properties of cellular systems are indicated as being predictable from and formally analogous with the well-understood behavior of oil-and-water systems.

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*THE ONTOGENY OF CHICK EMBRYO HEMOGLOBIN**

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A fundamental issue in cellular differentiation is the mechanism by which a portion of the genome is selectively expressed. Differential gene activity in embryogenesis may be indirectly approached by a study of the origin of protein synthesizing machinery. Hemoglobin is a suitable protein to use as an index of activity of a particular protein synthesizing system. Its structure, function, genetic regulation, physiological role, and mode of synthesis are better understood than those of any other protein of the metazoa. The present investigation is a description of the time and place of origin of globin, heme, and hemoglobin in the developing chick embryo. The results serve as a foundation from which to launch investigations on the mode of control of synthesis of hemoglobin. Furthermore, the present analysi presents some interesting features of the ontogeny of proteins. First, as other investigators have shown,[1] a protein population characteristic of a particular tissue differentiation may appear long before visible morphological differentiation occurs; second, the relative proportions of classes of proteins in a population may differ at different developmental ages; third, the relative rates of synthesis of an apoprotein and its prosthetic group may change during differentiation.

Methods.—(1) Preparation of hemoglobin (Hb). Hemoglobin was prepared from adult white Leghorn chicken blood by a procedure similar to Drabkin's.[2] After collection of citrated whole blood the erythrocytes were washed 7 times in saline, hemolyzed in saline containing 0.1% saponin, and stroma and ghosts removed by centrifugation. A crude Hb was obtained by precipitation with 2.8 *M* potassium phosphate buffer (pH 6.8), and further purified by three cycles of ammonium sulfate fractionation, the 60–70% saturated fraction containing Hb. The Hb was finally crystallized by dialysis against 2.8 *M* potassium phosphate buffer (pH 6.8). The extent of purification was estimated by spectrophotometric determination of heme to protein ratios,[3] DNA contamination,[4] and zone electrophoresis.[5] No further purification was obtained by ethanol precipitation, alumina gel cream adsorption, protamine sulfate precipitation, or DNase digestion. Protein was determined by the method of Lowry *et al.*[6]

(2) Hb antisera. Antisera to Hb were prepared in 8 rabbits. After a primary injection of 40 mg of Hb in Freund's adjuvant, followed by a 10-day rest, 20 mg of alum precipitated Hb was injected into the marginal ear vein on alternate days for 10 injections. After a 7-day rest, another 7 injections of alum precipitated Hb were made, and the rabbits were bled 5–12 days after the last injection. γ-globulin was prepared from antisera by 5 cycles of precipitation with 50% saturated ammonium sulfate. The final precipitate was dissolved in a volume of 0.9% saline equivalent to one-half the original volume of serum and dialyzed against 0.9% saline. γ-globulin preparations were exhaustively absorbed with lipid free egg yolk powder (Schechtman and Hoffman[7]). Interfacial ring tests were carried out according to Boyd.[8] Double diffusion Ouchterlony analyses were carried out in washed agar containing 0.9% NaCl, 0.01% merthiolate, and 0.01 *M* tris hydroxyl methyl amino methane buffer (pH 7.2).[9]

(3) Electrophoresis and immunoelectrophoresis on agar were carried out on microscope slides according to the method of Wieme.[10] Starch block electrophoresis was performed as recommended by Kunkel,[5] and starch gel electrophoresis carried out by the procedure of Smithies.[11]

(4) Culture of chick blastoderms in watch glass cultures was carried out by Spratt's technique.[12] All media contained 100 mg % glucose, 0.65% agar, Howard's chick saline, and either potassium phosphate–sodium bicarbonate buffer or diluted egg white (final pH 7.9). All embryos were from a single flock of white Leghorn chickens. Extracts of embryos were prepared by washing the blastoderm, or pieces of it, in 3 changes of saline, freezing and thawing of the embryos after macerating in a small volume of distilled water, and centrifugation for 30 min at 30,000 $\times$ gravity. The supernatant was carefully aspirated leaving the pad of lipid in the centrifuge tube.

(5) Double labeling studies were carried out by culturing blastoderms for 5 hr on Spratt's albumen medium containing H^3 leucine and $Fe^{59}Cl_3$. Extracts of blastoderms were reacted with control and immune globulins. After incubation for 1 hr at 37°C and 23 hr at 4°C, the antigen-antibody precipitates were isolated by centrifugation at 30,000 $\times$ gravity, and the precipitates washed 3 times with 0.9% saline buffered at pH 7.0 with 0.1 *M* potassium phosphate. The precipitates were dissolved by heating at 80° for 30 min in 0.05 ml of 1 *N* KOH and transferred to low K^{40} glass scintillation counting vials. Liquid scintillation counting procedures were based on the procedure of Brown and Badman.[13] The vials contained the antigen-antibody precipitate dissolved in 1 *N* KOH, 1.5 ml of 1 *M* Hyamine‡ hydroxide in methanol, 0.05 ml of 2 *N* HCl, and 8 ml of redistilled toluene containing 4 gm of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2(5-phenyloxazolyl)-benzene per liter. Counting was carried out on a split channel Packard Tri-Carb scintillation counter at a photomultiplier voltage of 880 volts. The lower pulse height discriminator registered pulses between 5 and 45 volts, giving an efficiency of 3.77% for H^3 and 36.3% for Fe^{59} with a background of 29.4 cpm. The upper pulse height discriminator collected pulses from 45–100 volts, counting H^3 with an efficiency of 0% and Fe^{59} with an efficiency of 16.7%, the background being 6 cpm. All vials were subsequently re-examined for quenching after counting by the addition of H^3 toluene and Fe^{59} standards.

Fe^{59} counts were corrected for decay from the time of culture using a half-life of 45.1 days.

Other methodological details are described in the text.

The Adult Hemoglobin.—An important detail in the preparation of avian hemoglobin is to avoid rupture of the erythrocyte nuclei with subsequent release of large amounts of DNA. The use of saponin for hemolysis satisfies this requirement. The final product had an absorption spectrum similar to adult human Hb. The oxyhemoglobin, methemoglobin, cyanomethemoglobin, carbonmonoxyhemoglobin, and reduced hemoglobin derivatives were prepared. They all showed absorption spectra characteristics similar to the same derivatives of human hemoglobin.[14] The preparation contained no detectable DNA (<0.001%). Analytical ultracentrifugation in the Spinco Model E centrifuge disclosed only one homogeneous component. Quantitative precipitation profiles determined by ammonium sulfate and potassium phosphate precipitation demonstrated apparent homogeneity.[15] No nonheme proteins (evaluated by comparing amido-black and benzidine staining) were present in zone electrophoretic analyses.

Moving boundary electrophoresis disclosed an initial sharp peak which, after prolonged electrophoresis, gave rise to two components of similar mobilities. Agar gel electrophoresis at pH values from 5.6–8.6 in potassium phosphate, sodium citrate, sodium barbituate, and boric acid buffers revealed only two components (Fig. 1). Methemoglobin, cyanomethemoglobin, and carbonmonoxyhemoglobin

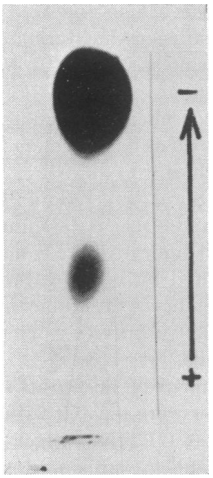

FIG. 1. — Adult chicken Hb was subjected to agar gel electrophoresis according to the procedure of Wieme.[10] (0.05 *M* veronal buffer, pH 8.6.)

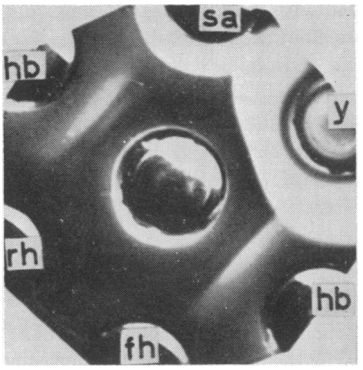

FIG. 2.—Gamma globulin from immune serum was placed in the central well in this double diffusion test of the specificity of the antibody. Chicken hemoglobin (hb), bovine serum albumen (sa), an aqueous extract of egg yolk (y), rat hemoglobin (rh), and frog hemoglobin (fh) were deposited in the peripheral wells.

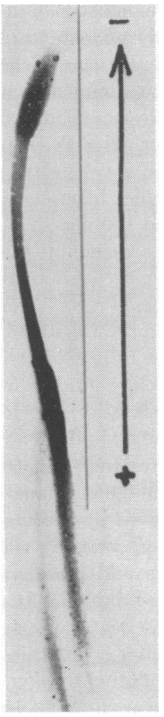

FIG. 3. — A typical microimmunoelectrophoretic pattern displayed by adult chicken hemoglobin was obtained after electrophoresis at 16 V/cm for 45′ in 1.5% agar containing 0.05 *M* veronal buffer (pH 8.5). Enlarged three times.

derivatives also displayed two components after agar gel electrophoresis. The mobilities of the methemoglobin derivatives were slightly lower than those of the components of oxyhemoglobin. Both of the components evident after zone electrophoresis contained heme. Starch gel electrophoresis and starch block electrophoresis in borate and barbital buffers at pH 8.6 revealed two components, although the separation was not as distinct as with agar gel electrophoresis. Huisman[16] has also reported two Hb fractions of adult chicken hemoglobin after chromatography on amberlite IRC-50, and Saha *et al.*[17] and Fraser[18] have described two components after paper electrophoresis.

The immunological response of rabbits to Hb was qualitatively similar in all 8 of the rabbits. Precipitating antibodies were present in substantial amounts in 6 of the 8 rabbits. The antisera and γ-globulins prepared from them showed no precipitin reaction in agar gel diffusion tests with bovine serum albumin, frog hemoglobin, rat hemoglobin, rabbit hemoglobin, or aqueous extracts of fresh egg yolk (Fig. 2). Interfacial ring tests with undiluted antisera showed clearly visible reactions with 0.001 per cent Hb, and reactions in agar gel diffusion tests using γ-globulin showed clear reactions with 0.1 μg of Hb. Agar gel double diffusion tests carried out over a wide range of antigen dilutions revealed two precipitation lines, one line

being much more dense than the other. The same two lines appeared when purified globin was used as the antigen (prepared by the method of Anson and Mirsky[19]), and Bjorklund inhibition tests[20] showed heme did not participate in the precipitin reaction. It is concluded that the use of these antisera results in the detection of two avian specific globins. There was never any reaction with control sera or γ-globulin fractions prepared from them.

Immunoelectrophoresis of the adult Hb disclosed that the major electrophoretic component gives rise to the dense precipitation line with antibody, and the minor component reacts to give a fainter and distinct line. There is, however, an invariable "tailing" of the heavy precipitation line into the region opposite the minor electrophoretic component, as shown in Figure 3. This is probably the result of incomplete electrophoretic resolution of the two components after agar gel electrophoresis.

In summary, the hemoglobin of the adult chicken is apparently a mixture of two similar globin populations, which differ slightly in electrophoretic mobility and immunological reactivity.

Embryonic Globin.—The time of appearance and type of globin present in the developing embryo were investigated by immunological methods employing the agar gel diffusion test. Extracts of 10–20 embryos in a volume of 0.3 ml were sufficient for an analysis. The extracts contained 0.5–1.5 mg protein per ml. Blastoderms of unincubated eggs contained two immunologically distinct reactants. The same two components, or precipitation lines, were present until 36–48 hours of development. One of the two precipitation lines gave reactions of identity with the minor adult immunological component; the other was distinct from both adult components. 48- and 72-hour blastoderms contained two components immunologically identical to the adult globins (Figs. 4–6). The interpretation is that the early chick blastoderm contains one component immunologically identical to the minor adult globin. A second distinct component which cross-reacts with the antibodies only persists until 48 hours of development. Furthermore, the evidence suggests that as this early component disappears a component immunologically identical to the major adult globin appears. All three of these globin-like molecules in embryo extracts precipitate between 60–70 per cent saturation with ammonium sulfate. Immunoelectrophoretic patterns from two- and three-day embryos are qualitatively identical to those of the adult. Embryos less than 36 hours old show a pattern quite similar after immunoelectrophoresis to adult patterns. However, with such small amounts of antigen it is necessary to deposit excessive amounts of protein in the agar gel, the conditions for resolution are not optimal, and the only prudent conclusion is that the patterns are similar. This does help strengthen the conclusion, however, that these immunological reactants are globins.

It was thought possible, with the use of these same methods, to map the distribution of globin in different areas of the blastoderm. However, the quantitative precision of agar gel diffusion analysis leaves much to be desired; only 5- to 10-fold differences in concentration can be detected by watching the time of appearance of precipitation lines. Anterior and posterior halves of entire early primitive streak and of 12- to 14-somite blastoderms were compared, as were the pellucid and opaque portions of the blastoderms of the same two groups. There was no apparent difference between anterior and posterior halves of the blastoderm, but the precipita-

tion lines appeared earlier from extracts of area opaca than of area pellucida. This was true at both stages examined. The antigen is apparently widely distributed, but is present in higher amounts in the area opaca. These crude localiza-

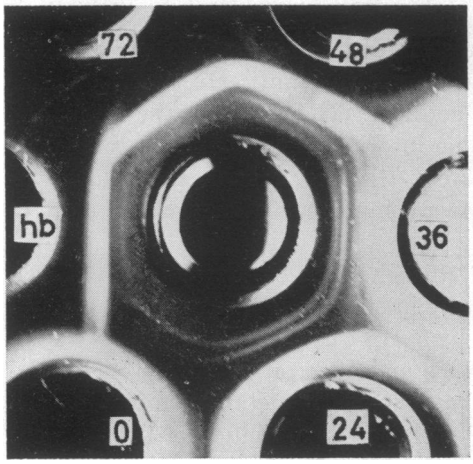

FIG. 4.—This agar gel diffusion plate summarizes the minimal number of hemoglobin antigens present in blastoderms of various stages of development. Immune γ-globulin was placed in the central wells. Hb is adult chicken hemoglobin; the numbers designate the number of hours of incubation of the eggs before preparation of the extracts of the blastoderm. Blastoderms from head process to the 2-somite stage were selected for the 24-hr extract; 8- to 11-somite blastoderms were selected for the 36-hr extract.

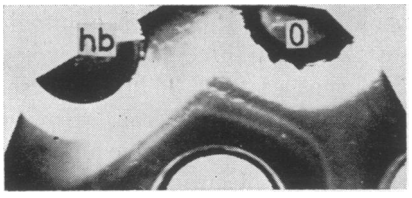

FIG. 5.—This agar gel diffusion plate shows the relation of the hemoglobin antigens of the unincubated blastoderm to adult hemoglobin.

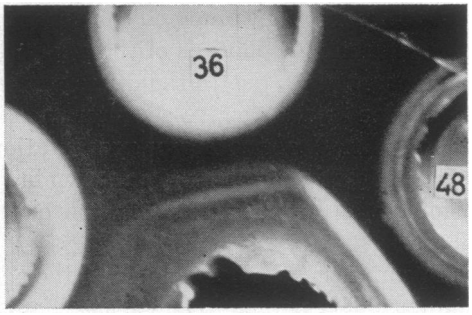

FIG. 6.—This agar gel diffusion plate shows the relation of the hemoglobin antigens of 36-hr and 48-hr blastoderms.

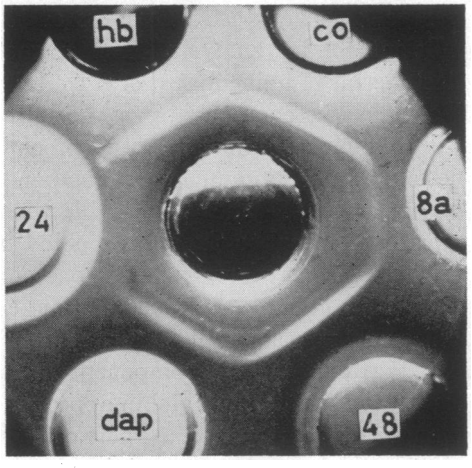

FIG. 7.—This agar gel diffusion plate shows the effect of 8-azaguanine on the development of the major adult hemoglobin antigen. (8A, embryos cultured in the presence of 8-azaguanine; co, embryos cultured on Spratt's albumen medium; dap, embryos cultured in the presence of 2,6-diamino purine; 48, uncultured 48-hr embryos; 24, uncultured 24-hr embryos; hb, adult chicken hemoglobin.) All the wells with embryo extracts contained about 1.5 mg of protein.

tion methods should be followed up by the use of fluorescent antibody staining, an area of research now under investigation.

The Formation of Hemoglobin.—Even though globin-like molecules are present in very early blastoderms, immunological methods do not distinguish globin from hemoglobin. O'Brien[21] has applied the very sensitive dimethoxy benzidine stain

for hemoglobin to developing chick embryos. The reaction is dependent upon heme for the pseudoperoxidase activity of hemoglobin. The reaction is first positive in the cells of the prospective blood islands in the area opaca at 7 somites, the nuclei staining more intensely than the cytoplasm. In a few hours the intensity of cytoplasmic staining increases and becomes more widely distributed through the area opaca. It has been possible to confirm the results of O'Brien except that the appearance of staining is first detected in 8- or 9-somite blastoderms. The results suggest that globin is present before heme incorporation into globin takes place. The finding by O'Brien that 8-azaguanine, applied prior to 7 to 9 somites of development, inhibits the appearance of benzidine staining seemed to provide a useful tool. Although 12 different nucleoside analogs were tested for their ability to inhibit hemoglobin development *in vitro* (5-bromodeoxyuridine, 5-bromodeoxycytidine, 5-fluorouracil, and thiouracil were effective inhibitors at definitive primitive streak stages), 8-azaguanine was selected for further study because of O'Brien's earlier findings. Embryos were explanted at 3 stages (definitive primitive streak, 6 to 7 somites, or 15 to 16 somites) on Spratt's minimal medium containing 10 mg per 100 ml of 8-azaguanine, and cultured for 20 hours. Selected living embryos were stained for hemoglobin[21] and subsequently fixed, the remainder being used to make extracts for immunological analysis. 8-Azaguanine completely abolished Hb formation at definitive primitive streak and 7-somite stages, and slightly reduced it at the 15-somite stage. Precipitation patterns of extracts from embryos cultured on 5-bromodeoxyuridine, 5-bromodeoxycytidine, 5-fluorouracil, and 2,6-diamino purine were identical to the controls. Extracts of embryos cultured on 8-azaguanine showed a definite reaction for the major adult globin even though no benzidine staining material could be detected (Fig. 7). This suggested a differential action of 8-azaguanine, i.e., heme and globin synthesis might be dissociable.

The hypothesis that there are differing rates of apoprotein synthesis and prosthetic group attachment at different developmental stages was put to the following critical test. Embryos at definitive primitive streak to 2-somite stage, 7- to 8-somite stage, and 15- to 18-somite stage were cultivated on Spratt's albumin-saline-agar medium containing mixtures of $Fe^{59}Cl_3$ and H^3 leucine. After 5 hours of development *in vitro*, the ratio of H^3/Fe^{59} into antibody precipitable protein was determined. Preliminary experiments on over 1,800 blastoderms using widely varying leucine to iron ratios were carried out. Precipitates were collected by filtration through Millipore filters or by centrifugation, the latter proving to be more reliable because the Millipore filters exhibited nonspecific adsorption of leucine. These experiments showed that almost no iron was incorporated into antibody precipitable material prior to the 7- to 8-somite stage, although some leucine was incorporated at this early stage. Table 1 presents the results of a duplicate pair of experiments using the final procedures outlined in the *Methods* section. The H^3/Fe^{59} ratio changes as a function of developmental age. Even though almost no iron is incorporated at the definitive primitive streak stage, leucine incorporation is definitely present. Between this stage and the 8-somite stage incorporation of leucine into globin on a per embryo basis rises at least 5-fold. Incorporation of iron rises even faster and consequently the H^3/Fe^{59} ratio decreases. If this same experiment is carried out in the presence of 8-azaguanine, the results are identical except the incorporation into antibody precipitable material is reduced about three-

fold in 7- to 8-somite embryos. There is no depression of incorporation at the 2-somite or 16-somite stage. The H^3/Fe^{59} ratios do not differ from those of control embryos cultured in the absence of 8-azaguanine. The previous suggestion that heme and globin synthesis are dissociable by 8-azaguanine is apparently not correct. But the hypothesis that at early stages globin synthesis occurs without substantial heme incorporation is supported.

TABLE 1

INCORPORATION OF Fe^{59} AND H^3 LEUCINE INTO EMBRYONIC HEMOGLOBIN

Experiment	Stage	H^3 leucine/embryo (cpm)	H^3/Fe^{59}
1	0–2 somites	38	22.2
1	7–8 somites	196	13.1
1	15–18 somites	164	8.0
2	0–2 somites	30	19.2
2	7–8 somites	194	11.7
2	15–18 somites	175	9.17

Embryos were cultured for 5 hr on media containing 10^7 cpm of H^3 leucine and 3.6×10^6 cpm of $Fe^{59}Cl_3$ per ml at the previously stated counting efficiencies. It was necessary to culture 45 embryos of the 0–2 somite stage, 30 embryos having 7–8 somites, and 15 embryos having 15–18 somites for each experiment at this isotope level. 100 μg of adult carrier hemoglobin was added to each extract. Counts recorded are the differences between precipitates from extracts to which was added excess immune and nonimmune γ-globulin. No visible precipitate was present in control tubes and counts did not deviate by more than 5% from background in control tubes. The maximum standard deviation in counting was 3%.

Discussion.—The finding that adult hemoglobin contains two distinct components is in agreement with several other studies. D'Amelio and Salvo[22] have, however, stated that a third hemoglobin component is present in chickens. This component is electrophoretically distinct from, but immunologically identical to, the major component. Differences in our findings could be due to differences in hemoglobin purification or strain differences in the birds. This component has not been observed in this laboratory using the methods of D'Amelio and Salvo. As D'Amelio has previously demonstrated, there are two adult components which are immunologically distinct.

Beard[23] has reported the definite presence of hemoglobin antigens at the 8-somite stage, and the possibility of globin antigens even earlier. The present studies demonstrate that a molecule whose immunological characteristics, behavior to ammonium sulfate, and electrophoretic mobility on agar are similar to the minor adult component is present in unincubated blastoderms. Furthermore, a cross-reacting component is present until at least 48 hours of development. Although this transient component behaves electrophoretically like the major adult component, it is immunologically distinguishable. D'Amelio and Salvo[22] have studied chick embryo hemoglobin from 47 hours until 11 days of incubation. They find the presence of two components at 48 hours of development which are similar to the two adult components; the embryonic components have a greater electrophoretic mobility than the adult components. The mobility differences are not great, however, and could be due to mobility differences between purified and unpurified hemoglobins. In this laboratory the electrophoretic mobilities on agar gel of the two components from 48-hour embryos (after partial purification by ammonium sulfate) are indistinguishable from the mobilities of the adult components. Fraser[18]

has shown the presence of the two adult Hb components, although in different relative concentrations, from 5 to 21 days of incubation.

O'Brien's and Beard's work shows, as do the present isotopic studies, that hemoglobin synthesis assumes a rapid rate at about the 8-somite stage, and that this occurs primarily in the blood islands. Some globin synthesis goes on prior to this time, although little heme iron is incorporated. 8-Azaguanine depresses but does not eliminate the increase in rate of synthesis at the 8-somite stage. Evidently the 8-azaguanine depression is substantial enough to reduce the eventual hemoglobin concentration below the threshold for benzidine staining.

A number of unanswered questions raised by this study may now be attacked experimentally. The identity of embryonic and adult globin is being determined by isotope dilution studies and fingerprinting. The precise distribution of heme and globin in the blastoderm is being analyzed by fluorescent antibody and autoradiographic studies. It is also feasible to begin experimentation on the nature of the control mechanisms in hemoglobin biosynthesis. The interrelations between the transient "embryonic" globin and the adult molecule, the possible interdependence of heme and globin synthesis, and the nature of the abrupt increase in synthesis at about 8 somites of development are issues which may be subjected to experimental analysis.

Summary.—An investigation of chick embryo hemoglobin synthesis using immunological and electrophoretic methods revealed 2 globin-like components in the unincubated blastoderm. One component is apparently identical to the minor adult globin component, the other is immunologically distinguishable from both adult components. This distinctive component disappears at approximately 36–48 hours of incubation concomitant with the appearance of the major adult component. Heme and globin synthesis proceed rapidly after the embryo has acquired 7 to 8 somites. Prior to this stage some globin synthesis occurs, but practically no heme iron is incorporated into hemoglobin.

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*EVIDENCE FOR THE COMPLETE DNA-DEPENDENCE OF RNA SYNTHESIS IN ISOLATED THYMUS NUCLEI**

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Ribonucleic acid synthesis in isolated calf thymus nuclei has already been demontreated to be partly DNA-dependent. This was shown by experiments in which deoxyribonuclease was added to nuclear suspensions and the ability of the DNAase-treated nuclei to incorporate C^{14}-labeled precursors into RNA was found to be impaired.[1, 2] Subsequent experiments showed this effect to be complex, and due, at least in part, to the fact that removal of the DNA from the nucleus also impairs its capacity for ATP synthesis.[3] The inhibition of ATP production can be prevented by replacing the DNA or by the additon of other polyanions (such as polyacrylic acid or polyethylene sulfonate) to the nuclear suspension. When ATP synthesis is restored in this way, many of the biosynthetic processes in the nucleus resume.[4]

It was a surprising observation that more than 60 per cent of the total DNA in a nuclear suspension could be replaced by a nonspecific polyanion, and yet the synthesis of RNA and protein could proceed at nearly normal rates, as long as ATP supplies were adequate.[4] This continuing capacity of DNA-depleted nuclei for RNA synthesis raised the question of whether such nuclei can synthesize RNA by a mechanism that does not require the direct participation of deoxyribonucleic acid. The experiments now to be described indicate, however, that probably *all of the*