series (Chui, Djaldetti, Marks & Rifkind, 1971) and the early synthesis of haemoglobin m-RNA (Djaldetti, Chui, Marks & Rifkind, 1970; Fantoni, DeLa-Chapelle, Rifkind & Marks, 1968; Wilt, 1965).

From these studies one might argue that transcriptional control ceases in early erythroblasts of the erythroid series. However, some of the tissue culture work (Fantoni et al. 1968) as well as my studies on enzymes associated with DNA synthesis suggest that transcriptional events could be important until the stage of the last cell division. It seems attractive to suggest that after this stage there is no further transcriptional control of the maturation processes and that the decline in macromolecular synthesis is governed simply by the half-life of the limiting factors involved. The idea that decline occurs due to lack of gene activation rather than specific repression seems more easily accepted in a situation where many activities are being lost simultaneously. A lack of specific repression is consistent with the reactivation of nuclear activity when mature erythrocytes are hybridized with cells fully competent for macromolecular synthesis (Harris, Sidebottom, Grace & Bramwell, 1969).

The possible failure of essential gene activation at the stage of the last cell division could be due to dilution of a control factor occurring throughout the series of erythroblast divisions (Weintraub, Campbell & Holtzer, 1971).

Tissue culture with the relatively homogeneous preparations of cells obtainable from anaemic avian bone marrow could provide the answers to some of the problems involved in understanding the mechanisms of the control of cell specialization seen in erythropoiesis.

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