CYTOPLASMIC RNA-DEPENDENT RNA SYNTHESIS IN MATURING CHICKEN ERYTHROCYTES.

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Thesis submitted in fulfilment of the
requirements for the degree of
Doctor of Philosophy in the
Faculty of Science, University of Cape Town.

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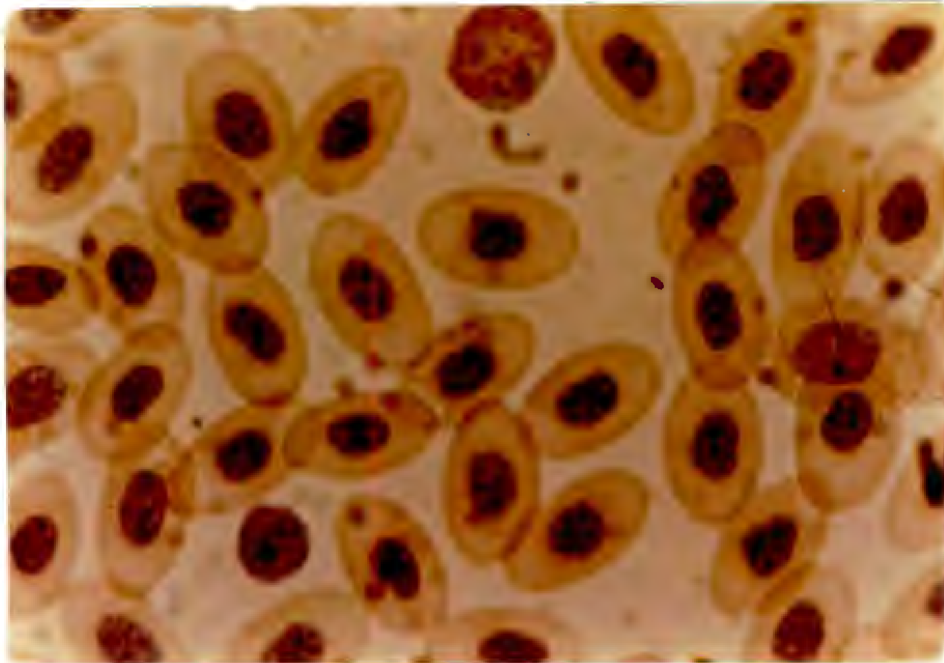

to pause, to wonder and stand rapt in
awe - the most beautiful thing we
can experience is the mysterious.

Albert Einstein.

CERTIFICATE OF SUPERVISOR

In terms of paragraph eight of 'General regulations for the degree of Ph.D.' I, as supervisor of the candidate, M.C.D. Boyd, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

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Frontispiece   Smear of normal chicken blood. Leishmans stain (x3000).

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SUMMARY

An RNA-dependent RNA polymerase from ribosomes of maturing chicken erythrocytes was investigated. Concurrent with an increase in globin and RNA synthesis during phenylhydrazine induced anaemia, the total and specific activities of ribosome bound RNA-dependent RNA polymerase increased 40 and 9.4 times respectively, indicating the possible involvement of this enzyme activity in the synthesis or control of synthesis of globin.

A partially purified RNA-dependent RNA polymerase was prepared from ribosomes of immature chicken erythrocytes. This preparation was shown to be a predominantly primer dependent enzyme activity, incorporating UTP, CTP and to a lesser extent ATP and GTP into homopolyribonucleotide material. The ribosome-bound enzyme preparation demonstrated in addition, the synthesis of heteropolyribonucleotide material.

A complementary DNA copy of chicken globin mRNA containing-9S RNA was prepared. Unlabelled complementary DNA was used to demonstrate that no globin mRNA replicase activity was detectable in the ribosome fraction of immature chicken erythrocytes. The radioactively labelled product obtained after incubation of ribosomes with [^{3}H]-UTP was shown by sucrose density gradient centrifugation to have a sedimentation coefficient of 4S; the ratio of non-terminal to terminal incorporation of UTP was 6.08. The product synthesized after incubation of intact cells in the presence of [^{3}H]-uridine and actinomycin D was shown to be very similar with respect to S-value and the ratio of UMP/uridine incorporated. This similarity suggests the presence of a ribosome bound RNA-dependent RNA polymerase activity in intact cells.

PART 1

INTRODUCTION

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1.1 CYTOPLASMIC RNA SYNTHESIS

The cytoplasmic synthesis of RNA in eukaryotic cells constitutes the central theme of this thesis. The presence of a number of biosynthetic systems in eukaryotic nuclear and cytoplasmic extracts, capable of incorporating one, two or all four ribonucleoside 5'-triphosphates into polynucleotide material was known in the early 1960s (1). The realization that nuclear DNA-dependent RNA synthesis represented the mechanism by which most of the molecules of cellular RNA are built up has resulted in the last decade, in a prodigious increase in our understanding of transcription of the eukaryotic genome. Relatively little by comparison is known about many of the functions and mechanisms of cytoplasmic RNA synthesis.

The intention in this introductory chapter is to briefly discuss the distribution of these RNA-dependent RNA polymerase activities in the cytoplasm and the mode of synthesis of the products as an introduction to the study of ribosome bound RNA-dependent RNA polymerase in maturing chicken erythrocytes. The discussion will be confined to cytoplasmic RNA-dependent RNA synthesis; the various DNA-dependent RNA polymerase activities thought to exist in the cytoplasm of eukaryotic cells (2,3,4,5) are beyond the scope of this introduction.

1.1.1 CYTOPLASMIC LOCALIZATION OF RNA SYNTHESIS

The various cytoplasmic RNA synthesizing systems are located in or associated with a number of constituents of the cytoplasm. Smellie and his colleagues (6,7,8) described the isolation of enzyme preparations from Ehrlich and Landschutz ascites tumour cells. Their results demonstrated that the microsomal fraction of Landschutz cells catalyzed the incorporation of UTP into polynucleotide material. RNA polymerase

activities have also been demonstrated in rat liver ribosomes (9), pigeon liver microsomes (10), rat brain microsomes (11), rabbit reticulocyte ribosomes (12) and sea urchin ribosomes (13,14).

Several workers have shown that ribonucleotides are also incorporated into polynucleotide material by post-ribosomal supernatant fractions. Klemperer (15) has partially purified an RNA polymerase activity from the pH 5 supernatant obtained from a 105 000 g supernatant of rat liver homogenate. Wilkie and Smellie (9) have described an RNA polymerase activity from the post-ribosomal supernatant of rat liver.

RNA-dependent RNA synthetic activities have also been shown to be associated with cytoplasmic organelles. Astier-Manifacier and Cornuet (16) have reported the presence of an RNA-dependent RNA polymerase in Chinese cabbage chloroplasts. Burkard and Keller (17) have shown RNA-dependent RNA polymerase activities in wheat chloroplasts. Mitochondria also contain RNA-dependent RNA polymerase activity (18,19). The location of RNA-dependent RNA polymerase activity is therefore fairly generally distributed throughout the cytoplasm and its various inclusions.

1.1.2 CYTOPLASMIC PRIMER-DEPENDENT RNA SYNTHESIS

Cytoplasmic, primer-dependent RNA synthesis results in the polymerization of particular ribonucleotides to form homo- or hetero-oligonucleotides of about 20 residues or less attached to one end of a primer molecule. Klemperer (15) demonstrated that a partially purified enzyme preparation from the pH 5 supernatant of a rat liver homogenate catalyzed the covalent attachment of UMP to the 3' end of a primer RNA. The presence of ATP, GTP and CTP slightly inhibited the incorporation of [^{3}H]-UTP into polynucleotide material. The synthesized oligouridylic acid had a chain length of about 3 residues. Hozumi et al. (11) have demonstrated that the microsomal fraction of rat brain effected the synthesis of homopolymers of UMP of up to 20 residues long. The addition of the other three ribonucleotides, if anything, inhibited the incorporation of [^{3}H]-UTP. The synthesis of homopolymers of adenylic acid (chain length

of about 20 residues) by a pH 5 supernatant fraction of rat liver has been demonstrated (20,21). The incorporation of [^{3}H]-ATP is inhibited in the presence of the other three unlabelled ribonucleotides. A similar inhibition of homopolyribonucleotide synthesis was observed by Burkard and Keller (17) who demonstrated the synthesis of poly(A) and poly(G) by an enzyme preparation from wheat chloroplasts. Wykes and Smellie (8) have shown the independent incorporation of radioactively labelled UTP, GTP, CTP and ATP by an enzyme preparation from the microsomal fraction of Landschutz ascites tumour cells. The presence of the unlabelled ribonucleotides inhibited the incorporation of the [^{3}H]-labelled ribonucleoside 5'-triphosphate. The synthesis of homopolymers of UMP, AMP, GMP and CMP by cytoplasmic enzyme preparations are therefore known to exist. A general feature of homopolyribonucleotide synthesis is the inhibition (of varying degree) of the incorporation of a particular ribonucleotide by the presence of the other three ribonucleoside 5'-triphosphates.

In contrast, Wilkie and Smellie (9) have shown that the individual incorporation of radioactively labelled GTP, CTP and UTP by an enzyme fraction from rat liver microsomes was enhanced by the presence of the other three unlabelled ribonucleotides (although the incorporation of GTP was relatively very low). This suggests heteropolymer formation and was confirmed by incorporation studies using [α-^{32}P]-UTP in the incubation mixture, followed by analysis of the radioactively labelled products by alkaline hydrolysis and paper electrophoresis. These experiments have shown, in the presence of unlabelled ATP, GTP and CTP in the incubation mixture, that a significant percentage of the ^{32}P incorporated into polynucleotide material was recovered in the ribonucleotide regions of the electrophoretogram corresponding to AMP, GMP and CMP. In the absence of unlabelled ATP, GTP and CTP, UTP was incorporated into homopolymers of UMP. The presence of all four ribonucleoside 5'-triphosphates results in the synthesis, by the enzyme preparation from rat liver microsomes, of heteropolyribonucleotide material rich in UMP.

The microsome fraction was further characterized by these workers (22) : the incorporation of $[^3H]$-UTP by an RNA-dependent RNA polymerase activity from the rough-surfaced vesicle (light) fraction (RSVL) was stimulated by the addition of ATP, GTP and CTP. The free ribosome fraction however incorporated predominantly UTP and to a lesser extent ATP and CTP into homopolyribonucleotide material. Whether the activity in the whole microsome fraction described above is analogous to the heteropolyribonucleotide synthesis noticed in the RSVL fraction is unclear; certainly the observed primer-dependent synthesis of polynucleotides by the free ribosome fraction differs from that of whole microsomes.

Heteropolyribonucleotide synthesis has been demonstrated by other workers: Strauss and Goldwasser (10) have shown that the incorporation of UTP by an enzyme fraction from pigeon liver microsomes was stimulated by the addition of the other three ribonucleoside 5'-triphosphates. The incorporation of CCA to the 3' end of tRNA represents another form of heteropolymer formation (2,23,24). These various hetero- and homopolyribonucleotides, synthesized by primer-dependent, cytoplasmic RNA polymerases are summarized in Table 1.1.

TABLE 1.1

Primer-dependent cytoplasmic RNA polymerases: products and functions of the products.

Product	Function	Reference
poly(G)	?	8,9,17
poly(A)	?	8,9,13,14,17,20, 21,22,25,26,27,28
poly(C)	?	8,22,9
poly(U)	?	8,9,11,15,22
poly(A,U,C,G)	?	9,10
CCA	Amino acid acceptance at 3' end of tRNA	2,23,24

The attachment of CCA to the 3' end of tRNA and the role of tRNA-CCA in protein synthesis is the only well understood cytoplasmic RNA biosynthetic system.

Brawerman and Diez (25,26) have demonstrated that the elongation of the poly(A) segment at the 3' end of mRNA is effected by a cytoplasmic RNA-dependent RNA polymerase. This is in confirmation of the earlier work of Wilt (13) and Slater and co-workers (14) and the suggestions of Perry et al. (29). Although the specific function of poly(A) remains uncertain, the elongation of poly(A) in the cytoplasm is certainly indicative of a cytoplasmic function for this adenylic acid polymer; whether this function is in determining mRNA stability (30,31) or some other function in the cytoplasmic control of translation of mRNA awaits further evidence (for discussion see Lewin (32) and Greenberg (33)). The modification of tRNA by a cytoplasmic primer-dependent RNA polymerase and the involvement of cytoplasmic enzymes in the processes of mRNA metabolism illustrate the potential importance of these RNA-dependent RNA polymerase activities in post-transcriptional processes.

The functions of the other cytoplasmic homo- and heteropolymers (Table 1.1) are completely obscure. The question of what RNA species could act as a primer for the addition of, for example, uridylic acid residues is equally unclear. Wilkie and Smellie (22) have demonstrated, using an enzyme preparation from the free ribosome fraction of rat liver, that tRNA, 5S, 18S and 28S rRNA were not active as primers for this RNA-dependent RNA polymerase reaction. The absence of oligo(U) sequences in cytoplasmic mRNAs (34) does not favour mRNA as a candidate for the primer-dependent post-transcriptional addition of uridylic acid. Precursor RNA molecules have not been excluded but no evidence is available pointing to the precursor of any RNA species as a likely primer for the RNA-dependent synthesis of oligo(U).

1.1.3 CYTOPLASMIC TEMPLATE DEPENDENT RNA SYNTHESIS

The template-dependent synthesis of RNA by cytoplasmic enzyme preparations from mammalian cells infected with an RNA-containing virus is

well known (35,36,37). RNA-dependent RNA polymerase, isolated from infected host cells would seem to be a virus-induced enzyme. This is supported by evidence indicating the existence of a period shortly after infection when subsequent synthesis of RNA is dependent upon the synthesis of protein (38,39). Furthermore, an increase in the synthesis of protein has been demonstrated in Krebs II ascites tumour cells shortly after infection with encephalomyocarditis virus (40). In addition Horton and co-workers (41) have shown that the enzyme present in infected cells and responsible for the template-dependent synthesis of viral RNA (36), is not present in uninfected cells.

Evidence for the existence of a template-dependent RNA polymerase in uninfected cells has been described by Astier-Manfacier and Cornuet (16). These authors reported the isolation of an RNA-dependent RNA polymerase from the chloroplasts of apparently uninfected Chinese cabbage plants. This enzyme preparation effected the incorporation of complementary ribonucleotides in the presence of the synthetic copolymers poly(U,C) and poly(A,C); in addition, the product appeared double-stranded. Assuming the absence of any latent virus infection, evidence to date does not favour the hypothesis that a host derived enzyme would be responsible for viral RNA replication.

What possible functions could a cytoplasmic template dependent RNA polymerase have in eukaryotic cells? Downey et al. (12) reported the isolation of an RNA-dependent RNA polymerase from rabbit reticulocyte ribosomes that showed, they concluded, a preference for globin mRNA as template. They proposed, on the basis of this evidence, the very attractive hypothesis that this enzyme activity is involved in the replication, or amplification of globin mRNA. The criterion of template-dependency relied upon the incorporation by the enzyme preparation of UTP and CTP only, in the presence of poly(A,G); although indicative of template-dependent synthesis, on its own, this evidence is not conclusive.

In summary therefore, it has been shown that the RNA-dependent synthesis of RNA is located fairly generally throughout the cytoplasm. The primer

dependent synthesis of homo- or heteropolyribonucleotides would seem to be the most prevalent forms of cytoplasmic RNA synthesis. The involvement of a cytoplasmic poly(A) polymerase in the elongation of the polyadenylic acid region of mRNAs illustrates the potential importance of cytoplasmic primer-dependent RNA synthesis in the processes of post transcriptional gene control.

The existence of template-dependent RNA polymerase activity in the cytoplasm remains unresolved. Although the evidence for template-dependent synthesis of RNA put forward by Astier-Manfacier and Cornuet is convincing, the question of a viral infection (or latent viral infection) cannot be excluded. The work of Downey et al. is only indicative of template dependent synthesis.

1.2 PLAN OF PRESENT RESEARCH

This investigation of cytoplasmic RNA-dependent RNA synthesis was undertaken to study : a) primer dependent cytoplasmic RNA synthesis in normal eukaryotic cells with the aim of establishing some hypotheses as to the function(s) of the lesser known RNA-dependent RNA synthesizing systems, especially those responsible for the incorporation of UTP into polynucleotide material. b) the question of cytoplasmic mRNA replication. An investigation aimed at providing more direct and conclusive evidence than has been shown by previous workers (12) for the existence of such an enzyme is relevant to considerations of function of cytoplasmic RNA-dependent RNA polymerases.

A number of RNA-dependent RNA polymerase activities have been shown (section 1.1.1) to be associated with the ribosome or microsomal fraction of eukaryotic cells. In addition, Downey et al. (12) have indicated the possible presence of a mRNA replicase, attached to ribosomes. Ribosome-bound RNA synthesis seemed therefore a likely candidate for the investigation of the two aspects of cytoplasmic RNA-dependent RNA synthesis mentioned above.

The biological process chosen for this study of ribosome bound RNA-dependent RNA synthesis was the maturation of the avian erythrocyte. During maturation of the nucleated avian erythrocyte there is a progressive reduction in nuclear DNA-dependent RNA synthesis and protein synthesis (42,43,51). Investigations of cytoplasmic RNA-dependent RNA synthesis have centred, to date, around their enzymology and products. The present study focuses in part on changes in the levels of RNA-dependent RNA polymerase activities during maturation of avian erythrocytes and the temporal relationship of such changes to RNA and protein synthesis in maturing chicken erythrocytes.

The erythrocyte, in addition, is ideally suited for the purpose of testing the hypothesis of cytoplasmic mRNA amplification. The immature erythrocyte is a highly differentiated cell in which globin is the predominant protein synthesized; nuclear mRNA synthesis is restricted to the predominant synthesis of the messenger for globin (45). It is reasonable to assume therefore that, should a ribosome bound mRNA replicase exist in immature erythrocytes, its product would be globin mRNA. A complementary copy of globin mRNA forms the basis of an assay to test for the synthesis of chicken globin mRNA sequences by a ribosome bound RNA-dependent RNA polymerase.

The changes in cellular protein synthesis, RNA synthesis (predominantly DNA-dependent RNA synthesis) and ribosome bound RNA-dependent RNA synthesis in maturing chicken erythrocytes are considered in the first section of this thesis (Part 2). The purification and an investigation of a number of properties of an RNA-dependent RNA polymerase from ribosomes of immature chicken erythrocytes, in particular the question of whether the enzyme preparation is a primer or template dependent activity, are reported in Part 3.

The preparation of a complementary DNA copy of chicken 9S RNA and the use of this globin mRNA probe to test for cytoplasmic globin mRNA replication are described in Part 4.

It remains possible that a ribosome bound RNA-dependent RNA polymerase

activity is nothing more than an artefact created by the conditions of assay or isolation. Part five investigates the existence of the product(s) of ribosome bound RNA-dependent RNA polymerase in intact cells.

PART 2

RIBOSOME BOUND RNA-DEPENDENT RNA POLYMERASE: CHANGES IN TOTAL AND SPECIFIC ACTIVITY DURING MATURATION OF AVIAN ERYTHROCYTES

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2.1 INTRODUCTION

The blood of the domestic fowl (Gallus domesticus) is made up predominantly of erythrocytes; thrombocytes, non granular leucocytes (which are subdivided into lymphocytes and monocytes) and granular leucocytes (heterophils, eosinophils and basophils) are also present, suspended in plasma (for nomenclature see Lucas and Jamroz (46)). Table 2.1 illustrates a typical distribution of cell types in 1.0 ml of whole blood taken from the wing vein of a normal, adult cock (figures taken from Freeman (47)).

Of the erythrocytes, the largest proportion (>95%) is represented by mature cells; mature erythrocytes support virtually no macromolecular synthesis at all (48,49,50). A small percentage of immature cells corresponding to different stages of development of the red cell make up the rest of the total erythrocyte complement in normal chicken blood. Most of these immature cells are late polychromatic erythrocytes but mid- and early polychromatic erythrocytes and erythroblasts can be found in the circulating blood of normal chickens. These are the cells active in the synthesis of globin and RNA (51,52) (the erythroblasts being the most active: see 'definitions'). Large quantities of immature cells can be easily obtained from the blood of chickens which have been rendered anaemic by phenylhydrazine hydrochloride treatment. The blood from such anaemic chickens shows a marked decrease in total cell count but an increase in immature erythrocytes. The proportion of polychromatic erythrocytes rises, as a result of this treatment, up to levels of about 40-50% of total cells; erythroblasts can reach levels of a few percent in some instances. In contrast, the total levels and proportions of granular and non-granular leucocytes do

TABLE 2.1

Distribution of cell types in the blood of normal, adult chickens

Cell type	Number of cells ($x\ 10^{-9}$/ml)	% of total cells
Erythrocytes	3.24 - 3.8	97.03
Thrombocytes	0.035 - 0.040	1.03
Nongranular leucocytes		
a. lymphocytes	0.040 - 0.080	1.65
b. monocytes	0.00033 - 0.00167	0.03
Granular leucocytes		
a. heterophils	0.003 - 0.012	0.21
b. eosinophils	~0.00091	0.025
c. basophils	~0.00091	

not change appreciably as a result of phenylhydrazine treatment (51).

During the maturation of this immature erythrocyte population, shortly after termination of phenylhydrazine treatment, the cellular levels of RNA and protein synthesis decrease markedly (53,54,55,56). Attardi and co-workers (57) and Scherrer et al. (58) have studied the patterns of synthesis of the various classes of RNA during avian erythrocyte maturation. The increased synthesis of 9S RNA (containing the mRNA for globin) has been demonstrated by Attardi et al. (57). The synthesis of HnRNA and its informational precursor-product relationship with globin mRNA has been reviewed by Scherrer ((59);see also ref. (60)). The formation of rRNA and tRNA in immature circulating erythrocytes was slower than that found in other animal cells (57). The isolation of a minor species of mRNA, histone F2c 9S mRNA from immature chicken

erythrocytes has also been reported (61,62).

Kabat and Attardi (52) have shown that immature erythrocytes are more active in the synthesis of the two avian haemoglobins than mature cells. The ratio of the rates of synthesis of these two haemoglobins during cellular development remains constant, suggesting the co-ordinate regulation of synthesis of these two proteins.

A good deal therefore is known about the process of cellular maturation and the patterns of DNA-dependent RNA synthesis and protein synthesis in maturing avian erythrocytes. Nothing by comparison is known about the ribosome bound synthesis of RNA during avian erythrocyte maturation. The experiments therefore, to be described below, illustrate the changes in cytology, cellular RNA and protein synthesis in relation to the levels of ribosome bound RNA-dependent RNA polymerase activity in maturing chicken erythrocytes, with the aim of obtaining some ideas of the functions of such an enzyme activity.

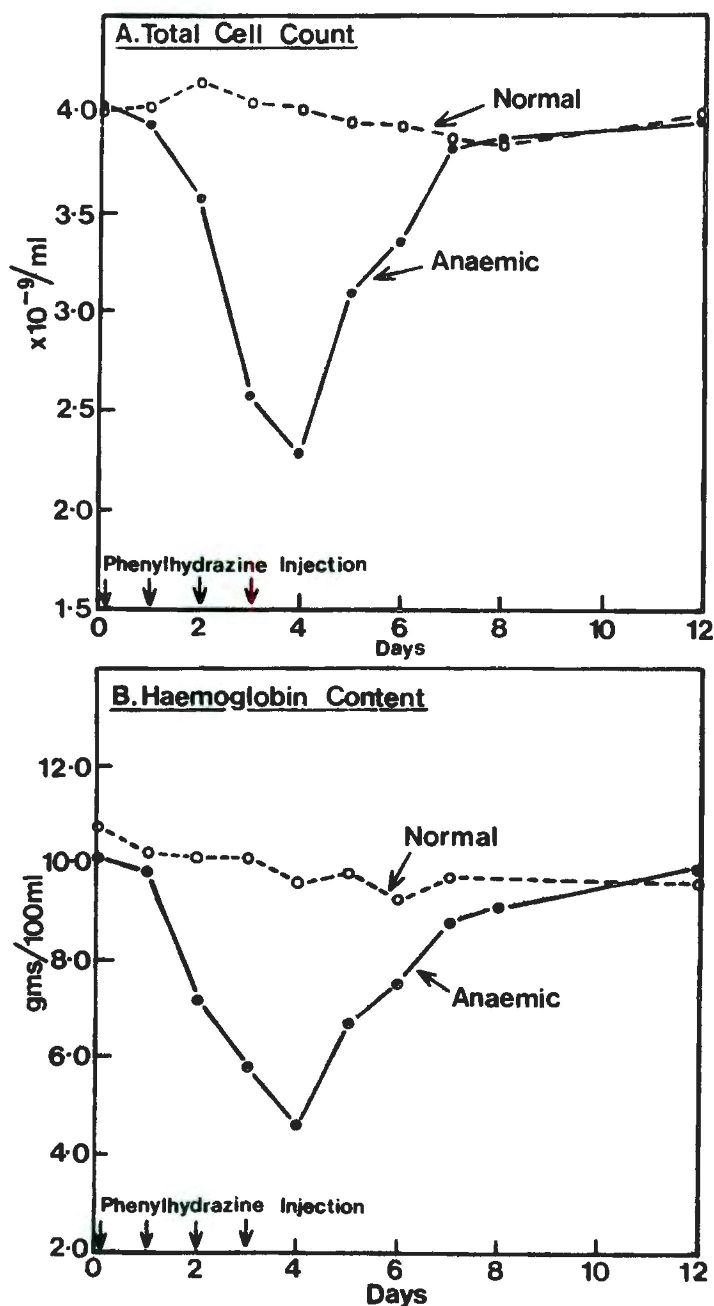
2.2 RESULTS AND DISCUSSION

2.2.1 CYTOLOGICAL CHANGES DURING ANAEMIA

The four daily injections of phenylhydrazine resulted in a decrease in total cell count and haemoglobin content of whole blood (Fig. 2.1). The average cell count in normal chickens was 3.99 x 10^9 cells/ml blood. This dropped to 2.3 x 10^9 cells/ml blood by day 4 of anaemia (blood from chickens injected with phenylhydrazine will be referred to as anaemic blood); recovery to normal values was complete by day 12. The blood haemoglobin concentration dropped from an average of 9.8% (w/v) to 4.6% (w/v) on day 4; recovery was again complete by day 12. Although total cell count decreased during phenylhydrazine induced anaemia, the proportion of immature erythrocytes increased dramatically

FIG. 2.1

Total blood cell count and haemoglobin content during anaemia and subsequent recuperation. Five chickens were injected with phenylhydrazine as described in Materials and methods 7.2.1. Aliquots of blood were removed daily from the wing vein of each and pooled; the pooled sample was used for the determination of the parameter concerned. Blood, pooled from 5 uninjected, normal chickens, was used as the control. Total cell count and haemoglobin concentration were determined as described (7.2.1).



(Fig. 2.2; the term 'reticulocyte' will be used to refer to all cytologically immature erythrocytes i.e. those erythrocytes whose cytoplasm contains basophilic blue granules when stained with brilliant cresyl blue). In contrast, no significant change in the proportion of 'non-erythrocytes' (leucocytes and thrombocytes) was evident. Fig. 2.3 and 2.4 illustrate typical smears of anaemic and normal blood (stained with Leishman's stain and brilliant cresyl blue respectively). Mature erythrocytes (using Leishman's stain) are characterized by a highly condensed granulated nucleus which is intensely basophilic and lacks a nucleolus. The cytoplasm is strongly acidophilic (staining orange-red) due to its high haemoglobin concentration. Of all the immature erythrocytes present in anaemic blood, the late polychromatic erythrocyte predominates. This cell is slightly oval with a moderately acidophilic cytoplasm and a nucleus that is less condensed than that of a mature cell. The mid- and early polychromatic erythrocytes tend towards a more basophilic (staining blue) cytoplasm and less condensed nucleus.

In earlier studies of this maturation system (63), I used chickens weighing 3 Kg or more. Their response to phenylhydrazine was slower than the smaller birds used subsequently, although dosage was according to weight. These larger and heavier chickens were injected for 3 successive days with 8 mg phenylhydrazine-HCl/Kg body weight. This schedule for inducing anaemia resulted in similar proportions of circulating immature erythrocytes as described above where smaller chickens and a different schedule were used (Materials and methods 7.2.1).

2.2.2 RNA AND PROTEIN SYNTHESIS DURING ANAEMIA

The levels of total protein and RNA synthesis in blood cells during the development of anaemia and the subsequent recuperation phase were determined (Fig. 2.5). The incorporation of [^{3}H]-uridine and [^{3}H]-histidine into TCA-insoluble material is always significantly higher in cells from anaemic blood than in normal blood although the extent of incorporation of either labelled precursor by cells from anaemic blood varied from

FIG. 2.2

Total reticulocyte, leucocyte and thrombocyte counts during anaemia and subsequent recovery. Reticulocyte count was determined after staining cells with brilliant cresyl blue and the percentage of leucocytes and thrombocytes was determined after staining cells with Leishman's stain (Materials and methods 7.2.1). Refer to legend of Fig. 2.1 for details of pooled blood samples.

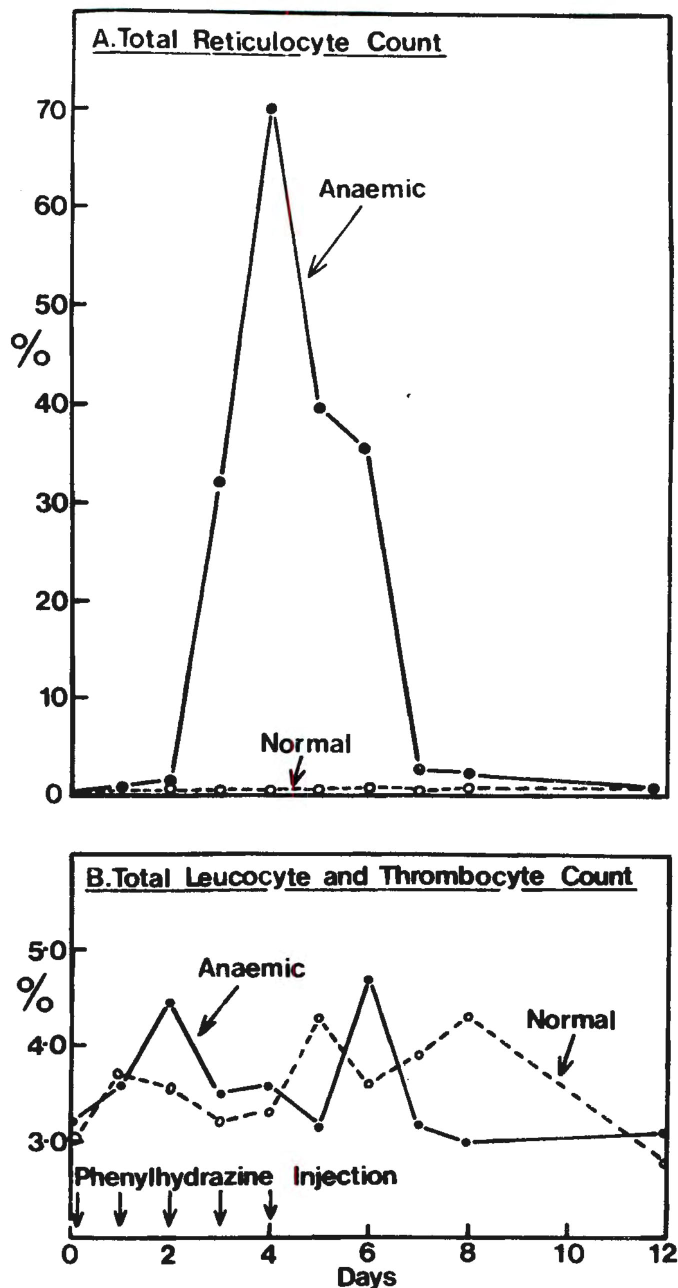
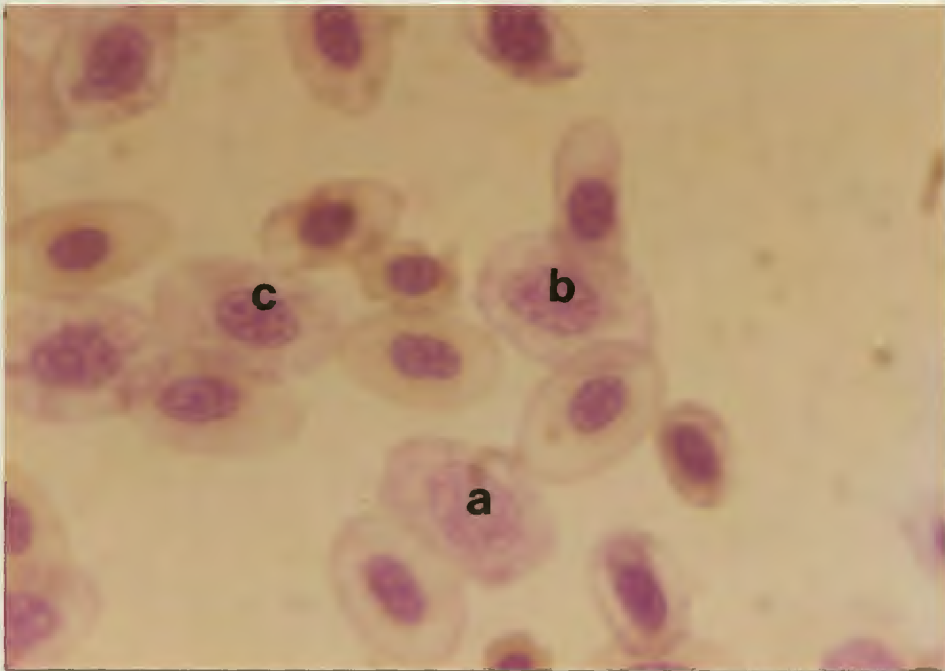


FIG. 2.3

Smears of anaemic and normal blood using Leishman's stain (x 3 000).
a,b,c. Early, mid and late polychromatic erythrocytes respectively.
d. lymphocyte (non-granular leucocyte). e. heterophil (granular leucocyte). f. thrombocyte. g. mature erythrocytes.

I. Anaemic blood



II. Normal blood

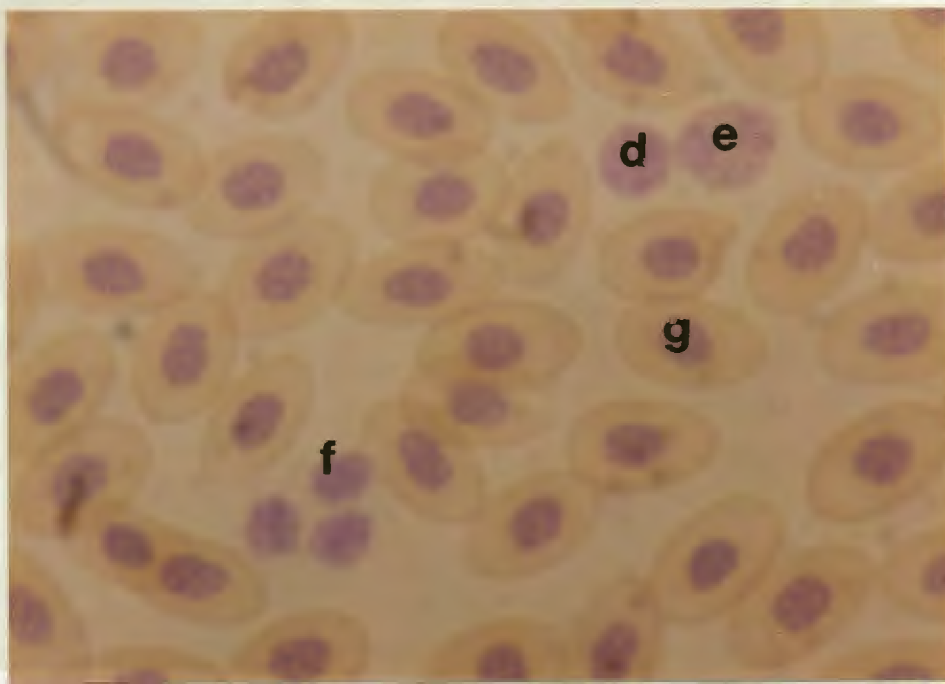
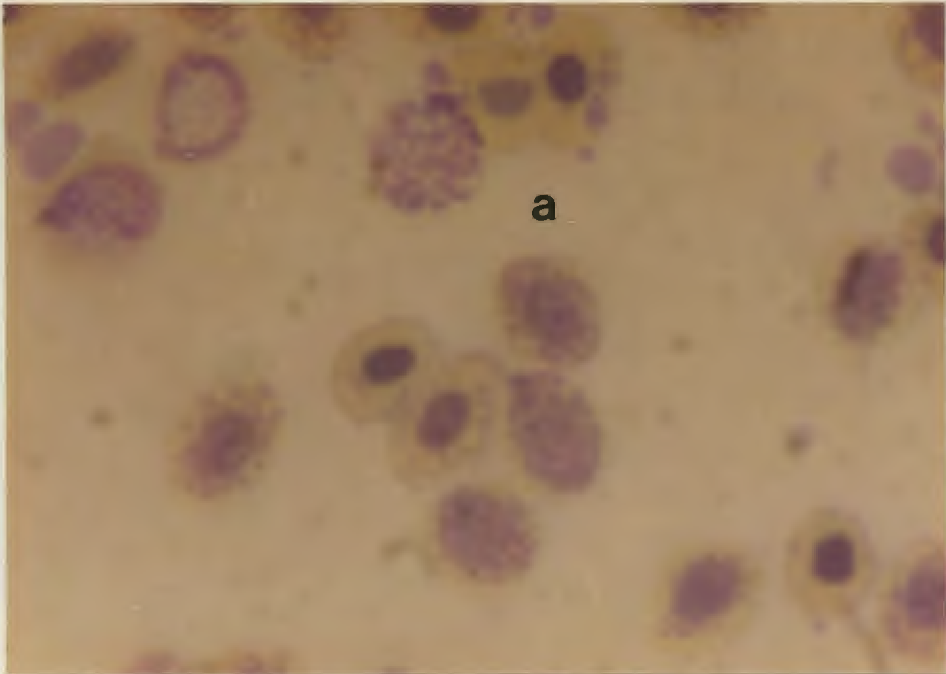


FIG. 2.4

Smears of anaemic and normal blood using brilliant cresyl blue (x 3 000). a. Reticulocyte. b. erythrocyte.

I. Anaemic blood



II. Normal blood

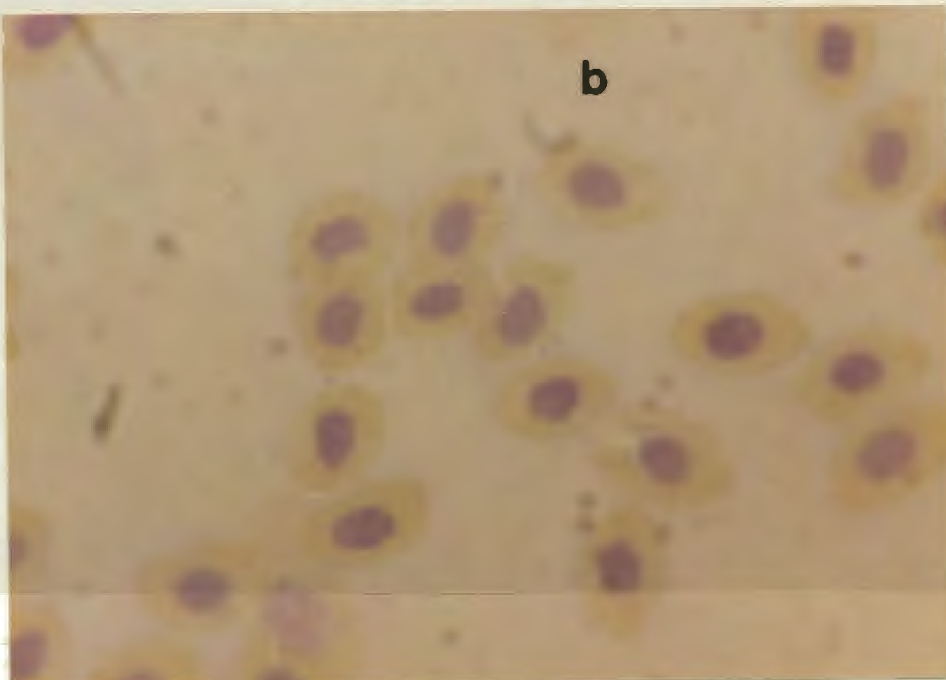
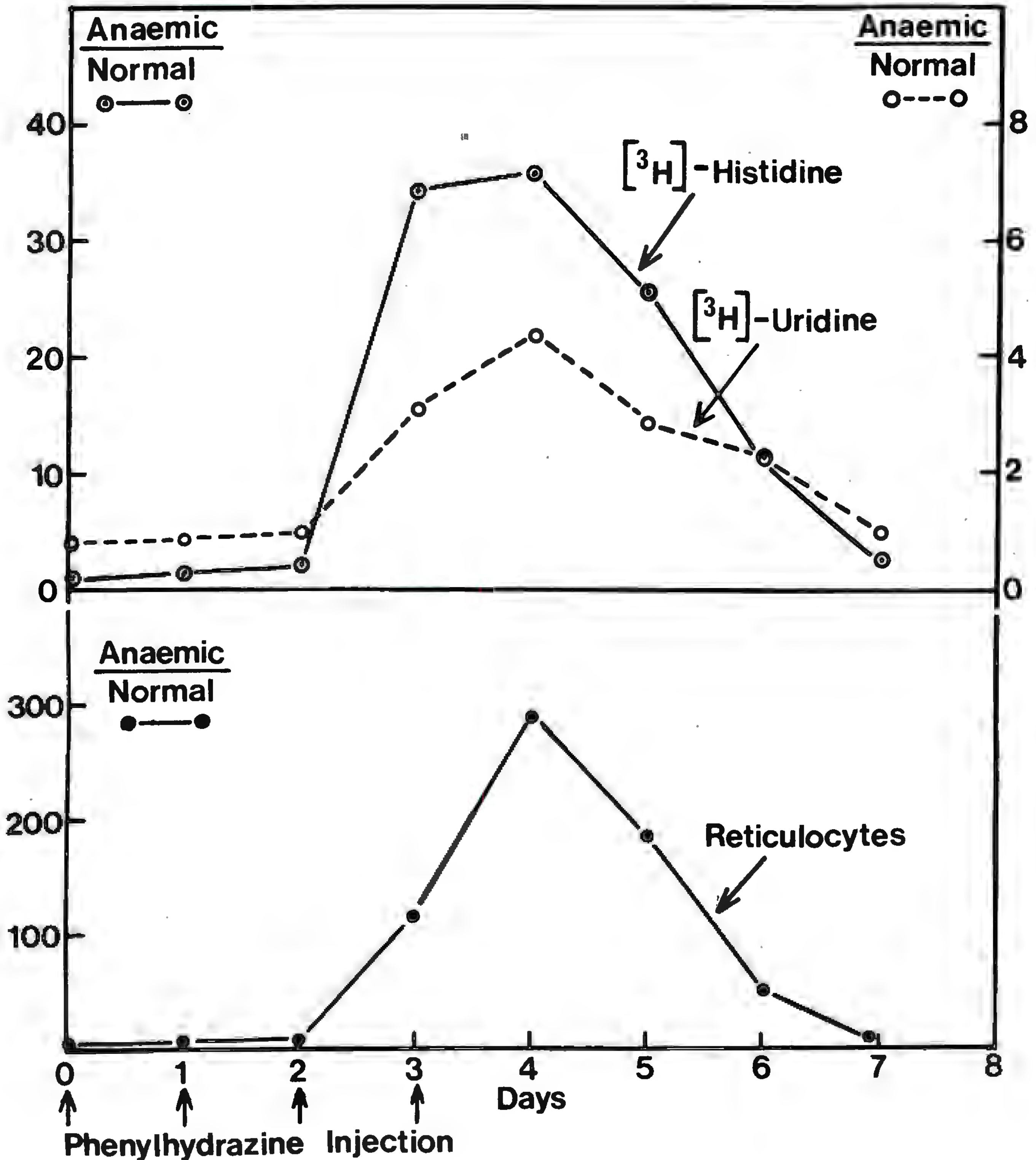


FIG. 2.5

RNA and protein synthesis in red blood cells during anaemia. Average values of three control and three experimental animals. Blood was removed daily and incubated with the respective radioactively labelled precursors [^{3}H]-uridine and [^{3}H]-histidine as described in Materials and methods (7.2.2.1.1) and (7.2.2.2.1). All results are expressed as a ratio of the incorporation per 10^9 cells from anaemic chickens to the incorporation per 10^9 cells from normal chickens.



one experiment to the next. The increase in the number of reticulocytes during anaemia (expressed as an anaemic/normal ratio) is shown in Fig. 2.5 to illustrate the concomitant cytological changes during the onset of anaemia and the subsequent recovery.

Anaemic blood is more active in protein and RNA synthesis than normal blood; anaemic blood, in addition, has a far higher proportion of immature erythrocytes than does normal blood. It has been shown by a number of workers (52,58) that immature erythrocytes are the cells responsible for this macromolecular synthesis. To demonstrate this, in this laboratory, cells from anaemic blood were fractionated by buoyant density gradient centrifugation on discontinuous gradients of bovine serum albumin (BSA). This procedure, initially formulated by Leif and Vinograd (64) separates erythrocytes of varying stages of maturation. The cell types in the various layers can then be assayed for their RNA and protein synthesizing ability. Fig. 2.6 illustrates the distribution, during the onset of anaemia and subsequent recovery, of blood cells in the various discontinuous layers of BSA. A comparison of Fig. 2.2(a) with this series of cell separations conducted daily during anaemia, revealed qualitatively, that as the number of reticulocytes in whole blood increases, so does the proportion of less buoyant cells present. In collaboration with a colleague (D.R. van der Westhuyzen), I determined, using a similar discontinuous BSA gradient, the RNA synthesizing capacity of cells in the various layers. The results show (Fig. 2.7) quite clearly that the more immature cells have a greater ability to incorporate labelled ribonucleotide into TCA insoluble material. We were also able to show that the incorporation by total leucocytes (including thrombocytes) is very low compared with that of the red blood cells (these cells sediment above the 27% (w/w) BSA layer).

To determine the percentage of total protein synthesis present as haemoglobin synthesis in these immature erythrocyte populations, a post mitochondrial supernatant was prepared from cells of anaemic blood (day 4), incubated with [^{3}H]-histidine; an aliquot was applied to a carboxymethyl cellulose column and the two haemoglobins were eluted through a linear gradient of phosphate buffer (Fig. 2.8). 92% of the acid-

FIG. 2.6

Buoyant density centrifugation of blood cells during anaemia and subsequent recovery. 2 ml aliquots of blood pooled from 5 chickens (blood was collected as described in Materials and methods 7.2.1) being treated with phenylhydrazine was applied to discontinuous gradients of BSA; the various 2 ml layers are indicated in the figure using the tube for day 8 as an example. Preparation of BSA and method of centrifugation of gradients is described in section 7.2.3.

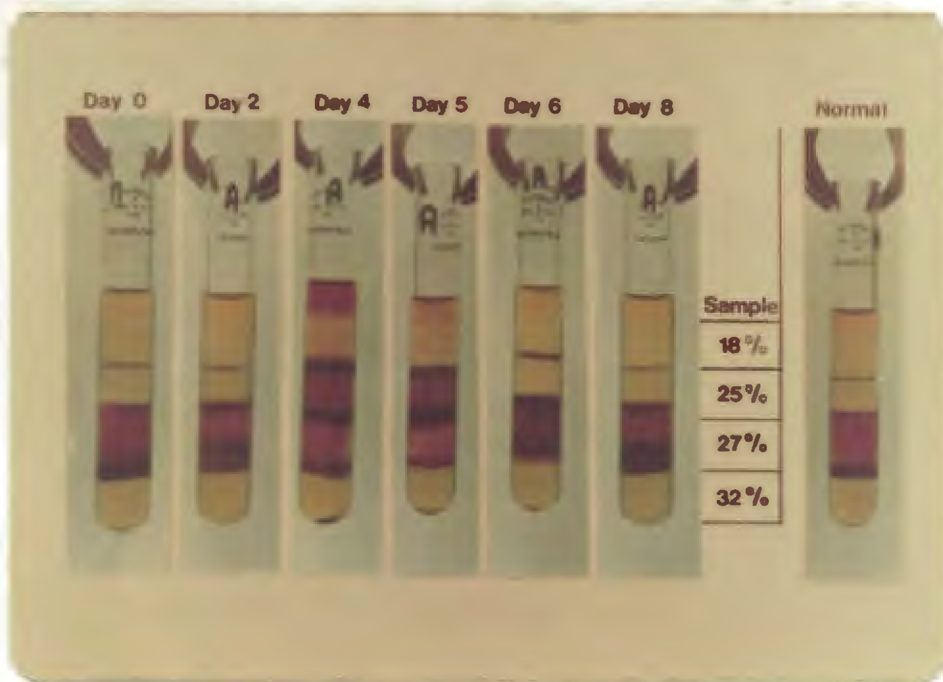


FIG. 2.7

$[^{14}C]$-adenine incorporation by subfractions of blood cell populations from anaemic chickens. Buoyant density centrifugation of cells from anaemic blood and incubation of subfractions was carried out as described in sections 7.2.3 and 7.2.2.1.3 respectively. Corresponding fractions from different tubes were pooled. Cells layering above 27% and 25% BSA were pooled into one fraction. Results are expressed as incorporation (dpm) per 10^9 cells. The distribution of total cells is shown as well as the distribution of erythrocytes in the various maturation stages : mature erythrocytes (●---●); late polychromatic erythrocytes (x——x); mid-polychromatic erythrocytes (⊙——⊙); early polychromatic erythrocytes (⊘---⊘).

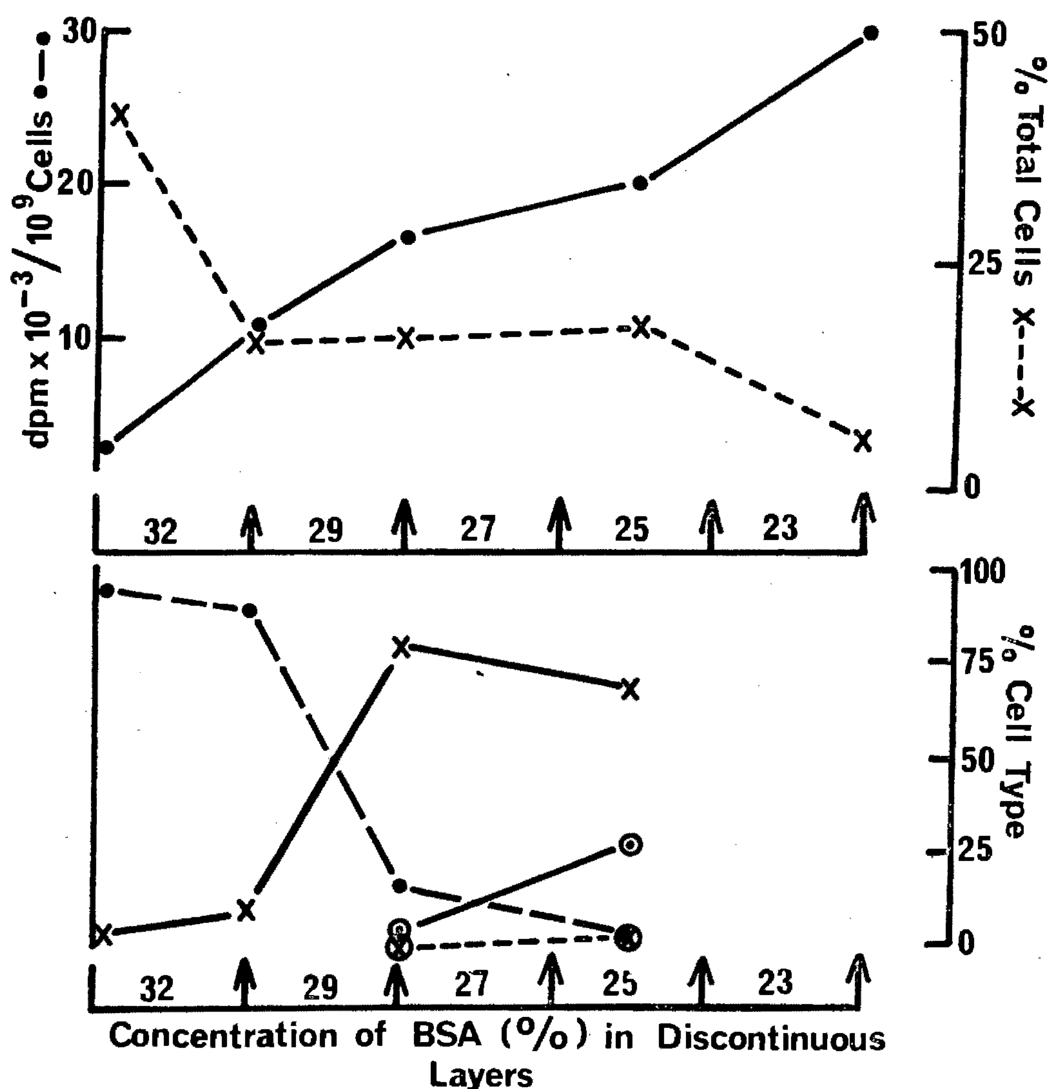
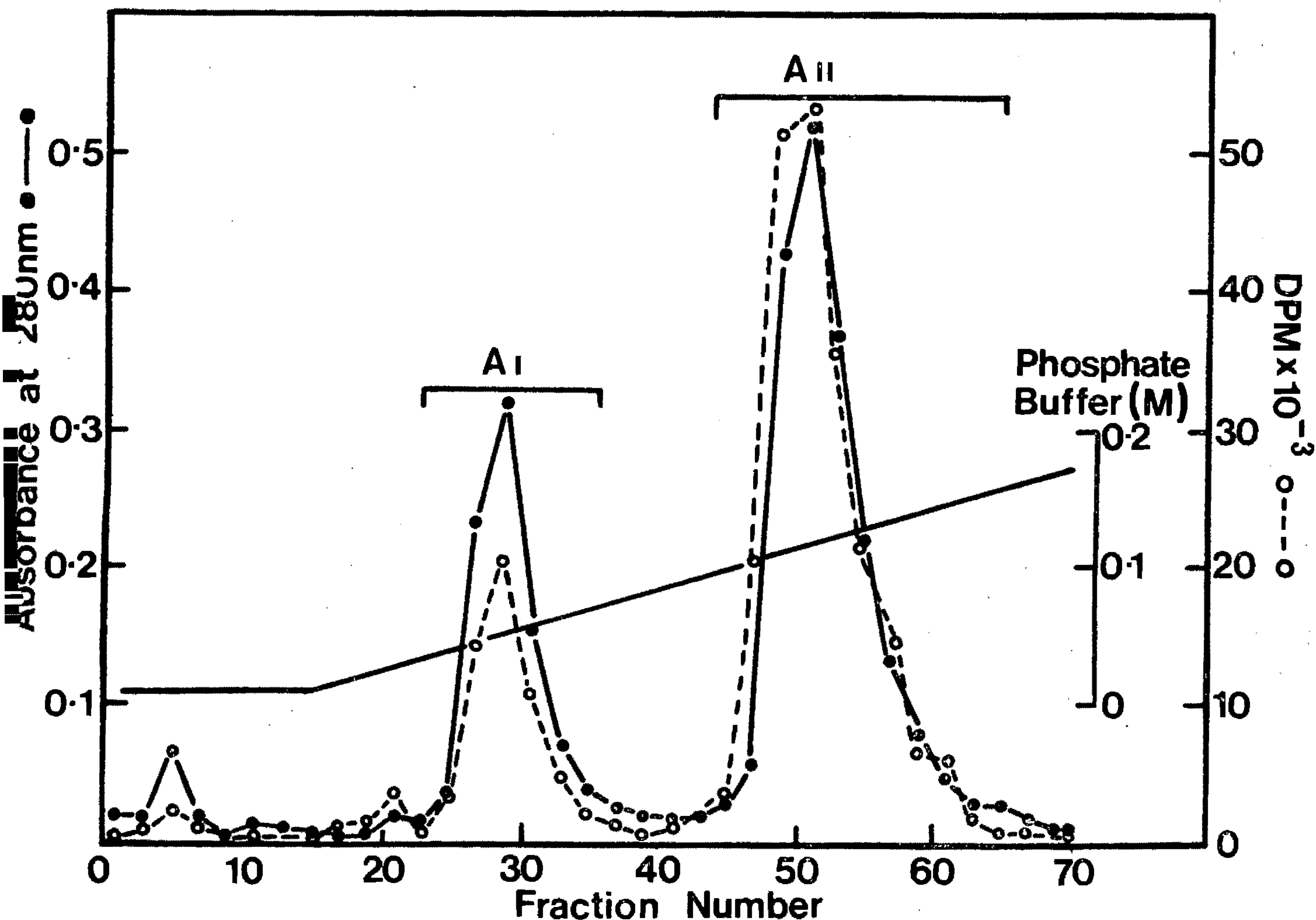


FIG. 2.8

Separation of chicken haemoglobins on carboxymethyl cellulose. 1.0 ml of anaemic blood suspension (Materials and methods 7.2.2.2.2) was incubated with 10 μCi [^{3}H]-histidine at 37°C for 1 hour. After incubation 0.1 ml aliquots were removed, 1.0 ml 5% TCA was added and acid-insoluble radioactivity was determined. A post-mitochondrial supernatant was prepared from the rest of the cell suspension. 29 $OD_{280\ nm}$ units were applied to a 1.3 x 11 cm column of CM-52 previously equilibrated with 0.01 M potassium phosphate buffer pH 6.3. Protein was eluted with a 250 ml linear gradient of 0.01 M - 0.2 M phosphate buffer pH 6.3 at a flow rate of 66 ml/h. 3 ml fractions were collected and assayed spectrophotometrically at 280 nm; acid-insoluble radioactive material was determined in appropriate fractions as described in section 7.2.2.2.2(a).



insoluble radioactivity recovered from whole cells incubated with $[^3H]$-histidine was recovered in the fractions (23-36) (AI) and fractions (44-65) (AII); (nomenclature according to Matsuda et al. (65)). The presence of two haemoglobins from a post-mitochondrial supernatant prepared from chicken erythrocytes was confirmed by acrylamide gel electrophoresis (Fig. 2.9).

These experiments have defined the parameters of a system in this laboratory in which immature erythrocytes can be produced in significant amounts in the circulating blood of chickens by injection with phenylhydrazine. These immature cells, active in the almost exclusive synthesis of haemoglobin, mature in the course of a few days to cells almost completely inactive in terms of haemoglobin synthesis.

2.2.3 RIBOSOME BOUND RNA-DEPENDENT RNA POLYMERASE ACTIVITY DURING ANAEMIA

Ribosomes were prepared from aliquots of blood extracted on each day of anaemia (as well as from normal blood) and the yield was determined spectrophotometrically (Fig. 2.10). Ribosome yield increased almost ten times during anaemia (0.0108 mg/10^9 cells (normal) to 0.1079 mg/10^9 cells (anaemic, day 4)). The parallel increase in reticulocyte count and cellular ribosome content is consistent with the observation (Fig. 2.5) that these immature cells were more active in globin synthesis.

Ribosomes were then assayed for RNA-dependent RNA polymerase activity. Total ribosome bound RNA-dependent RNA polymerase activity rose some 40 times, from 0.66 units of enzyme activity/10^9 cells (normal blood; for definition of units see Materials and methods 7.6.5) to 26.51 units/10^9 cells (anaemic blood - day 5, Fig. 2.11). The specific activity also increased (Fig. 2.12) from 56.2 units of enzyme activity/mg ribosomes (normal blood) to 528.6 units/mg ribosomes on day 7 (an anaemic/normal ratio of 9.4 on day 7).

These results clearly show, for the first time, the presence of a

FIG. 2.9

Polyacrylamide gel electrophoresis of total chicken haemoglobin and haemoglobin separated on CMC. Preparation and electrophoresis of post-mitochondrial supernatant from immature chicken erythrocytes was as described in 7.2.2.2.2(b).

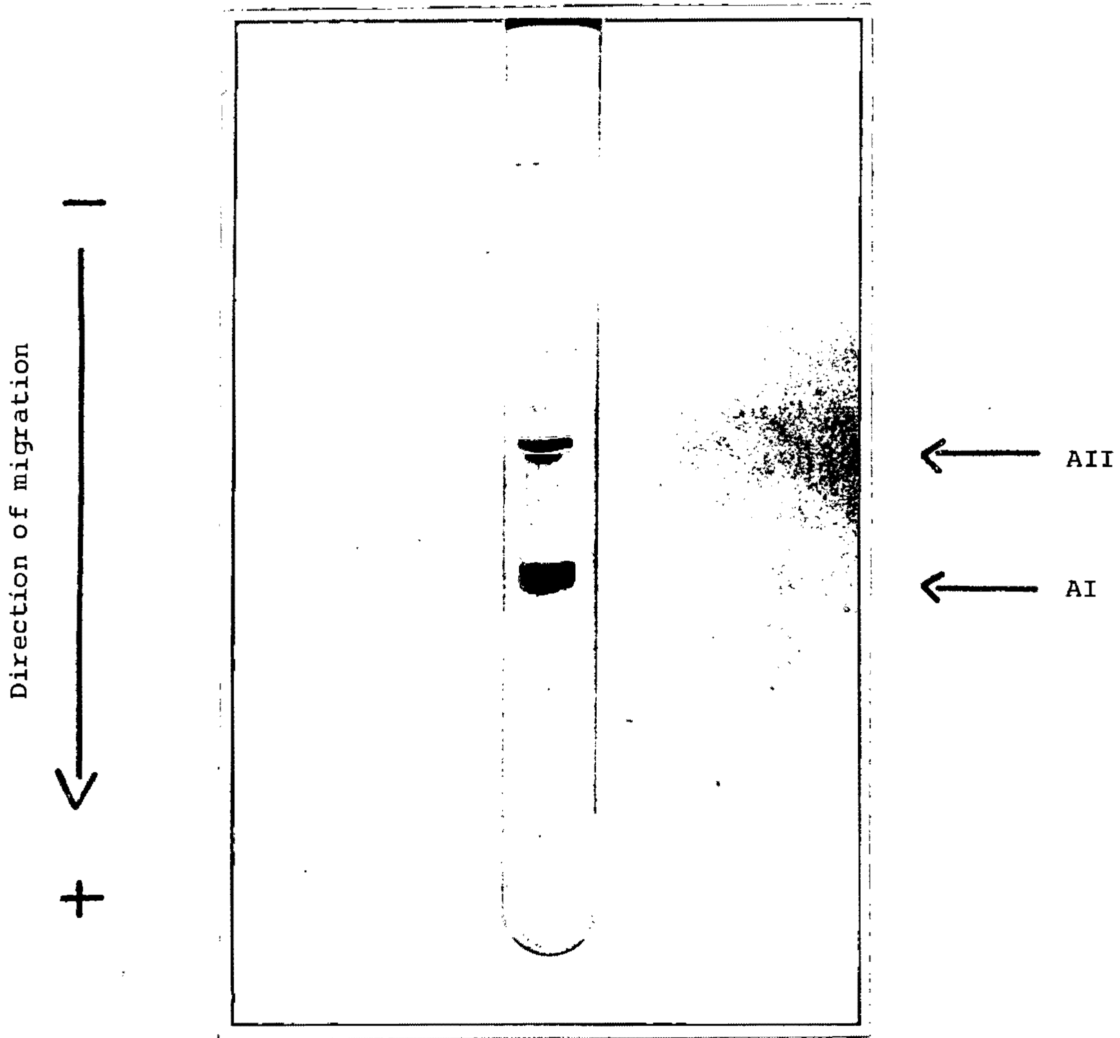


FIG. 2.10

Ribosome content in red blood cells during anaemia. Average values of 3 control and 3 experimental animals. Blood was removed daily and ribosomes isolated as described in Materials and methods (7.6.2). Results are expressed as a ratio of the yield of ribosomes per 10^9 cells from anaemic chickens to the yield of ribosomes per 10^9 cells from normal chickens. ●----● Ribosome yield (mg/10^9 cells); ●——● Reticulocyte count. Ribosome yield was evaluated from the relationship : 1.0 mg ribosomes/ml ——> $A_{260\ nm}^{1\ cm}$ = 10.

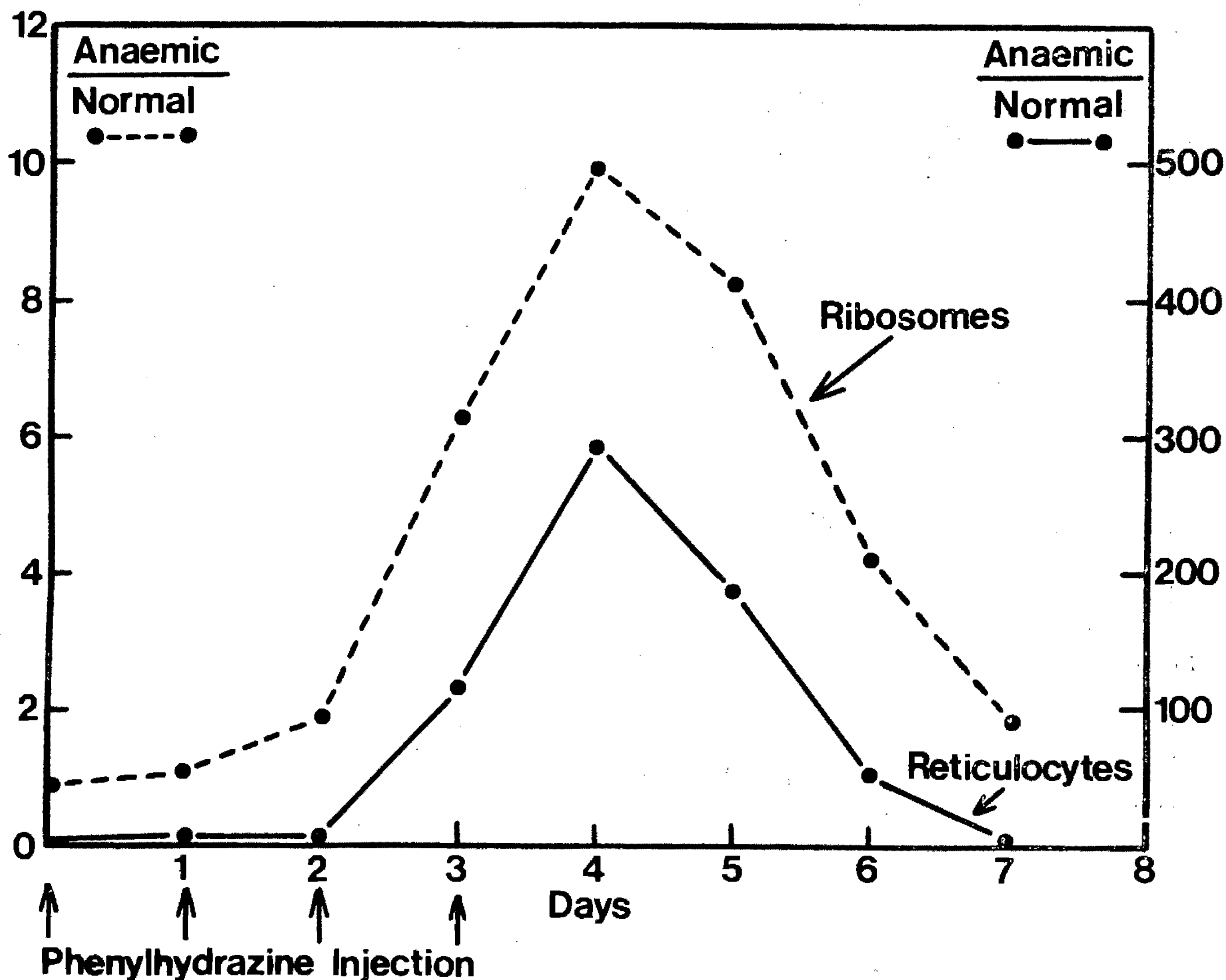


FIG. 2.11

RNA synthesis, globin synthesis and total ribosome-bound RNA-dependent RNA polymerase activity in red blood cells during anaemia. Average values of 3 control and 3 experimental animals. Blood was removed daily and ribosomes isolated as described in Materials and methods (7.6.2). The ribosomal pellets were suspended in 50 mM Tris-HCl pH 7.8, 1.0 mM DTT, 1.0 mM EDTA, 0.25 M sucrose and RNA polymerase activity determined in standard incubation mixtures (no added 18S RNA) (Materials and methods 7.6.5). Globin and RNA synthesis were determined as described in the legend to Fig. 3.5. All results are expressed as a ratio of the parameters found in anaemic and normal animals. ⊙——⊙ globin synthesis (dpm/10^9 cells); o----o total cellular RNA synthesis (dpm/10^9 cells); x----x RNA-dependent RNA polymerase activity (units/10^9 cells); ●——● reticulocyte count.

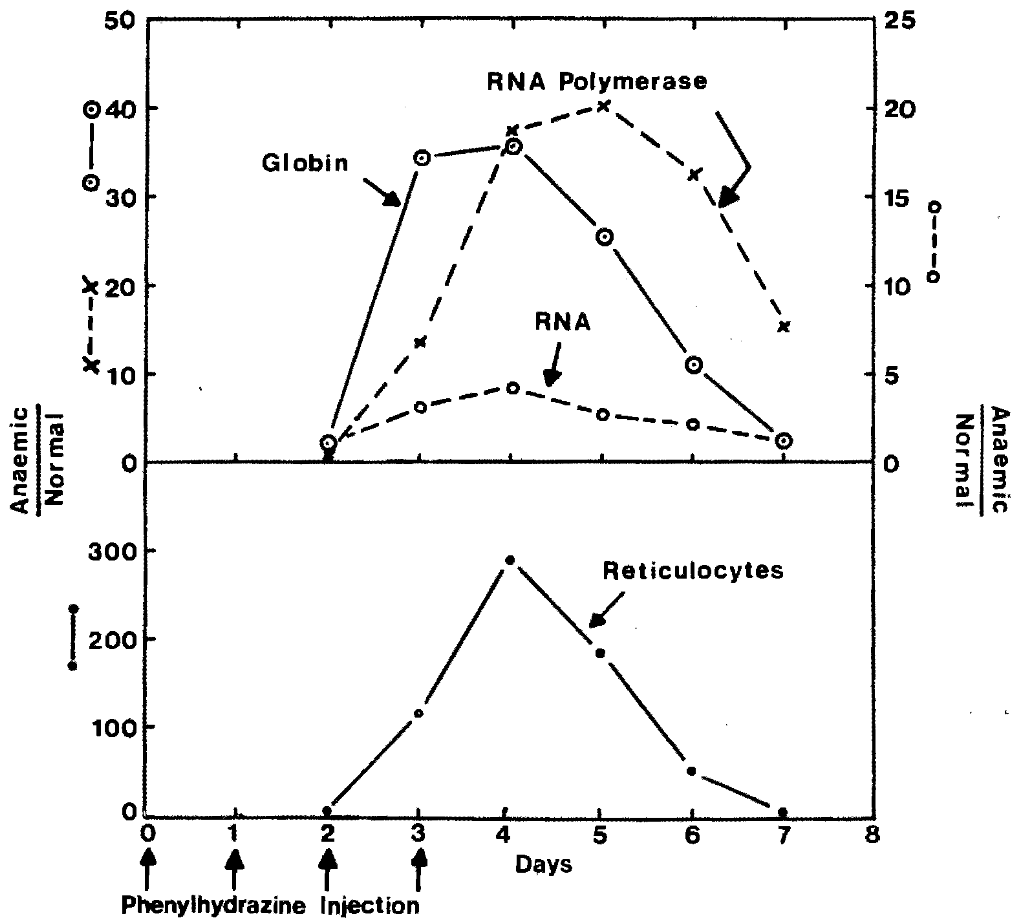
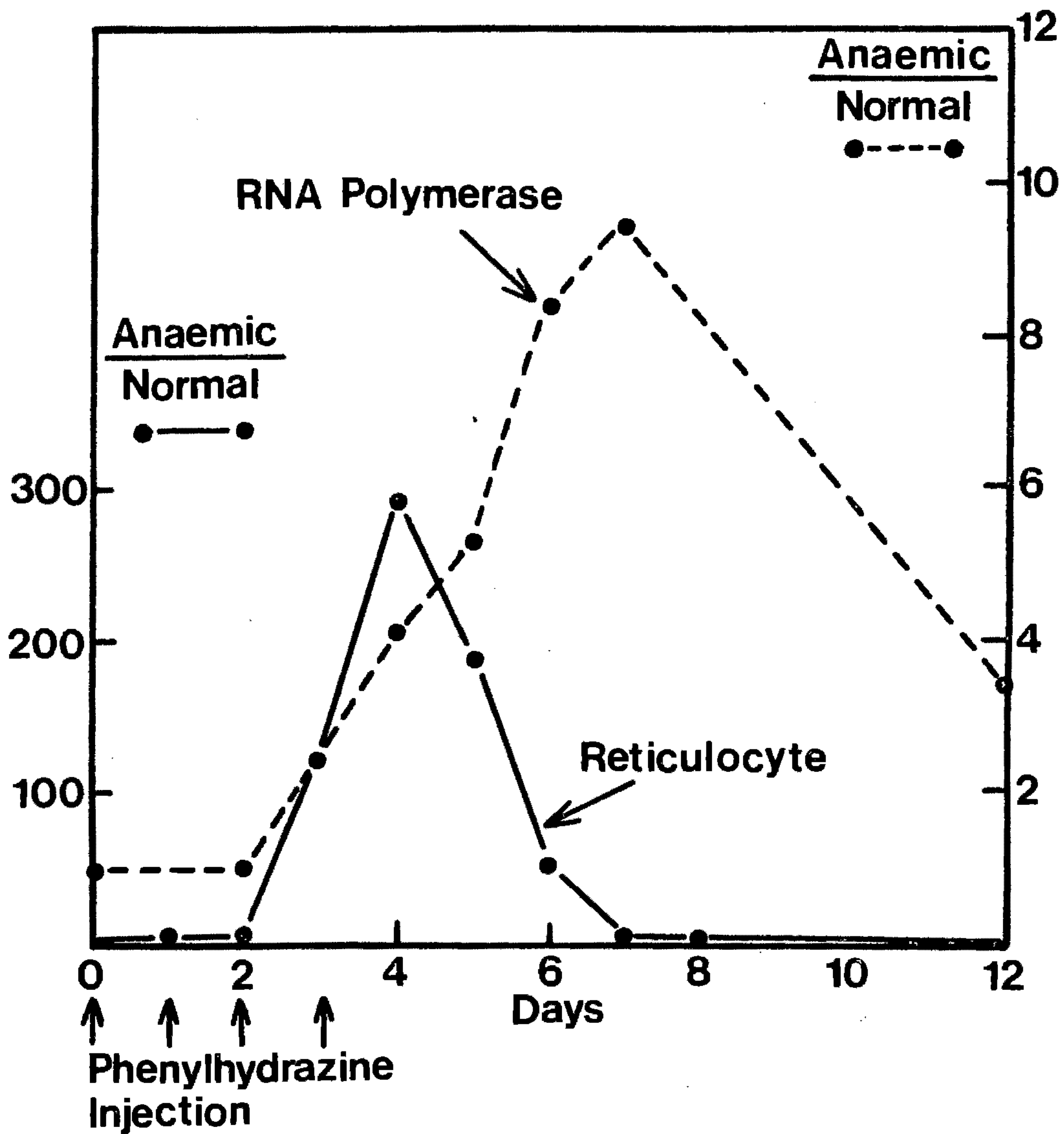


FIG. 2.12

Specific activity of ribosome-bound RNA-dependent RNA polymerase activity in red blood cells during anaemia. Refer to the legend of Fig. 2.11 for details. ●----● RNA-dependent RNA polymerase activity (units/mg ribosomes). ●——● reticulocyte count.



ribosome bound RNA-dependent RNA polymerase activity in maturing avian erythrocytes. The increase in total and specific activities of a ribosome bound RNA-dependent RNA polymerase during anaemia, paralleled by the increased synthesis of total cellular RNA and globin and a concomitant increase in many of the constituents closely associated with translation (total ribosome yield, Fig. 2.10; polyribosome content (66); 9S RNA content (57)) would seem to reflect the involvement of this enzyme in the synthesis or control of synthesis of globin.

Whether such a ribosome bound RNA-dependent RNA polymerase plays a role in the control of synthesis of all proteins is not known. Its presence however in the maturing chicken erythrocyte and its likely involvement in the synthesis of globin poses interesting problems as to the nature of a more specific role for this RNA-dependent RNA polymerase activity during avian erythrocyte maturation (this evidence, for example, is consistent with the hypothesis that such an RNA-dependent RNA polymerase may be involved in globin mRNA replication).

The activity of ribosome bound RNA-dependent RNA polymerase (units/mg ribosomes) is still 3.4 times normal values on day 12 (Fig. 2.12). This anaemic/normal ratio increased to 5.63 when activities per cell were compared. Globin synthesis, ribosome yield and reticulocyte count have returned to normal values by day 12. The change in cell count from day 7 to day 12 was negligible (Fig. 2.1(a)). If one assumes that the decrease in cellular levels of ribosome bound RNA-dependent RNA polymerase is due to enzyme catabolism, and that no synthesis of the enzyme occurs in circulating immature erythrocytes (see below), the half-life of this RNA-dependent RNA polymerase can be estimated as being equal to 40 h.

The specific activity of ribosome bound RNA-dependent RNA polymerase increased during days 6 and 7 (Fig. 2.12), a period in which total RNA-dependent RNA polymerase activity, ribosome yield and reticulocyte count were all clearly decreasing (Fig. 2.10 and 2.11). The long half-life of this enzyme would not be an obvious explanation for this observation. An alternative suggestion is a decrease in ribosome yield reflecting the selective degradation of ribosomes in almost completely

Ribosome yield (mg/10^9 cells) increased almost ten times during anaemia. Total ribosome-bound RNA-dependent RNA polymerase activity (units of enzyme/10^9 cells) increased 40 times over the same period; the specific activity (units of enzyme activity/mg ribosomes) rose significantly during the same period.

These increases in RNA-dependent RNA polymerase activity, paralleled by increases in components known to be closely associated with the synthesis of globin has led me to conclude that such an RNA-dependent RNA polymerase activity may play a role in the synthesis of globin or the translational control of globin synthesis. Although ribosome bound RNA-dependent RNA polymerase activities, responsible for the incorporation of UTP into polynucleotide material have been demonstrated in a variety of eukaryotic cells (see Section 1.1) the general occurrence of such enzyme activities in all cells is not known. In addition, whether such ribosome bound RNA polymerase activities are a general requirement for the synthesis of all proteins or only the synthesis of proteins in highly differentiated tissue (as in the immature erythrocyte) is equally unclear.

mature cells, resulting in an apparently higher titre of enzyme per mg ribosomes isolated from anaemic blood containing immature erythrocytes in the final stages of maturation (indistinguishable from mature erythrocytes using brilliant cresyl blue). The synthesis of RNA-dependent RNA polymerase in the circulating erythrocyte is another speculative possibility. In the context of the latter suggestion, the following correlation can be made : 12S RNA has been shown to be present in haemoglobin-producing polyribosomes (67). It is certainly present in immature chicken erythrocyte ribosomes (Figs. 4.3, 4.4 and Table 3.4). The nature of 12S RNA is unknown, circumstantial evidence (67) points to a function as a messenger RNA - but for what protein ? The molecular weight of 12S RNA is in the region of approximately 400 000 (67,68). A mRNA of this size would code for a polypeptide of molecular weight of about 40 000. The molecular weight distribution of proteins from a partially purified RNA-dependent RNA polymerase preparation separated by SDS-acrylamide gel electrophoresis is from 11 000 to 50 000 daltons (Fig. 3.8) with many of the proteins sedimenting in the 40 000 molecular weight region. A correlation between molecular weights of particular proteins from an impure preparation of this enzyme activity and the molecular weight of a tentative mRNA product could be completely fortuitous; it is however interesting to speculate that the 12S RNA might direct the synthesis of protein(s) with RNA-dependent RNA polymerase activity.

2.3 CONCLUSION

Induction of anaemia in adult chickens by subcutaneous injection of phenylhydrazine was the method used in this laboratory to produce immature erythrocytes for the purpose of studying the occurrence of ribosome-bound RNA-dependent RNA polymerase. A number of parameters associated with this maturational system were defined, in confirmation of previous reports. These included reticulocyte and total leucocyte count, protein and RNA synthesis. 92% of total protein synthesis in immature erythrocytes was shown to be haemoglobin synthesis. Leucocyte count did not change during anaemia nor did the RNA and protein synthesizing activity of these cells contribute significantly to overall RNA and protein synthesis in anaemic blood.

PART 3

PURIFICATION AND PARTIAL CHARACTERIZATION OF RIBOSOME BOUND RNA-DEPENDENT RNA POLYMERASE FROM IMMATURE CHICKEN ERYTHROCYTES

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3.1 INTRODUCTION

The results from the previous chapter have shown the presence of a ribosome bound RNA-dependent RNA polymerase in immature chicken erythrocytes and indicated the possible involvement of this enzyme in the synthesis of globin. An important prerequisite in the elucidation of a more specific role for this RNA-dependent RNA polymerase is that of template dependency: is this RNA-dependent RNA polymerase activity a template or primer-dependent phenomenon? In order to answer this question it was necessary to purify the ribosome bound RNA-dependent RNA polymerase preparation to the extent where meaningful studies could be carried out.

This chapter describes the purification of RNA-dependent RNA polymerase from ribosomes of immature chicken erythrocytes and characterization of the isolated enzyme fraction in terms of nucleotide and nucleic acid requirements and template dependency; other aspects including polynucleotide phosphorylase and ribonuclease activity are also considered.

3.2 RESULTS AND DISCUSSION

3.2.1 PURIFICATION OF RIBOSOME BOUND RNA-DEPENDENT RNA POLYMERASE AND A TEST FOR TEMPLATE DEPENDENCY

An RNA-dependent RNA polymerase was isolated from ribosomes of immature chicken erythrocytes and enriched 158 fold (Table 3.1). The KCl extraction and subsequent precipitation with ammonium sulphate, although effecting little increase in specific enzyme activity was nevertheless

TABLE 3.1

Preparation of RNA-dependent RNA polymerase from 385 ml of anaemic blood. For details of purification procedure see Materials and methods 7.6.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein	Enzyme recovery (%)	Purification	$\frac{E_{280\ nm}}{E_{260\ nm}}$
Postmitochondrial supernatant	10248	53128	5.20	100	1	1.10
Ribosome suspension	77.51	29208	376	55	72	0.62
60% saturated $(NH_4)_2SO_4$ precipitate in TM-30 buffer	36.50	14530	398	27	76	0.78
DEAE-Sephadex chromatography (fractions 38-50)	11.00	9064	824	17	158	1.91

important as it solubilized this RNA-dependent RNA polymerase activity (no activity was found in the KCl-extracted ribosome pellet) and facilitated further purification on DEAE-Sephadex.

The elution of RNA-dependent RNA polymerase from a DEAE-Sephadex column using a linear gradient of ammonium sulphate is shown in Fig. 3.1. In a typical experiment, 185 $OD_{260\ nm}$ units were applied to the column of DEAE-Sephadex. The material eluted from the column has an absorbance maximum at 280 nm and consists predominantly of protein; the yield of protein off the column (as determined by the method of Lowry et al. (167)) was about 90%. Most of the RNA in the ammonium sulphate fraction (see Fig. 3.2) must therefore remain bound to the column. This resulted in a marked increase in the $E_{280\ nm}/E_{260\ nm}$ ratio of the pooled fraction (frac. 38-50, Table 3.1; see also absorbance spectra, Fig. 3.2). The elution profile of RNA-dependent RNA polymerase activity illustrated in Fig. 3.1 exhibited a degree of heterogeneity that was independent of the physical dimensions of the column or column bed. Although chromatography of the 60% saturated ammonium sulphate fraction using a shallower gradient of ammonium sulphate (0-0.5 M) was attempted, the problem of more than one RNA-dependent RNA polymerase activity remains unresolved.

Chromatography of this avian RNA-dependent RNA polymerase on phosphocellulose, a method used by Downey et al. (12) for the purification of the rabbit enzyme, proved unsuccessful in my hands; equally, little or no RNA-dependent RNA polymerase could be eluted off CM-Sephadex. The reasons for this are unknown.

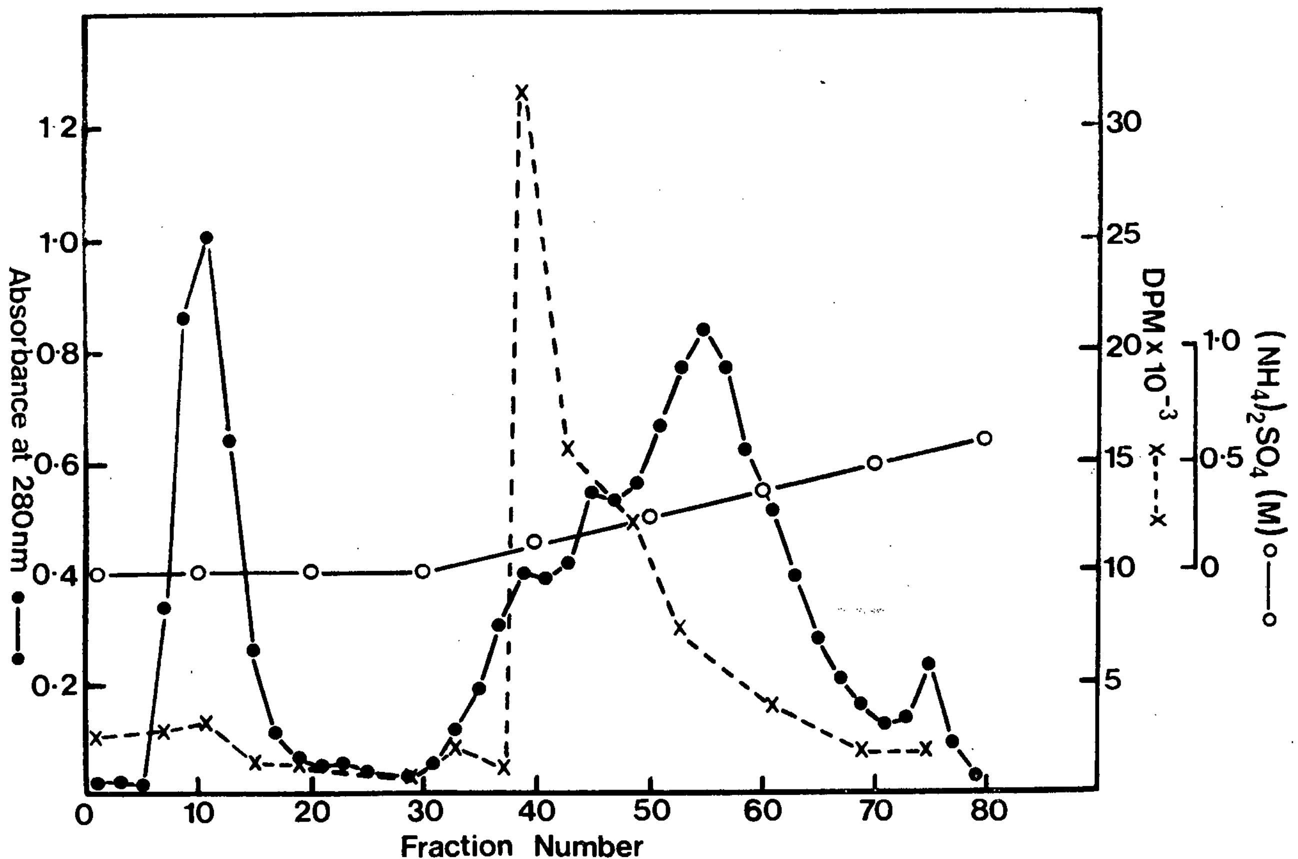
RNA-dependent RNA synthesis by the enriched enzyme preparation (Fractions 38-50, Table 3.1) was almost completely (90%) dependent on added RNA (Table 3.2). DNA had no effect on the incorporation of [^{3}H]-UTP at all.

This enzyme preparation was used to test, by measuring the incorporation into RNA of the individual tritiated ribonucleoside triphosphates in the presence of various ribonucleotide polymers, whether the incorporation of ribonucleotides into polynucleotide material by this RNA-

FIG. 3.1

DEAE-Sephadex chromatography of RNA-dependent RNA polymerase. A 60% saturated-$(NH_4)_2SO_4$ precipitate (prepared from ribosomes of an anaemic blood type), dissolved in TM-30 and dialyzed against the same buffer (see Materials and methods 7.6.4), was applied to a 1.3 x 11 cm column of DEAE-Sephadex A-25, previously equilibrated with TM-30 buffer. Protein (OD 280 nm ●——●) was eluted with a 60 ml linear gradient of 0-1.0 M $(NH_4)_2SO_4$ in TM-30 at a flow rate of 34 ml/h.

1.0 ml fractions were collected and 0.10 ml aliquots assayed for enzyme activity in standard incubation mixtures with no added $(NH_4)_2SO_4$ (dpm incorporated x 10^{-2} x-----x).



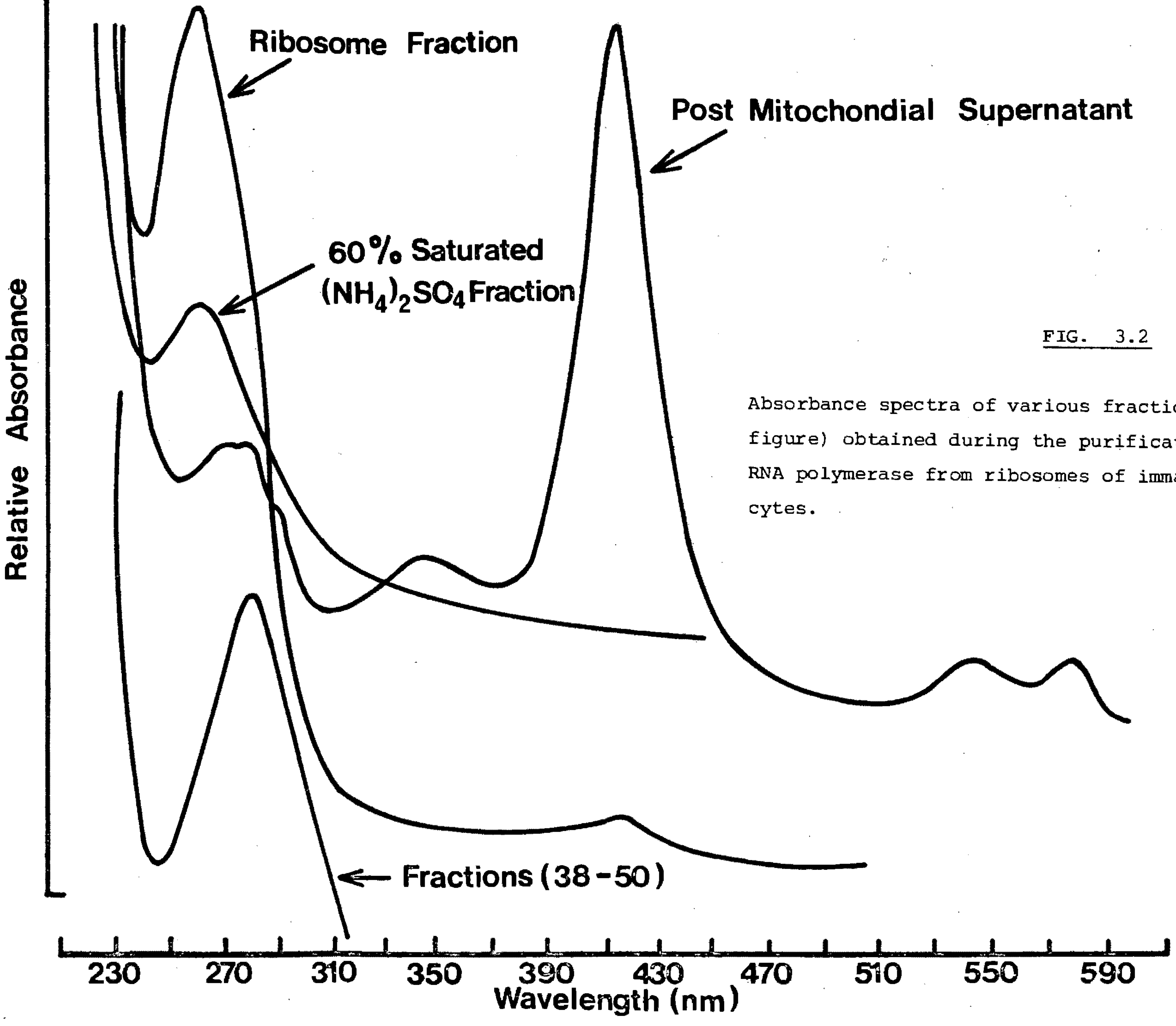


FIG. 3.2

Absorbance spectra of various fractions (indicated in the figure) obtained during the purification of RNA-dependent RNA polymerase from ribosomes of immature chicken erythrocytes.

TABLE 3.2

Incorporation of [^{3}H]-UTP by purified enzyme fraction in the presence of added RNA and DNA. 20 μg immature chicken erythrocyte 18S rRNA or 20 μg chicken erythrocyte DNA and 25 μg enzyme preparation (fractions 38-50, Table 3.1) were added per assay. See Materials and methods 7.6.5 for further details.

Nucleic acid	dpm	Relative activity (%)
18S rRNA	10522	100
DNA	922	8.8
no added nucleic acid	950	9.0

dependent RNA polymerase preparation was a template or primer-dependent phenomenon (Table 3.3).

It was clear from these data that this enzyme preparation incorporated predominantly [^{3}H]-CTP and [^{3}H]-UTP into TCA-precipitable material regardless of which ribonucleotide polymer was present. This suggests the primer-dependent synthesis of a polynucleotide, in contrast to the template dependent synthesis of RNA shown by Downey and co-workers (12). These authors concluded that the enzyme preparation from immature rabbit erythrocytes was a template dependent RNA polymerase, from evidence that demonstrated the incorporation of [^{3}H]-UTP and [^{3}H]-CTP into TCA-insoluble material in the presence of poly(A,G) only. The unambiguous demonstration (Table 3.3) that a similar incorporation of UTP and CTP by an enzyme preparation from immature chicken erythrocytes, regardless of which synthetic polymer was present, indicates that their conclusion of the template dependency of a ribosome bound RNA-dependent RNA polymerase was incorrect. As both enzyme preparations exhibit similar nucleotide incorporation patterns, it is unlikely that the species difference will affect this conclusion. It is unclear why Downey et al. (12) were not able to demonstrate appreciable incorporation of [^{3}H]-UTP in the presence

of synthetic polynucleotides other than poly(A,G). They have shown, for example, that poly(A) is 4.2 times less effective in the incorporation of [^{3}H]-UTP than poly(A,G) (2:1). Although I have used poly(A,G) (1:1), the results from Table 3.3 demonstrate that poly(A) was twice as effective as poly(A,G) in this respect.

TABLE 3.3

[^{3}H]-ribonucleoside triphosphate incorporation into RNA using various ribonucleotide polymers. Reaction conditions were as described in Materials and methods (7.6.5) except for addition of individual [^{3}H]-ribonucleoside triphosphates (all at a specific activity of 250 Ci/mol) and ribonucleotide polymers (70 μg/assay) as indicated. Amount of enzyme added as in Table 3.2. The incorporation figures obtained on incubation of the enzyme preparation with each of the four [^{3}H]-ribonucleotides in the absence of added ribonucleotide polymer represent the background value that was subtracted from incorporation data obtained in the presence of added polynucleotide to give the values shown.

Ribonucleoside triphosphate present	Ribonucleotide polymer			
	Poly(U)	Poly(A)	Poly(A,G)	Poly(U,C)
	Incorporation (dpm)			
[^{3}H]-ATP	224	756	810	751
[^{3}H]-GTP	0	951	2945	911
[^{3}H]-CTP	2779	28253	21684	8951
[^{3}H]-UTP	2795	38377	18803	12406

Analysis of [^{3}H]-ATP and [^{3}H]-GTP by thin layer electrophoresis (Materials and methods 7.7.2.2) have shown that contamination of these radioactively labelled ribonucleotide preparations with [^{3}H]-UTP or [^{3}H]-CTP was less than 1-2%. The data in Table 3.3 therefore would seem to indicate real incorporation of ATP and GTP into polynucleotide material. The incorporation of all four ribonucleotides suggests several possibilities:

a) The RNA-dependent RNA polymerase preparation might be a mixture of template and primer-dependent RNA polymerase activities, the lattter being the dominant one.

b) The enzyme preparation might be a primer-dependent RNA polymerase activity synthesizing homopolymers of all four ribonucleotides. Wykes and Smellie (8) have demonstrated the existence of such enzyme activities in the microsomal fraction of Landschutz ascites tumour cells.

c) The enzyme preparation might be a primer-dependent RNA polymerase activity incorporating all four [^{3}H]-ribonucleotides into heteropolyribonucleotide material.

Wilkie and Smellie ((9) and Section 1.1.2) have shown that a microsomal fraction from rat liver effects the incorporation of all four ribonucleoside 5'-triphosphates into heteropolyribonucleotide material. From Table 3.3, it is not apparent whether the enzyme preparation effected homo- or heteropolyribonucleotide synthesis. It is clear however, that although the template-dependent synthesis of RNA cannot be excluded, the primer-dependent synthesis of polynucleotide material is the predominant activity present in this cytoplasmic RNA-dependent RNA polymerase preparation.

From Table 3.3 it is evident that ribonucleotide polymers containing adenylic acid stimulate the greatest incorporation of [^{3}H]-CTP and [^{3}H]-UTP. The significance of these results however is unclear; evidence to date would not implicate, for example, the involvement of the poly(A) segment at the 3'-end of mRNA in cytoplasmic RNA synthesis.

3.2.2 FURTHER CHARACTERIZATION OF THE PURIFIED RNA-DEPENDENT RNA POLYMERASE PREPARATION

3.2.2.1 RIBONUCLEIC ACID REQUIREMENTS

Table 3.2 demonstrated that the ribosome bound RNA polymerase preparation from immature chicken erythrocytes was indeed RNA-dependent. To investigate whether this enzyme preparation showed any specificity towards any particular RNA, the incorporation of [^{3}H]-UTP by this RNA-dependent RNA polymerase preparation was tested in the presence of various RNA species isolated from ribosomes of immature chicken erythrocytes (Table 3.4). Downey et al. (12) reported that 9S RNA and 18S rRNA stimulated incorporation of [^{3}H]-UTP into TCA-insoluble material to the greatest extent. Although the incorporation of [^{3}H]-UTP by the chicken enzyme preparation in the presence of all the various RNA species shown in Table 3.4 supports this finding, in view of the substantial incorporation of UTP in the presence of 5S, 12S and 28S rRNA, the incorporation in the presence of 18S rRNA and 9S RNA cannot be said to be selective for globin mRNA sequences in these two fractions. Kabat (69) has shown the presence of globin 9S mRNA in 28S rRNA fractions prepared from immature rabbit erythrocytes by mild procedures (70), that could be removed by heat treatment. As all the RNA fractions prepared in this laboratory were heated (Materials and methods 7.3.3) prior to application to sucrose density gradients, the presence of 9S mRNA in the various fractions would seem unlikely. Imaizumi et al. (71), in addition, have shown that immature avian erythrocyte 12S polysomal RNA does not contain any 9S globin mRNA sequences i.e. this RNA species is not a precursor to globin mRNA. Therefore, although it cannot be excluded that the incorporation in the presence of 5S RNA could be due to the presence of degraded globin mRNA, taken in toto, these results clearly do not support the conclusions of Downey and co-workers (12).

TABLE 3.4

RNA requirements of RNA-dependent RNA polymerase from ribosomes of immature chicken erythrocytes. Reaction conditions for RNA synthesis were as described in 7.6.5 except for addition of RNA as indicated (20 μg/assay) and 25 μg of enzyme preparation per assay. Each RNA species was isolated from ribosomes of immature chicken erythrocytes by extraction with phenol and repeated sucrose density gradient centrifugation. Each RNA species was homogeneous as judged by sucrose gradient centrifugation (see section 7.6.6 for details). A background of 950 dpm (endogenous activity) has been subtracted to obtain these figures.

RNA	dpm	Relative activity (%)
18S RNA	9570	100
9S RNA	8820	92
12S RNA	8130	85
5S RNA	6220	65
28S RNA	6130	64

A lack of preference for 9S mRNA or 18S aggregates thereof however, is not necessarily inconsistent with the hypothesis of globin mRNA replication. The specificity of such an RNA replicase could depend on the proteins associated with its mRNA template; removal of these proteins could destroy the template specificity of the enzyme.

3.2.2.2 DEOXYRIBONUCLEOTIDE INCORPORATION

A DNA-dependent DNA polymerase activity has been isolated from rabbit reticulocyte ribosomes (72). The chicken RNA-dependent RNA polymerase preparation does not incorporate DNA precursors into acid-insoluble material using either added DNA or 18S rRNA (Table 3.5). This enzyme preparation therefore contains no detectable levels of either DNA-dependent or RNA-dependent DNA polymerase activity.

TABLE 3.5

Incorporation of [^{3}H]-radioactively labelled ribonucleotides by RNA-dependent RNA polymerase preparation from ribosomes of immature chicken erythrocytes. Reaction conditions for DNA synthesis were as described in Materials and methods 7.6.5 except that the [^{3}H]-precursor (8 μM) was used at a specific activity of 250 Ci/mol. Amounts of added nucleic acid and enzyme added per assay were as in the legend to Table 3.2.

Nucleic acid	[^{3}H]-precursor	Relative activity (%)
18S rRNA	UTP	100
18S rRNA	TTP	0
Chicken erythrocyte DNA	TTP	0

3.2.2.3 REQUIREMENTS FOR RNA-DEPENDENT RNA SYNTHESIS

Using the assay for RNA polymerase activity described by Downey et al. (12) (for details see Materials and methods 7.6.5), the RNA directed synthesis of RNA by the purified enzyme preparation showed a requirement for Mn^{++} (Table 3.6). In the absence of Mn and EDTA, 28% of total activity was observed. Present in the enzyme buffer, TM-30, was 5 mM $MgCl_2$; the final Mg^{++} concentration in each incubation (0.05 ml aliquots of enzyme fraction per assay) was 1.0 mM. In the absence of a chelating agent and Mn^{++} therefore, the recorded activity was due to the presence of Mg^{++}. Removal of EDTA only, results in a 50% drop in [^{3}H]-UTP incorporation presumably due to excess Mn^{++} in the incubation (EDTA chelates Mn^{++} in preference to Mg^{++} (73)).

A preference towards either cation is not evident from these studies. Previous reports (12, 9) concerning the cation requirements of ribosome or microsome-bound RNA-dependent RNA polymerase activities have demonstrated either Mn^{++} or Mg^{++} to be the preferred cation. Further investigation is necessary to determine cation preference and optimum concentrations of either cation in the incubation, in the absence of EDTA.

TABLE 3.6

Requirements for RNA-dependent RNA synthesis. Reaction conditions were as described in section 7.6.5 except that the individual components of the reaction mixture were omitted as indicated. 18S rRNA was the added RNA (30 μg).

Reaction conditions	Relative activity (%)
Complete	100
-Mn	0
-Mn, -EDTA	28
-EDTA	48
-Dithiothreitol	11
-ATP, GTP, CTP	122

The presence of dithiothreitol was essential for activity. Reduced sulfhydryl groups presumably play an important role in enzyme activity (compare with nuclear DNA-dependent RNA polymerase (75)).

The absence of ATP, GTP and CTP from the incubation mixture stimulates the incorporation of [^{3}H]-UTP into polynucleotide material (or alternatively (Section 1.1.2), the presence of the three unlabelled ribonucleotides inhibited the incorporation of [^{3}H]-UTP). This suggests homopolyribonucleotide synthesis in the presence of the other ribonucleotides. This was further confirmed by incubating each respective radioactively labelled ribonucleotide with purified RNA-dependent RNA polymerase, in the presence of the other three unlabelled nucleoside 5'-triphosphates, and comparing the incorporation of labelled nucleotides in the absence of the other three (Table 3.7). In all cases, the incorporation of a particular ribonucleotide was inhibited by the presence of the other three. This is similar to the homopolymer synthesis reported by Wykes and Smellie (8) by an enzyme preparation from the microsomal fraction of Landschutz ascites tumour cells. Taken together therefore, the results from Table 3.3 and 3.7 indicate the presence of a primer dependent enzyme activity (or activities) synthesizing homopolymers of AMP, GMP, CMP and UMP, the synthesis of poly(U) and poly(C) predominating.

The incorporation of [^{3}H]-CTP is inhibited to a greater extent by the addition of unlabelled ribonucleoside 5'-triphosphates than the incorporation of radioactively labelled UTP, GTP or ATP (Table 3.7). The relative incorporation of UTP therefore is higher when all 4 ribonucleotides are present. The significance of this result is unclear.

Although the results shown in Table 3.7 were obtained using added poly(A), similar results could be demonstrated using 18S rRNA. The increased relative incorporation of [^{3}H]-UTP therefore is not due to template-dependent synthesis; poly(A) was used in these studies as it stimulated the greatest incorporation of [^{3}H]-UTP into TCA-insoluble material.

3.2.2.3.1 TIME DEPENDENCE OF THE REACTION

The incorporation of [^{3}H]-UTP by the purified RNA-dependent RNA polymerase preparation was linear for 30 min and slowly declined after that (Fig. 3.3).

TABLE 3.7

The incorporation of [^{3}H]-ribonucleotides by the purified RNA-dependent RNA polymerase preparation in the presence and absence of the other three unlabelled nucleoside 5'-triphosphates. Each ribonucleotide (labelled or unlabelled) was added to a final concentration of 8 μM; poly(A) was used throughout (70 μg/assay). All further details as described in 7.6.5.

Ribonucleotides present				Incorporation (dpm)	Relative activity (%)
[^{3}H]-UTP	CTP	ATP	GTP	22820	100
UTP	[^{3}H]-CTP	ATP	GTP	5127	23
UTP	CTP	[^{3}H]-ATP	GTP	476	2.1
UTP	CTP	ATP	[^{3}H]-GTP	495	2.2
[^{3}H]-UTP	-	-	-	38377	100
-	[^{3}H]-CTP	-	-	28253	74
-	-	[^{3}H]-ATP	-	951	2.5
-	-	-	[^{3}H]-GTP	756	1.9

3.2.2.3.2 DEPENDENCE OF RNA-DEPENDENT RNA SYNTHESIS ON ENZYME CONCENTRATION

The incorporation of [^{3}H]-UTP was linear between 10 μg and 25 μg enzyme protein added per incubation (Fig. 3.4). Non-linearity below this figure may be due to a dilution affect in the absence of added carrier protein.

3.2.2.3.3 DEPENDENCE OF RNA-DEPENDENT RNA SYNTHESIS ON THE CONCENTRATION OF ADDED RNA

FIG. 3.3

Time dependence of RNA synthesis catalyzed by RNA-dependent RNA polymerase. Assays were performed as described in section 7.6.5 (25 μg enzyme protein per assay) except that poly(A) (70 μg/assay) was used and the time of incubation was varied as shown. Each experiment was done in duplicate and the range of values between duplicates is indicated.

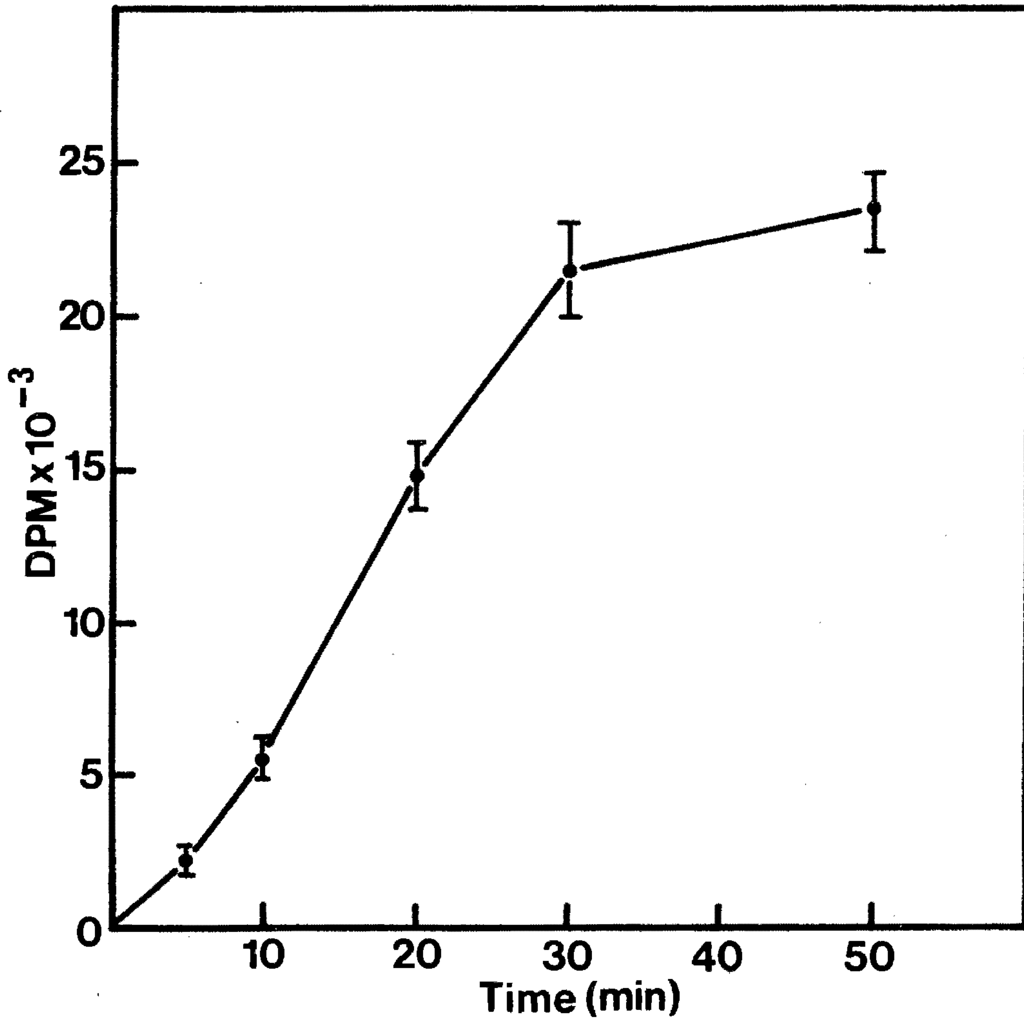
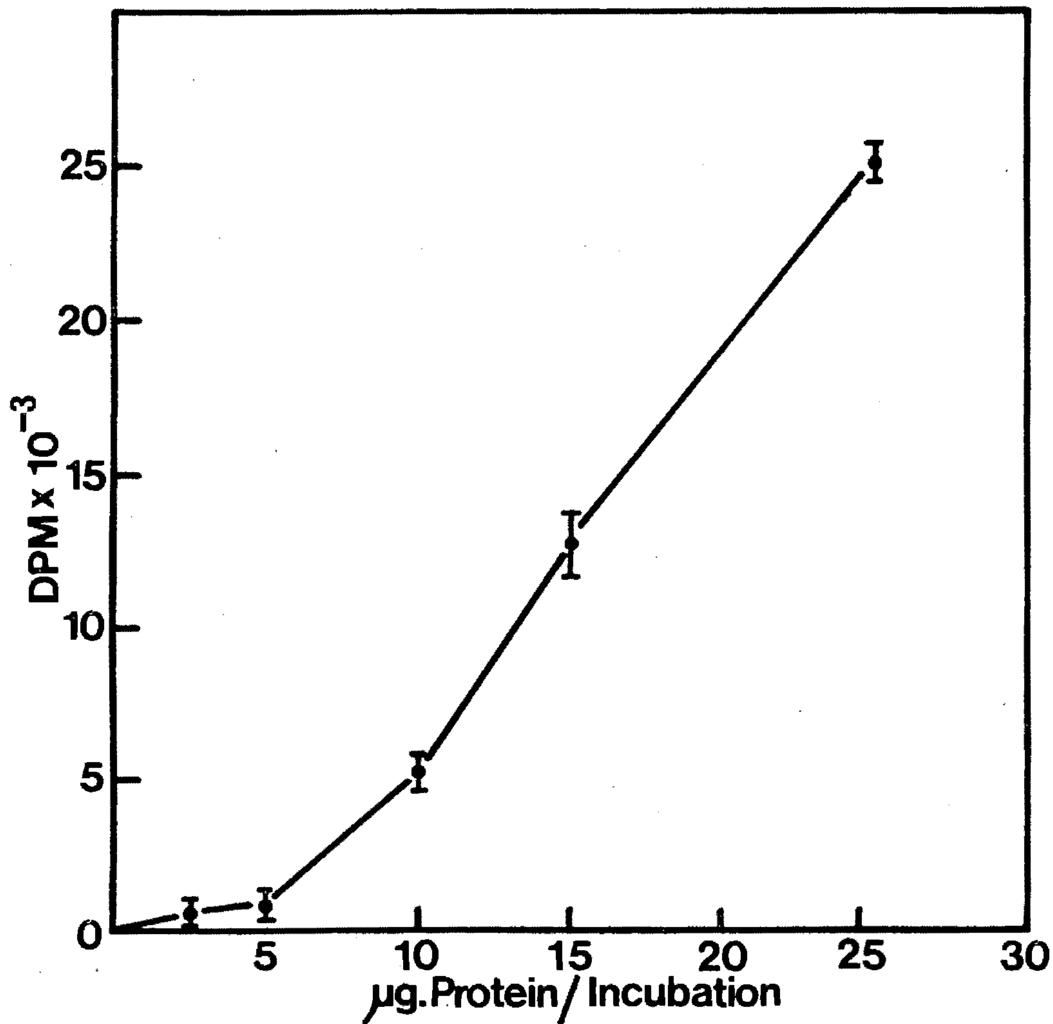


FIG. 3.4

The rate of RNA synthesis at various concentrations of RNA-dependent RNA polymerase. Reaction conditions were as previously described (legend to Fig. 3.3) except that incubation was for 30 min and the concentration of protein added per assay was varied as shown.



The incorporation of $[^3H]$-UTP by the RNA-dependent RNA polymerase preparation was proportional to the concentration of added RNA up to 50 μg RNA per incubation (Fig. 3.5), and then tails off markedly. This experiment was conducted by my colleague Derek Woods, using a mixture of 18S and 28S rRNA from sea urchin embryos. In my experience, no difference in the stimulation of $[^3H]$-UTP incorporation into TCA-insoluble material was found between a mixture of 18S and 28S rRNAs from rabbit or chicken reticulocyte ribosomes or from ribosomes of sea urchin embryos.

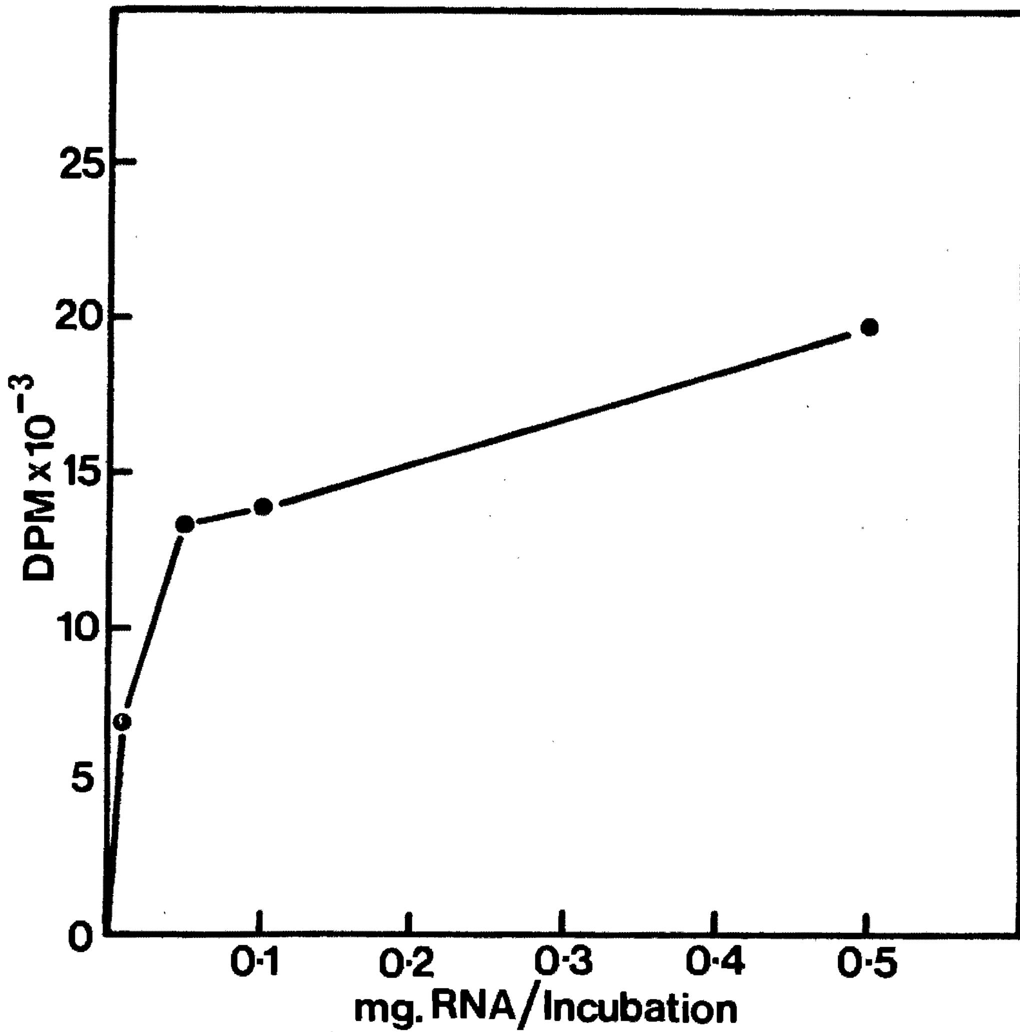
3.2.2.4 DETERMINATION OF RIBONUCLEASE ACTIVITY IN AN RNA-DEPENDENT RNA POLYMERASE PREPARATION

Although reticulocytes have been found to be markedly free of ribonuclease activity (74), the demonstration of phosphodiesterase activity in an RNA-dependent RNA polymerase preparation from rat liver (22) stimulated an investigation of levels of ribonuclease activity from immature chicken erythrocytes. Aliquots of this RNA-dependent RNA polymerase preparation were incubated with $[^3H]$-poly(U) and $[^3H]$-polysomal RNA (for preparation of the latter see section 7.7.1) (Table 3.8). No reduction below control values of TCA-precipitable radioactivity was observed in either case. Pancreatic ribonuclease rendered most or all $[^3H]$-poly(U) TCA soluble. This demonstrated that no ribonuclease-like activity was detectable in the RNA-dependent RNA polymerase preparation. The basis for this assay, TCA-precipitation, does not exclude the presence of a specific endonuclease however. Derek Woods tested this by incubating total rat liver ribosomal RNA in the presence and absence of the RNA-dependent RNA polymerase preparation (Fig. 3.6). 16S and 28S rRNA (Fig. 3.6(b)), incubated in the absence of the enzyme preparation, were degraded in its presence to a heterogeneous mixture with an average S-value of 11 (Fig. 3.6(a); although not shown, the profile of TCA-precipitable radioactivity parallels this broad absorbance peak).

That no reduction in TCA-precipitable radioactivity occurs on incubation of this RNA-dependent RNA polymerase preparation with $[^3H]$-poly(U) and

FIG. 3.5

The effect of the concentration of added RNA on the incorporation of [^{3}H]-UTP by RNA-dependent RNA polymerase. Reaction conditions were as described in Materials and methods 7.6.5 (25 μg enzyme protein per assay) and the amount of the added mixture of sea urchin embryo 18 and 28S rRNA was varied as shown.



yet 18 and 28S rRNA are markedly broken down by the enzyme preparation demonstrates, conclusively, the presence of endonuclease activity. It would seem (see below) that this endonuclease activity is a contaminant rather than a secondary activity of this RNA-dependent RNA polymerase.

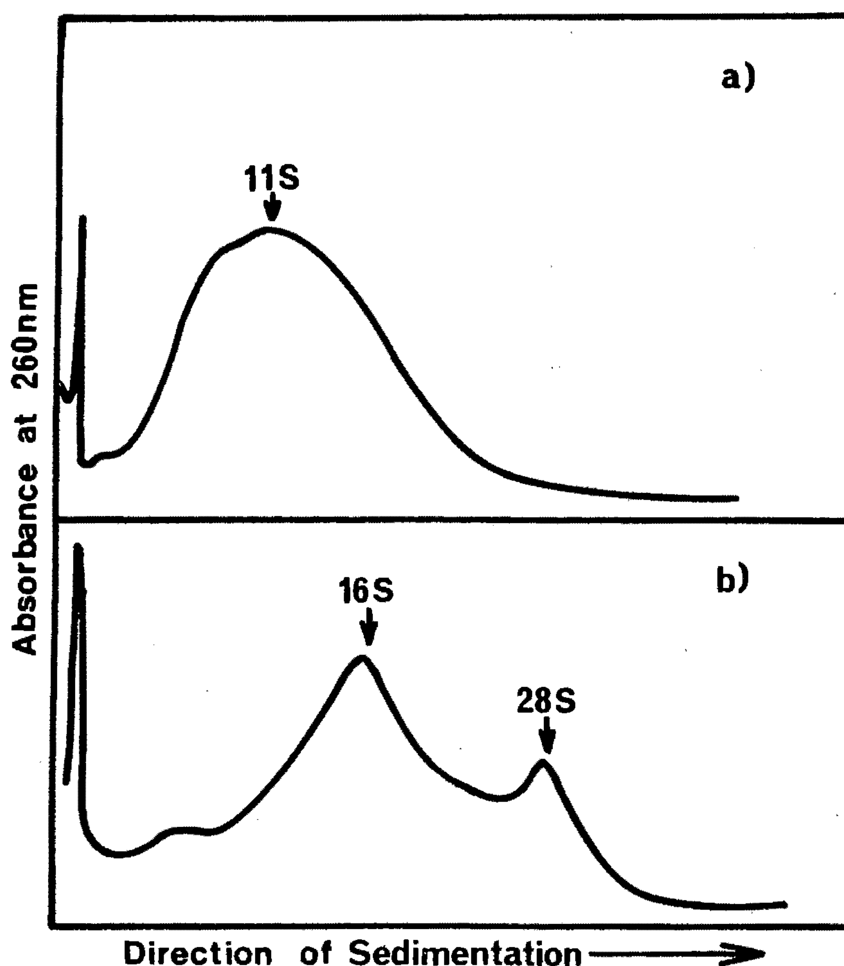
TABLE 3.8

Incubation of RNA-dependent RNA polymerase (RDRP) from ribosomes of immature chicken erythrocytes with [^{3}H]-poly(U) (Schwarz Bio-Research) and [^{3}H]-polysomal RNA. 0.05 ml aliquots of pooled fractions (38-50) (Fig. 3.1) were incubated with 46 µg [^{3}H]-polysomal RNA (346 dpm/µg) or 78 pmoles [^{3}H]-poly(U) (12.7 mCi/mmole) in 0.2 M NaCl, 0.02 M sodium citrate at 37°C for 60 min in a volume of 0.3 ml. Where indicated 50 µg pancreatic ribonuclease (Sigma) were added. 0.5 ml 10% (w/v) TCA was added and TCA-precipitable radioactivity determined as described in 7.6.5.

RNA added	Enzyme added	Relative amounts of TCA-insoluble radioactivity (%)
[^{3}H]-poly(U)	-	100
"	RDRP	100
"	Pancreatic ribonuclease	3
[^{3}H]-polysomal RNA	-	100
	RDRP	103

FIG. 3.6

Sucrose density gradient centrifugation of rat liver ribosomal RNA incubated in the presence and absence of an RNA-dependent RNA polymerase preparation from immature chicken erythrocyte ribosomes. 0.10 mg rat liver ribosomal RNA (prepared as described by Kirby (61)) was incubated in the presence and absence of 0.05 ml RNA-dependent RNA polymerase preparation (25 μg protein) as described in 7.6.5. After incubation, SDS was added to a final concentration of 0.3% (w/v). The incubates were then heated to 65°C for 10 min, cooled rapidly to room temperature and applied to 15-30% sucrose gradients. Centrifugation was for 2 hours at 420 000 g (SW65LTi rotor). Gradients were analyzed as described in 7.2.2.1.2. (a) Rat liver ribosomal RNA incubated in the presence of RNA-dependent RNA polymerase preparation and (b) in the absence of this enzyme preparation.



3.2.2.5 POLYNUCLEOTIDE PHOSPHORYLASE ACTIVITY

Orthophosphate inhibits polynucleotide phosphorylase (1) but not RNA polymerases utilizing nucleoside triphosphates. The complete inhibition of RNA-dependent RNA polymerase activity by pyrophosphate is shown in Table 3.9; orthophosphate did not significantly affect the incorporation of [^{3}H]-UTP by this enzyme preparation at all. This RNA-dependent RNA polymerase preparation from ribosomes of immature chicken erythrocytes will therefore not incorporate nucleoside diphosphates into polynucleotide material; furthermore, as no polynucleotide phosphorylase activity was detected, it would seem likely that the endonuclease activity reported in 3.2.2.4 is a contaminant rather than an activity of the RNA polymerase per se.

3.2.2.6 INHIBITORS OF RNA SYNTHESIS

Actinomycin D has no effect on the incorporation of [^{3}H]-UTP by the purified RNA-dependent RNA polymerase preparation (Table 3.10). α-amanitin, the specific inhibitor of the DNA-dependent RNA polymerase B (75,95), the only nuclear DNA-dependent RNA polymerase known to be present in immature chicken erythrocytes (63), does not effect RNA-dependent RNA polymerase activity at all. The presence of haemin and ribonuclease result in almost complete inhibition of RNA-dependent RNA polymerase activity. The significance of the inhibition of this enzyme by haemin (12), a compound known to stimulate protein synthesis (77,78,79,80) is unknown.

3.2.2.7 POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA-DEPENDENT RNA POLYMERASE PREPARATION

Aliquots of pooled fractions (38-50) (Fig. 3.1) were analyzed by polyacrylamide gel electrophoresis in the absence and presence of SDS (Fig. 3.7). A number of protein bands were obtained on electrophoresis in the presence of SDS (see also Fig. 3.8) ranging in molecular weight from 16 000 to about 50 000 daltons.

TABLE 3.9

A test for polynucleotide phosphorylase activity in an RNA-dependent RNA polymerase preparation from ribosomes of immature chicken erythrocytes. Reaction conditions were as described in Materials and methods 7.6.5 except poly(A) was added (70 μg/assay); where indicated ortho- or pyrophosphate were present at a final concentration of 8.0 mM.

Additions	Relative activity (%)
-	100
Sodium orthophosphate	95
Sodium pyrophosphate	0.18

FIG. 3.7

Polyacrylamide gel electrophoresis of RNA-dependent RNA polymerase preparation in the presence and absence of SDS. Preparation of gels and electrophoresis of samples were as described in Materials and methods 7.6.7. Molecular weight markers used were as follows: horse haemoglobin (65 000 with no SDS (170) ;15 500 in the presence of SDS (169)). Pepsin (35 000 (169)).

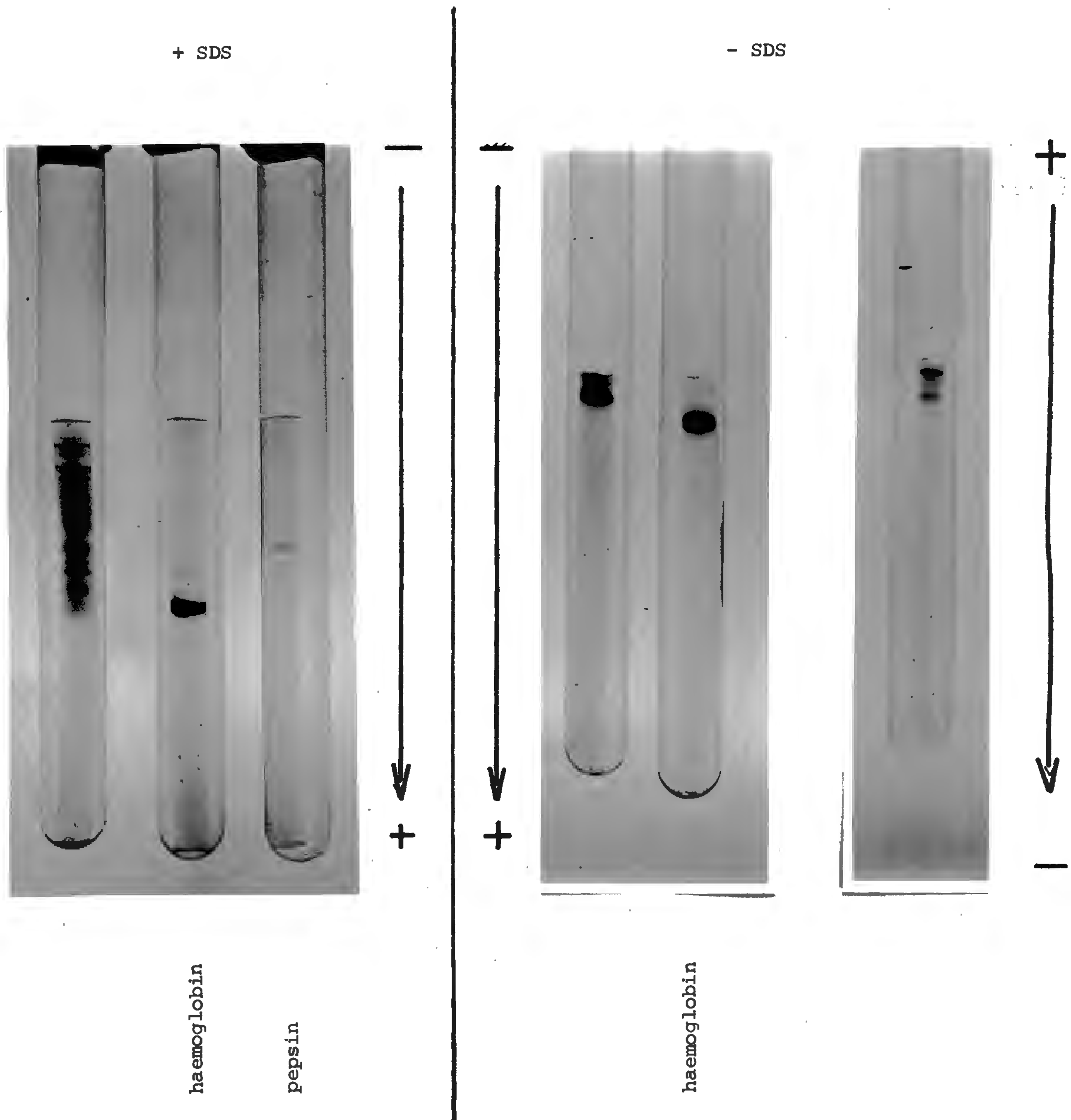
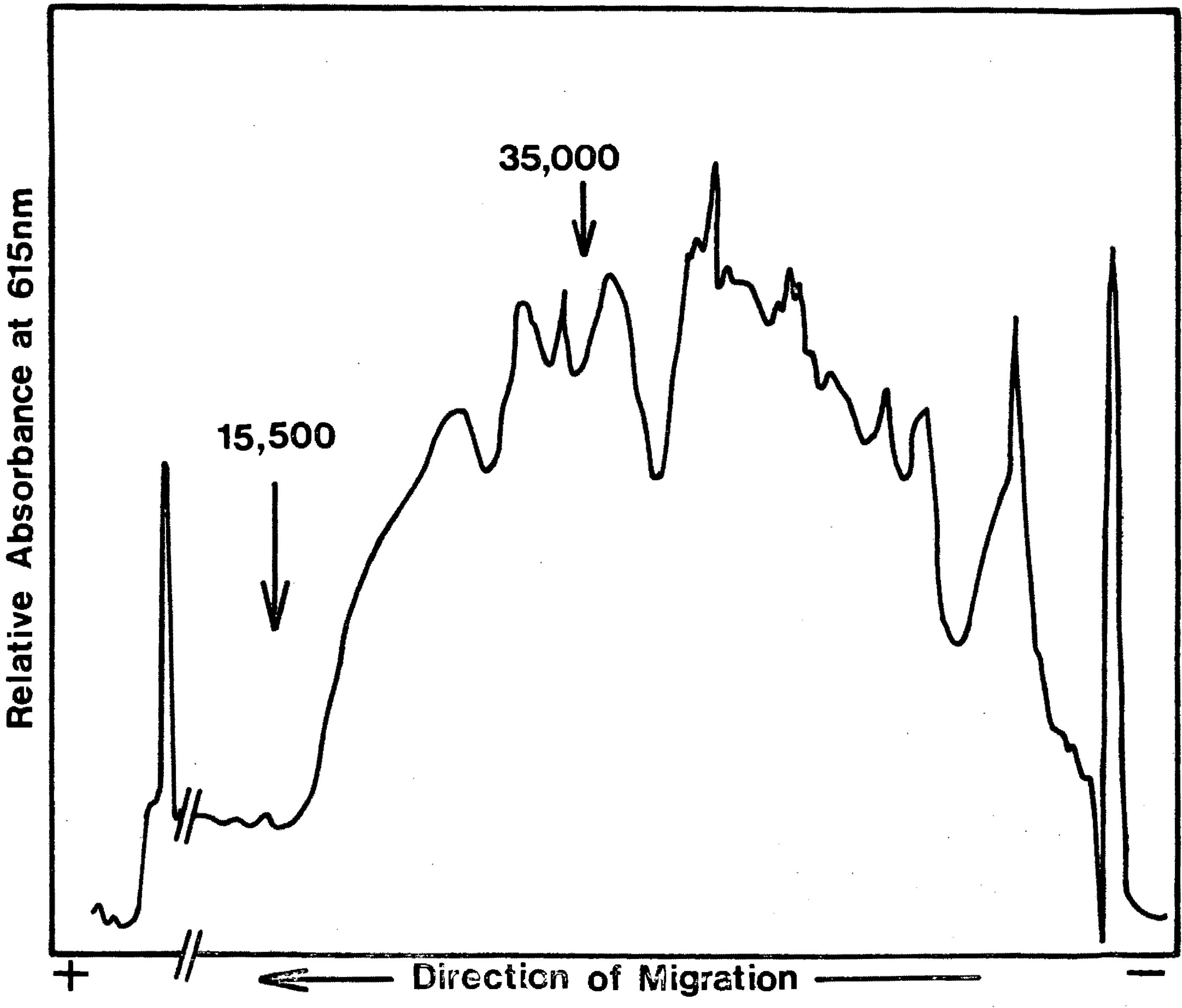


FIG. 3.8

Densitometric scan of RNA-dependent RNA polymerase preparation after electrophoresis on polyacrylamide in the presence of SDS (gel I from Fig. 3.7).



3.2.3 RIBOSOME BOUND RNA-DEPENDENT RNA POLYMERASE (ACTIVITY *IN SITU*)

As many of the experiments to be described in subsequent chapters deal with the RNA-dependent RNA polymerase at the stage of still being associated with the ribosome pellet, it is relevant to discuss the similarities and differences between this enzyme preparation and the purified and predominantly RNA-dependent preparation.

Ribosomes, isolated from immature chicken erythrocytes, contain 280 $\pm$ 67 units of RNA polymerase activity per mg (assayed as in 7.6.5 with no added RNA). The incorporation of [^{3}H]-UTP was stimulated by the addition of RNA; DNA had no affect (Table 3.11). The incorporation of [^{3}H]-UTP was, furthermore, enhanced by the addition of ATP, GTP and CTP. This latter finding was confirmed by the results from Table 3.12 in which is illustrated the incorporation of each particular [^{3}H]-ribonucleotide by the ribosome bound enzyme in the presence and absence of added unlabelled ribonucleoside 5'-triphosphates. This is in contrast to the observation, using a purified RNA-dependent RNA polymerase preparation where addition of the three nucleotides inhibited the incorporation of [^{3}H]-UTP (Table 3.6 and 3.7). The incorporation of [^{3}H]-CTP, [^{3}H]-ATP and [^{3}H]-GTP (Table 3.12) are relatively unaffected by the addition of unlabelled ribonucleotides. Taken together, the results from Table 3.12 do however indicate heteropolymer synthesis. Heteropolyribonucleotide synthesis by the more purified enzyme preparation was not detectable (Table 3.7).

By a comparison of Tables 3.7 and 3.12, it is not clear whether the similarity of U/C ratios in the presence and absence of added ribonucleotides is of any significance.

TABLE 3.10

Effects of inhibitors on RNA-dependent RNA synthesis. Reaction conditions as described in 7.6.5 except for addition of inhibitors as indicated. Haemin prepared as described by Labbe and Nishida (81). A zero time incorporation of 1 220 dpm has been subtracted to obtain these incorporation figures.

Inhibitor	Final concentration in incubation	Incorporation dpm	Relative activity (%)
No inhibitors	-	10407	100
Actinomycin D	16 μg/ml	10255	98
α-amanitin	8 μg/ml	10237	98
Pancreatic ribonuclease	20 μg/ml	79	0.8
Haemin	18 μM	1077	10

3.3 CONCLUSIONS

RNA-dependent RNA polymerase isolated from ribosomes of immature chicken erythrocytes was shown to be a predominantly primer dependent enzyme activity effecting the incorporation, mainly of UTP and CTP but also ATP and GTP into polynucleotide material. This enzyme preparation showed no significant preference for any particular RNA primer isolated from ribosomes of immature erythrocytes. Within the limitations mentioned in 3.2.2.1, these data do not support the conclusions of Downey et al. (12) that such an RNA-dependent RNA polymerase was primarily

TABLE 3.11

Incorporation of $[^3H]$-UTP by ribosome-bound RNA-dependent RNA polymerase from immature chicken erythrocytes. Ribosomal pellets, isolated as described in 7.6.2 were suspended in 50 mM Tris-HCl pH 7.8, 1.0 mM dithiothreitol, 1.0 mM EDTA, 0.25 M sucrose. 0.2 mg were included in a reaction mixture as described in 7.6.5 with no added RNA; where indicated 50 μg 18S rRNA or chicken erythrocyte DNA were added. The final incubation concentration of actinomycin D, where relevant, was 20 μg/ml.

Conditions of incubation	Incorporation (dpm)	Relative activity (%)
Complete (no added RNA)	40922	100
+ 18S rRNA	55654	136
+ DNA	40103	98
- ATP, GTP, CTP	22507	55
Actinomycin D	42149	103

TABLE 3.12

The incorporation of [^{3}H]-ribonucleotides by a ribosome bound RNA-dependent RNA polymerase preparation in the presence and absence of the other three unlabelled nucleoside 5'-triphosphates. Each ribonucleotide (labelled or unlabelled) was added to a final concentration of 8 μM; no RNA was added. 0.08 mg ribosomes were included in each reaction mixture. All further details were as described in 7.6.5.

Ribonucleotides present				Incorporation (dpm)	Relative activity (%)
[^{3}H]-UTP	CTP	ATP	GTP	11309	100
UTP	[^{3}H]-CTP	ATP	GTP	4085	36
UTP	CTP	[^{3}H]-ATP	GTP	275	2.3
UTP	CTP	ATP	[^{3}H]-GTP	265	2.4
[^{3}H]-UTP	-	-	-	6267	100
-	[^{3}H]-CTP	-	-	4833	77
-	-	[^{3}H]-ATP	-	286	2.6
-	-	-	[^{3}H]-GTP	160	4.6

dependent on globin mRNA for activity. Hozumi and co-workers (11) concluded that an RNA-dependent RNA polymerase from rat brain microsomes demonstrated preference towards high molecular weight ribosomal RNAs. Although the two enzymes might be unrelated, the data from Table 3.4 clearly demonstrate that RNA-dependent RNA polymerase from ribosomes of immature chicken erythrocytes shows no preference for high molecular weight RNA.

This purified RNA-dependent RNA polymerase from ribosomes of immature chicken erythrocytes showed a requirement for a divalent cation

and a reducing agent (Table 3.6). An endonuclease, probably a contaminant, was found in this RNA-dependent RNA polymerase preparation (Table 3.8 and Fig. 3.6). No polynucleotide phosphorylase activity could be detected (Table 3.9).

The purified primer-dependent RNA polymerase preparation demonstrated the predominant incorporation of all four ribonucleotides into homopolyribonucleotide material. Little or no template-dependent RNA polymerase activity would seem to be present. However, the heteropolymer synthesis shown to be present in the ribosome bound RNA polymerase preparation introduces the possibility that a template dependent RNA polymerase was not detected in the purified enzyme preparation as KCl extraction and DEAE-Sephadex chromatography destroyed or changed its characteristics. These experiments, although not supporting the conclusions of Downey and co-workers (12), do not exclude the possible existence of a template dependent globin mRNA replicase (82).

PART 4

THE PREPARATION OF DNA COMPLEMENTARY TO CHICKEN 9S RNA AND ITS USE AS AN ASSAY FOR THE DETECTION OF REPLICATED GLOBIN mRNA

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4.1 INTRODUCTION

The nucleated erythrocyte series is a terminally differentiated cell line synthesizing one predominant protein, globin, in large quantities (52). How does the erythrocyte genome effect such a disproportionately large synthesis of one protein ? The reiteration of certain genes required for the synthesis of specific products would be one method. Multiple copies of the genes for rRNA, tRNA, 5S RNA (83,84) and histone mRNA (85) are known to be present in the genome of many organisms. However, the genes for globin in a variety of tissues are unique or nearly so (less than 5 copies per genome (86,87,88). Nuclear gene amplification (the selective replication of unique or reiterated gene sequences during the development of some differentiated tissues) is another potential method. A clear example of this is the amplification of the genes coding for ribosomal RNA in amphibian oocytes (90). However Packman et al. (91) have shown no difference between the globin gene content of duck reticulocyte nuclear DNA and DNA from the nuclei of duck liver cells, indicating no specific amplification of globin genes during erythroid differentiation. Similar results have been obtained by Paul et al. (92) using DNA from mouse erythropoietic tissue and sperm.

Gene reiteration and nuclear gene amplification do not play a role therefore, during erythrocyte development, in the expression of the globin gene. Cytoplasmic amplification has however not been excluded. The synthesis of multiple DNA copies of the globin gene and their localization in the cytoplasm where they could be further amplified and act as templates for the synthesis of globin mRNA is a possibility (93). Although cytoplasmic DNA-dependent DNA polymerase and DNA-dependent RNA polymerase activities have been demonstrated in eukaryotic tissue

(72,75), Bishop and Freeman (89) using DNA prepared from immature duck erythrocytes by a method which resulted in a 100% yield of thymidine-labelled DNA from Hela cells, have provided evidence indicating no detectable amplification of globin gene sequences in total erythrocyte DNA. Therefore, although the possibility cannot be totally excluded, it would seem unlikely that the globin gene, in the immature erythrocyte, is amplified by this mechanism. An alternative possibility is the replication of globin mRNA by a cytoplasmic mRNA replicase. The results from incorporation studies using a purified RNA-dependent RNA polymerase from ribosomes of immature chicken erythrocytes (Part 3) are not consistent with the conclusions of Downey et al. (12) concerning the existence of a specific globin mRNA replicase. However, due to the heteropolymer-like synthesis of polynucleotide material by the ribosome-bound enzyme preparation, it remains relevant to consider a different and more direct approach to test this hypothesis.

If an RNA-dependent RNA polymerase, bound to globin synthesizing ribosomes and capable of making multiple copies of globin mRNA exists, then, by incubating ribosomes with [^{3}H]-UTP and extracting the labelled RNA, one should be able to detect radioactively labelled globin mRNA sequences using an unlabelled DNA complement of globin mRNA. Immature avian erythrocyte 9S RNA is known to contain the mRNA for globin (68, 96,97,98). A complementary DNA copy of this poly(A) containing mRNA can be prepared using RNA-dependent DNA polymerase as outlined by several authors (71,87,96). This chapter describes the isolation and translation of chicken globin mRNA and the preparation of its DNA complement using the commercially available RNA-dependent DNA polymerase from avian myeloblastosis virus. Finally this chapter considers the existence of a ribosome bound globin mRNA replicase using unlabelled complementary DNA as an assay for the detection of radioactively labelled globin mRNA.

4.2 RESULTS AND DISCUSSION

4.2.1 ISOLATION OF GLOBIN mRNA

Ribosomes were isolated from immature chicken erythrocytes (as described

in section 7.3.2). Sedimentation analysis on sucrose gradients showed a typical polysome pattern (Fig. 4.1). This ribosomal preparation consisting mainly of polysomes, had an $E_{260\ nm}/E_{280\ nm}$ ratio of 1.63 $\pm$ 0.13 (for spectrum see Fig. 3.2), indicating a relatively pure ribosomal preparation (145). Ferritin (47) and haemoglobin were minor contaminants of the ribosomal pellets. The yield of ribosomes from an immature erythrocyte population was between 0.1079 - 0.2168 mg/10^9 cells. These considerable variations in the yield of ribosomes from anaemic blood depended on the response of different batches of chickens to phenylhydrazine.

The isolation of ribosomes from immature erythrocytes has frequently been reported as being conducted at 0-4°C (95,100). In my experience, washing blood cells from anaemic blood with cold (2°C) 0.14 M NaCl, 5 mM KCl, 1.5 mM $MgCl_2$ frequently effected disaggregation of polysomes resulting in ribosomal preparations consisting predominantly of monosomes (or single ribosomes) (Fig. 4.2(a)). In some instances disaggregation was so acute that no difference was observed between ribosomes from anaemic blood and normal blood which consisted mainly of single ribosomes (Fig. 4.2(b)). To obtain ribosome preparations therefore with a polyribosome content as shown in Fig. 4.1, it was necessary to wash cells at 20°C and as rapidly as possible. Similar methods have been reported by Spohr et al. (67) and Nijhof and Wierenga (101).

Although the positive effects of the ribonuclease free conditions used during all stages of the isolation of mRNA (Materials and methods 7.3.1) were not always apparent, polyribosome content of ribosomal preparations was less in the absence of these conditions.

The method finally adopted in this laboratory for the isolation of 9S RNA from immature chicken erythrocyte ribosomes was that described in section 7.3.3.1. This method involved the preparation of total polysomal RNA by deproteinization of ribosomes with a phenolic 'cocktail' (the reasons for the various additives are explained by Mathews (93)). Brawerman and co-workers (102) emphasized the importance of an alkaline pH to ensure that the poly(A)-containing mRNAs remained in the aqueous

FIG. 4.1

Sucrose density gradient centrifugation of a ribosomal preparation from immature chicken erythrocytes. 4 $OD_{260\ nm}$ units (0.04 ml) of a ribosomal preparation suspended in 30 mM Tris-HCl, pH 7.8, 30 mM KCl, 1.5 mM $MgCl_2$ were applied to a 15-30% (w/v) sucrose gradient and centrifuged at 420 000 g for 30 min at $2^{o}C$ (Beckman SW 65LTi rotor).

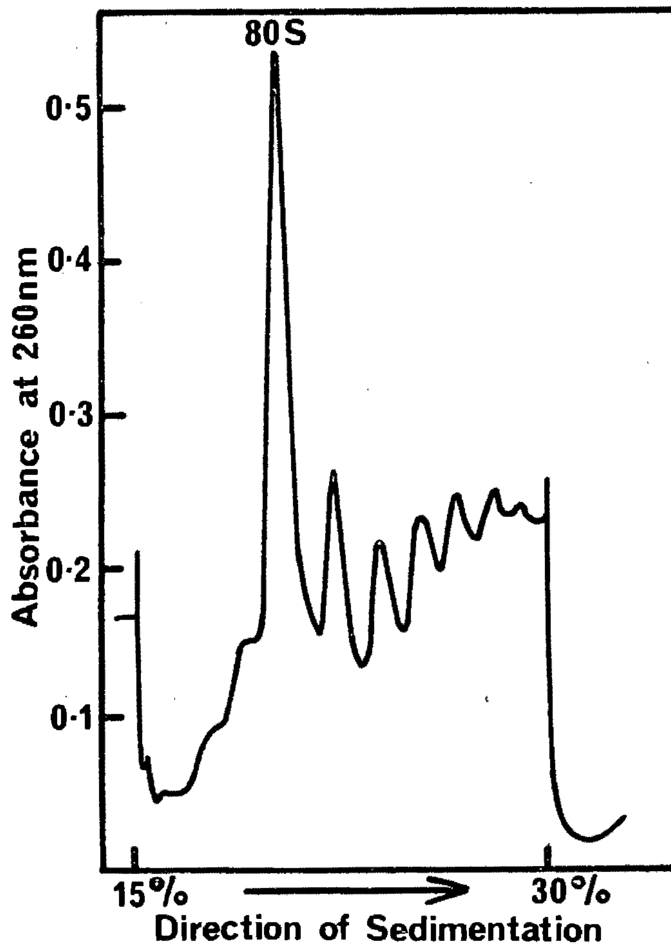
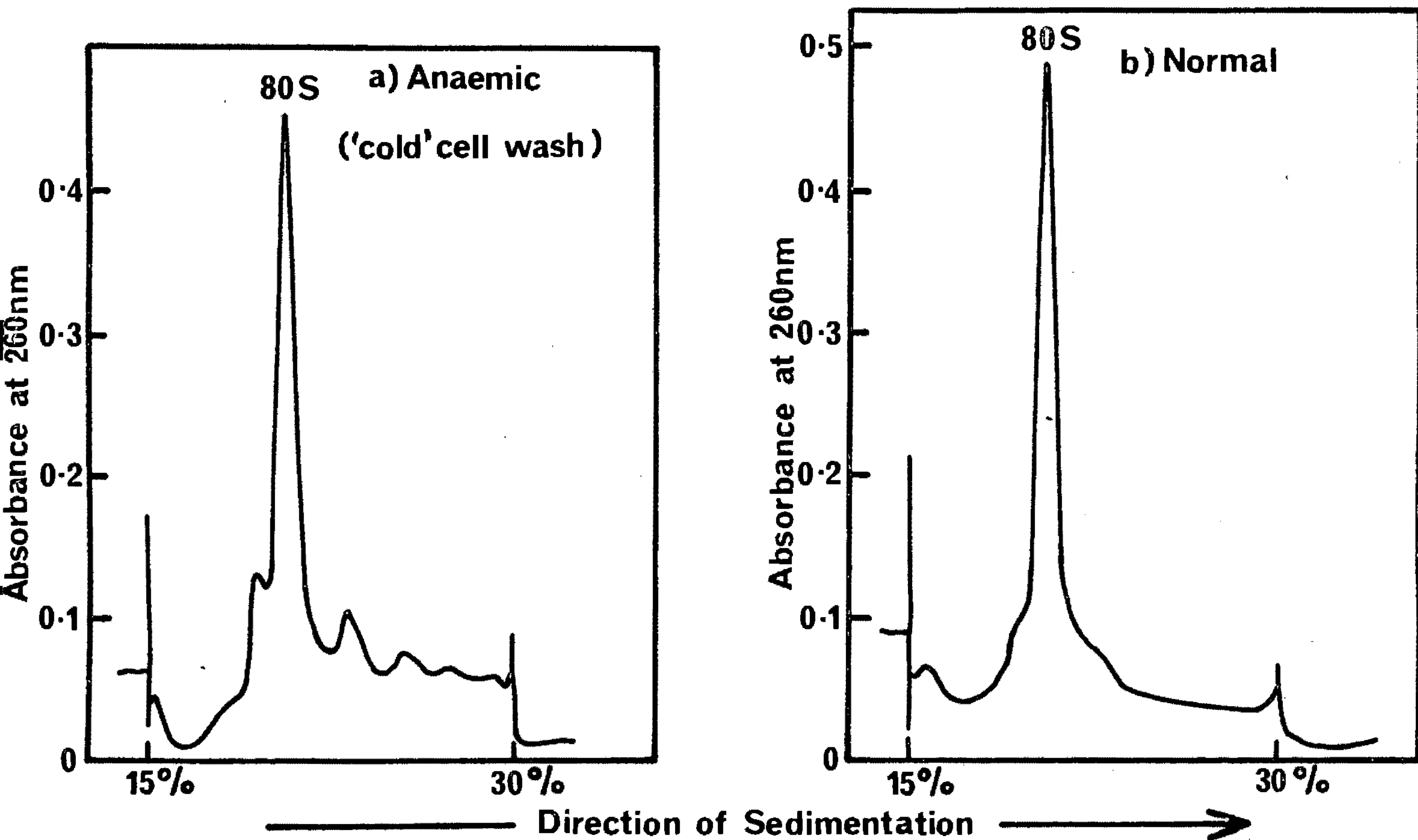


FIG. 4.2

Sucrose density gradient centrifugation analysis of ribosomal preparations from immature erythrocytes of anaemic blood (a) and ribosomes from erythrocytes of normal blood (b). Preparations from anaemic blood were isolated as described in 7.3.2 except that all solutions from the moment of collecting blood were kept on ice. Ribosomes from erythrocytes of normal blood were prepared according to 7.3.2. Centrifugation was as described in the legend to Fig. 4.1.



phase. Total polysomal RNA was dissolved in a buffer containing SDS and heated to 65°C. This heat treatment was found by McKnight and Schimke (103) to reduce aggregation of RNA. It was introduced into this isolation procedure as it improved the yield of 9S RNA (Fig. 4.3).

The isolation of a homogeneous peak of 9S RNA was achieved after two successive centrifugation steps (Fig. 4.4). The yield of 9S RNA was between 1.3 and 1.8% of total polysomal RNA. 9S RNA prepared by this method revealed 6 bands when analyzed by gradient polyacrylamide microgel electrophoresis (Fig. 4.5). A similar heterodisperse nature of chicken 9S RNA was reported by Barrett et al. (96). Some of these bands may represent the individual mRNAs for the various polypeptide chains of chicken globin (104). In addition, Pemberton and Baglioni (105) have demonstrated a difference in the size of the poly(A) sequence from newly synthesized and 'old' duck globin mRNA (150-200 and 85 nucleotides respectively). This variability in size of the poly(A) segment is another factor contributing to the heterogeneity of this 9S RNA preparation.

Polyvinyl sulphate (Sigma) was used as a ribonuclease inhibitor (106,107) in earlier attempts to isolate chicken 9S RNA. The use of this polymer was discontinued in later studies as it was found extremely difficult to remove from RNA preparations (it was necessary to separate polyvinyl sulphate from mRNA preparations as it is a known translational inhibitor (108)). A method utilizing chromatography on DEAE-Sephadex was devised to separate polyvinyl sulphate and 9S RNA but resulted in large losses of RNA (polyvinyl sulphate was assayed using the observation that the vinyl polymer changed the absorption maximum of toluidene blue from 640 nm to 525 nm (this colour change was sensitive down to about 0.5 µg polyvinyl sulphate per ml assay mixture).

Direct disruption of poly-ribosomes with SDS, a method used by several authors (74,110,111) for the isolation of mRNA, was also employed for

FIG. 4.4

Sucrose gradient centrifugation analysis of total polysomal RNA (——) and purified 9S RNA (----). See Materials and methods 7.3.3.1 for details.

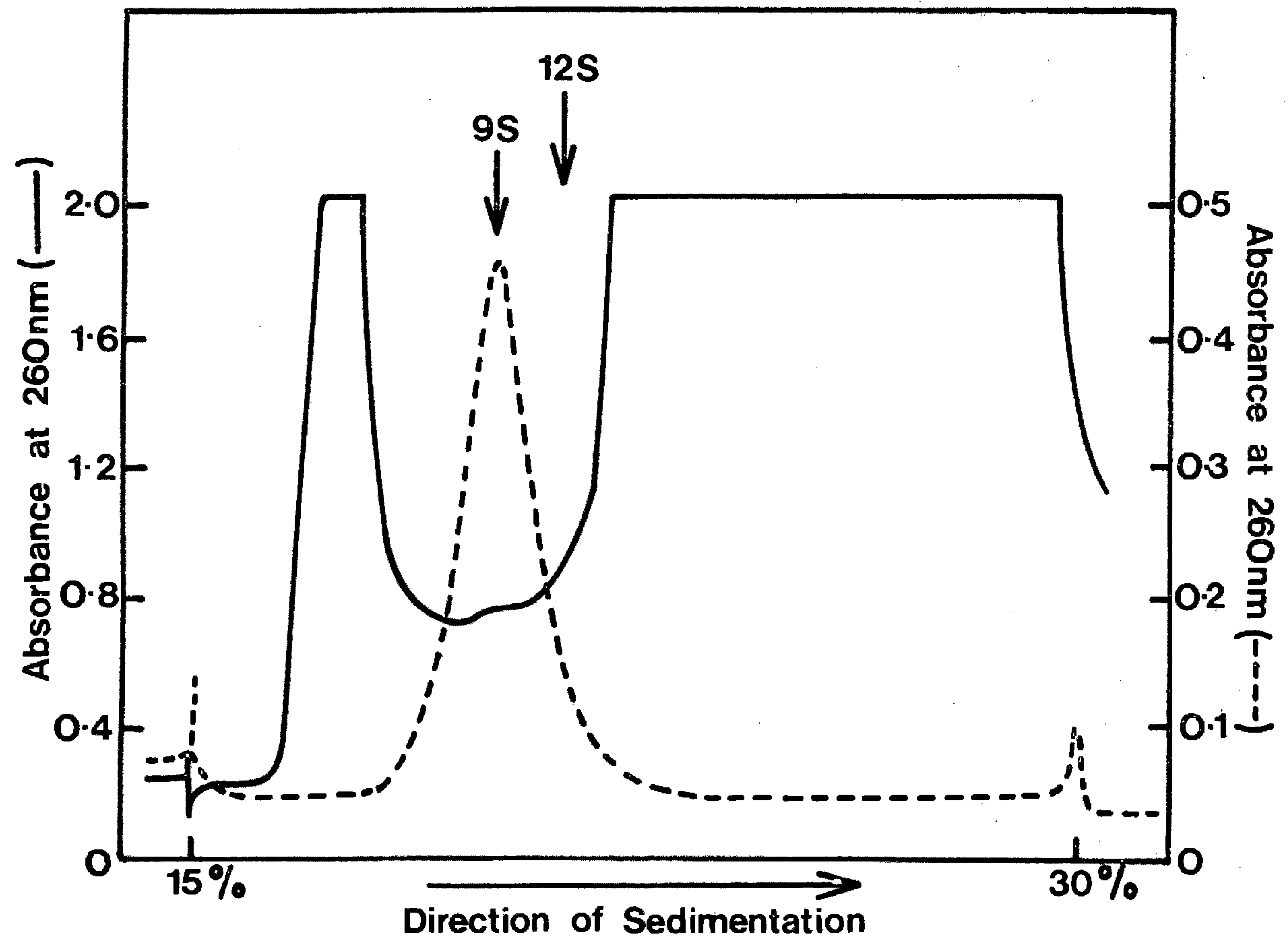
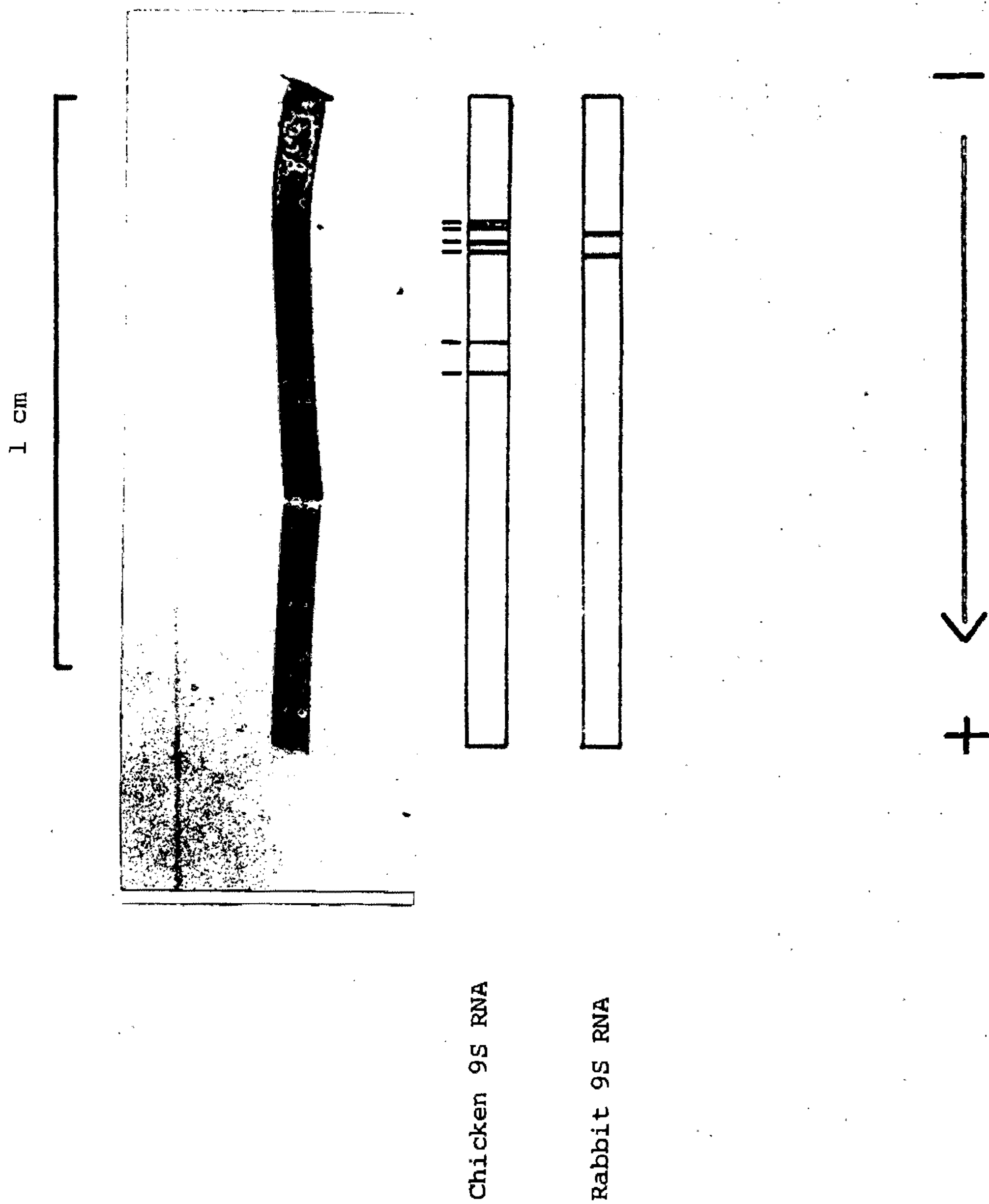


FIG. 4.5

Microgel polyacrylamide electrophoresis of chicken 9S RNA. Details of method described in Section 7.3.3.1.1. For purposes of comparison a drawing of the result obtained after electrophoresis of commercially available rabbit 9S RNA (prepared by oligo(dT)-cellulose chromatography) has been included.



the preparation of chicken 9S RNA; however, contamination of RNA fraction with protein (Fig. 4.6) proved to be a problem, even in the final sucrose density gradient centrifugation steps. Protein was removed by extraction of the final 9S RNA with phenol as described by Lane et al. (112). The yield of RNA after phenol extraction was about 80%; a lower yield of 9S RNA was obtained as a result. The initial removal of protein by extraction of ribosomes with phenol remained therefore the preferred method.

Two disadvantages of repeated gradient centrifugation as a method of obtaining pure 9S RNA are time and cost. Two alternative methods for the isolation of 9S RNA were investigated.

a. Oligo(dT)-cellulose chromatography

Affinity chromatography of poly(A) containing mRNA is a method that has been used in several laboratories for the isolation of globin mRNA (113,114). The method is rapid and involved application of total polysomal RNA (prepared by extraction with phenol) to oligo(dT) cellulose in a high salt buffer; unabsorbed RNA was removed by washing and bound RNA was eluted in 10 mM Tris-HCl, pH 7.8 (Fig. 4.7). In my hands, although the material eluted in low salt was clearly enriched with respect to 9S RNA (Fig. 4.8), the preparation of a homogeneous 9S RNA peak was obtained only after sucrose density centrifugation of this RNA preparation. In addition, the yield of 9S RNA was only 12% of the amount of 9S RNA obtained by repeated centrifugation of phenol extracted polysomal RNA. 9S RNA from immature avian erythrocyte ribosomes is known to contain histone F2c mRNA (115) (which does not bind to oligo(dT)-cellulose); other non-messenger, non poly(A)-containing RNA species may also be present; poly(A)-containing globin mRNA however is thought to be the predominant RNA species present (45). A yield of only 12% would indicate a substantial loss of globin 9S mRNA under these conditions.

b. Unsubstituted cellulose chromatography

This method, first described by Schutz et al. (116), was a second

FIG. 4.6

Centrifugation of crude 9S RNA obtained from SDS-treated polyribosomes. Centrifugation was for 43 hours at 90 000 g (Beckman SW 25.1 rotor) at 20°C using a 5-20% sucrose gradient in 10 mM Tris-HCl pH 7.8, 0.5% (w/v) SDS. The bracket indicates those fractions pooled for extraction with phenol.

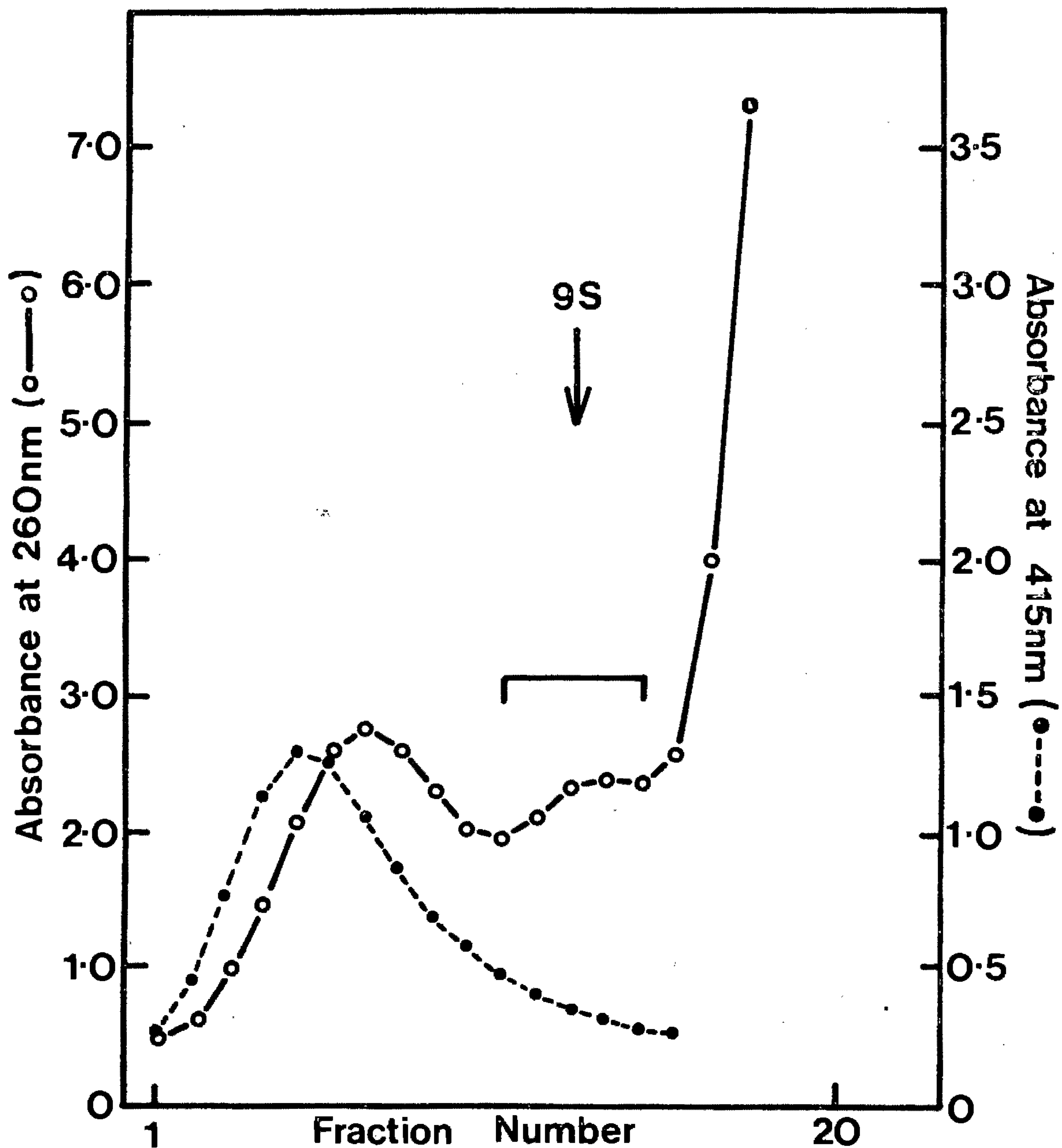


FIG. 4.7

Oligo(dT)-cellulose chromatography of polysomal RNA from immature chicken erythrocytes. For details see Materials and methods 7.3.4.1.

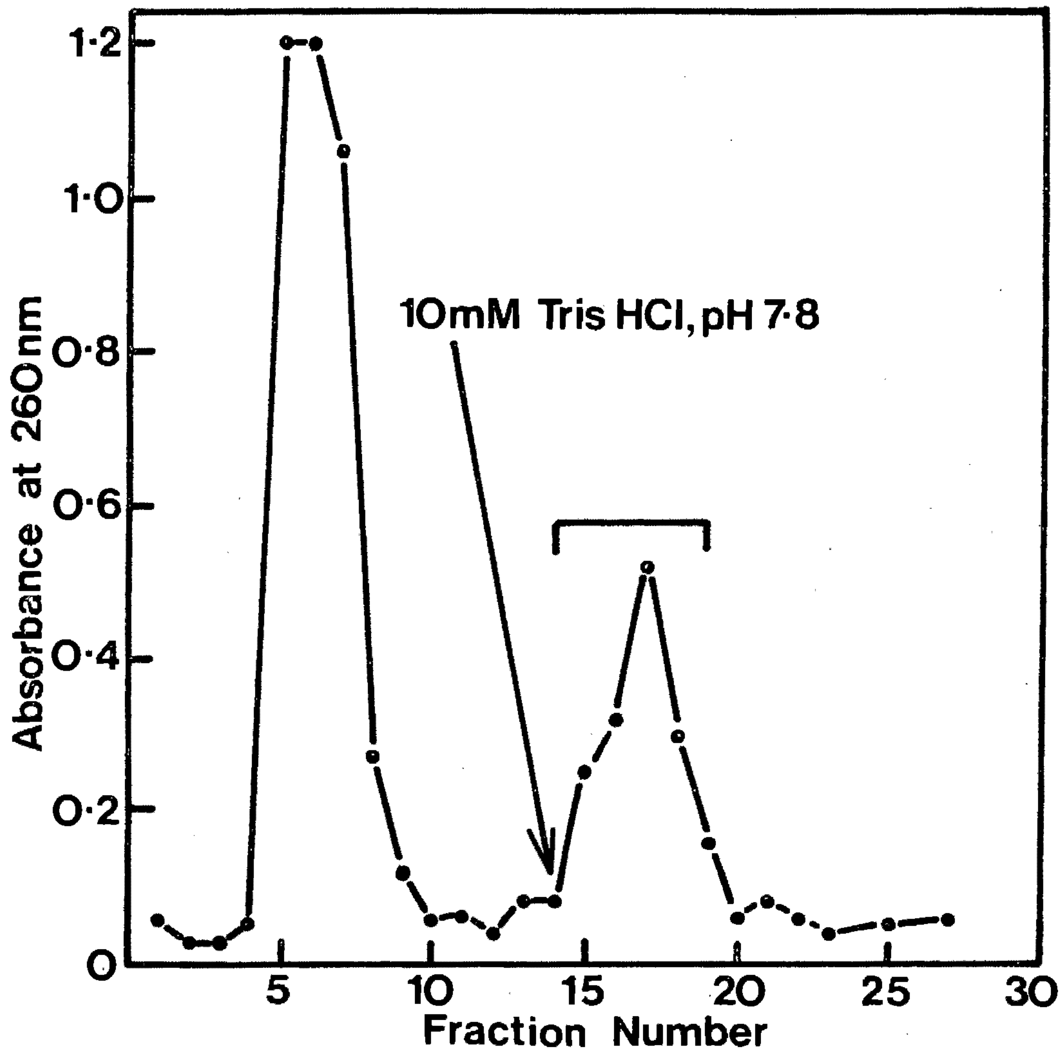
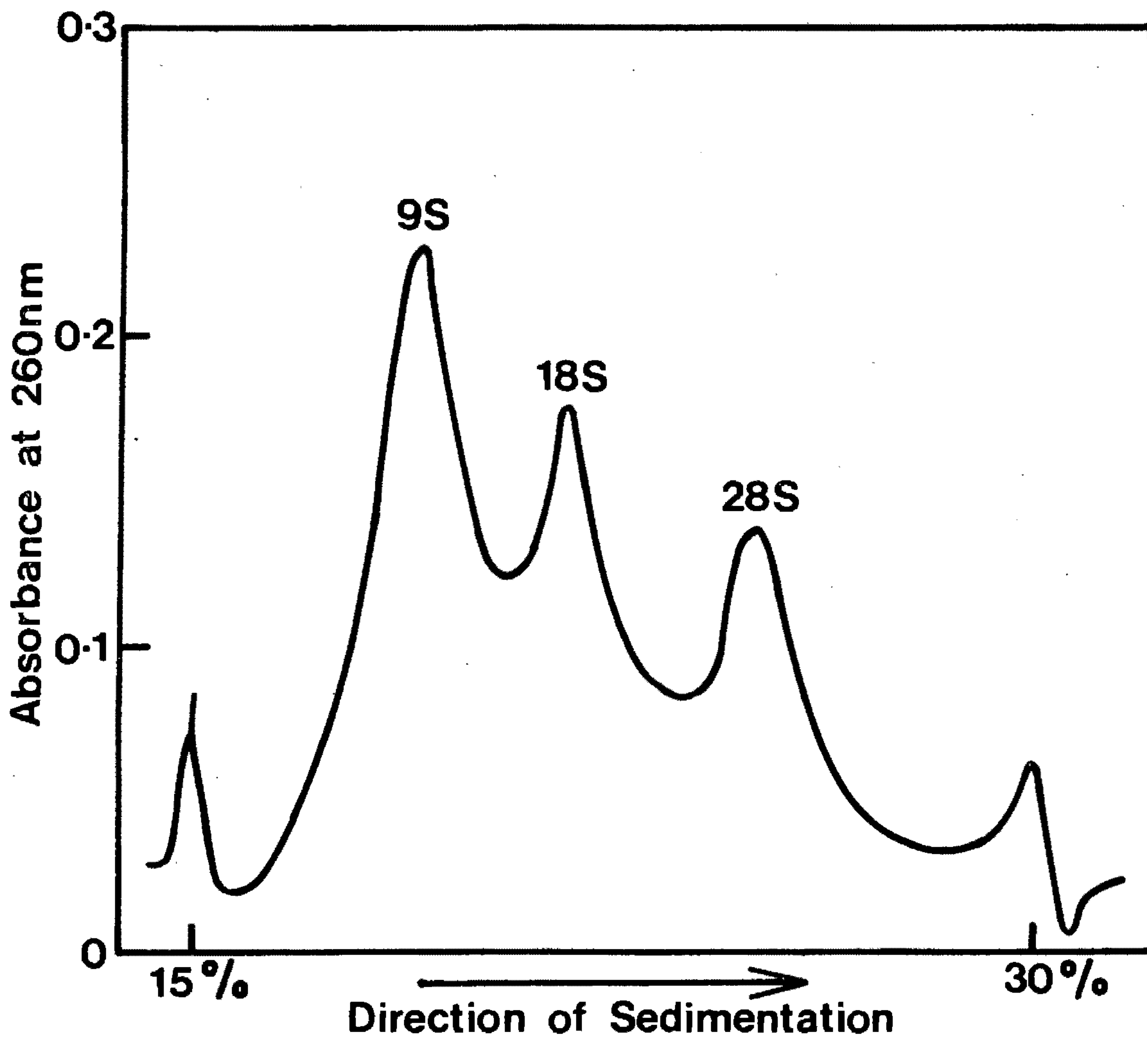


FIG. 4.8

Sucrose density gradient centrifugation analysis of RNA bound to oligo(dT)-cellulose and eluted with 10 mM Tris-HCl pH 7.8 (fractions 14-19). Centrifugation as in Section 7.3.4.1.



chromatographic procedure attempted for the rapid isolation of chicken 9S RNA. Low yields of 9S RNA, however, similar to those described above were obtained using unsubstituted cellulose (Fig. 4.9, 4.10). It was not clear why these two methods proved so inefficient in the isolation of 9S RNA in high yields. Polysomal RNA used in these experiments on the chromatographic isolation of 9S RNA was prepared without heat treatment (see Materials and methods 7.3.3.1). Recently, Kabat (69) has shown that poly(A)-containing globin mRNA isolated from rabbit reticulocyte polysomal RNA prepared by a mild procedure (70), sedimented at 9S, 18S and 28S positions in a sucrose gradient. Globin mRNA activity could be removed from the 18S and 28S RNA fractions by prior heat treatment and subsequent sucrose gradient centrifugation. It is possible therefore that the low yield of 9S RNA (as opposed to globin mRNA) as shown in my experiments could be due to a process of globin mRNA association with 18S and 28S rRNA, with less globin mRNA available for detection as 9S RNA, although little messenger activity was detectable in fractions other than 9S (Table 4.1). It is unlikely that the presence of 5S RNA in the RNA fraction eluted off unsubstituted cellulose (Fig. 4.10) could be explained in this manner. The constituent of the cellulose preparation thought to exhibit an affinity for poly(A) containing RNA is lignin (118), a compound which could well exhibit an affinity to hydrophobic regions of RNA other than poly(A) segments.

12S RNA, shown to be present in total polysomal RNA prepared by heat treatment (Fig. 4.3) could not be detected in polyosmal RNA used in these experiments; furthermore, the results from Fig. 4.3 demonstrated an increase in the amount of apparent 12S RNA after heat treatment of total polysomal RNA. The possibility exists, by analogy to the work of Kabat (69), that 12S RNA might be associated with other RNA species and was thus not detectable in the absence of any thermal dissociation of aggregated RNA.

Chromatography of 9S RNA obtained from polysomal RNA by repeated sucrose density gradient centrifugation using either of the two matrices described above was not considered necessary : 9S RNA was to be included in a reaction mixture with an RNA-dependent DNA polymerase specific for poly(A)-containing RNA; any non-poly(A)-containing RNA would not be copied.

FIG. 4.9

Unsubstituted cellulose chromatography of polysomal RNA from immature chicken erythrocytes. For details see Materials and methods 7.3.4.2.

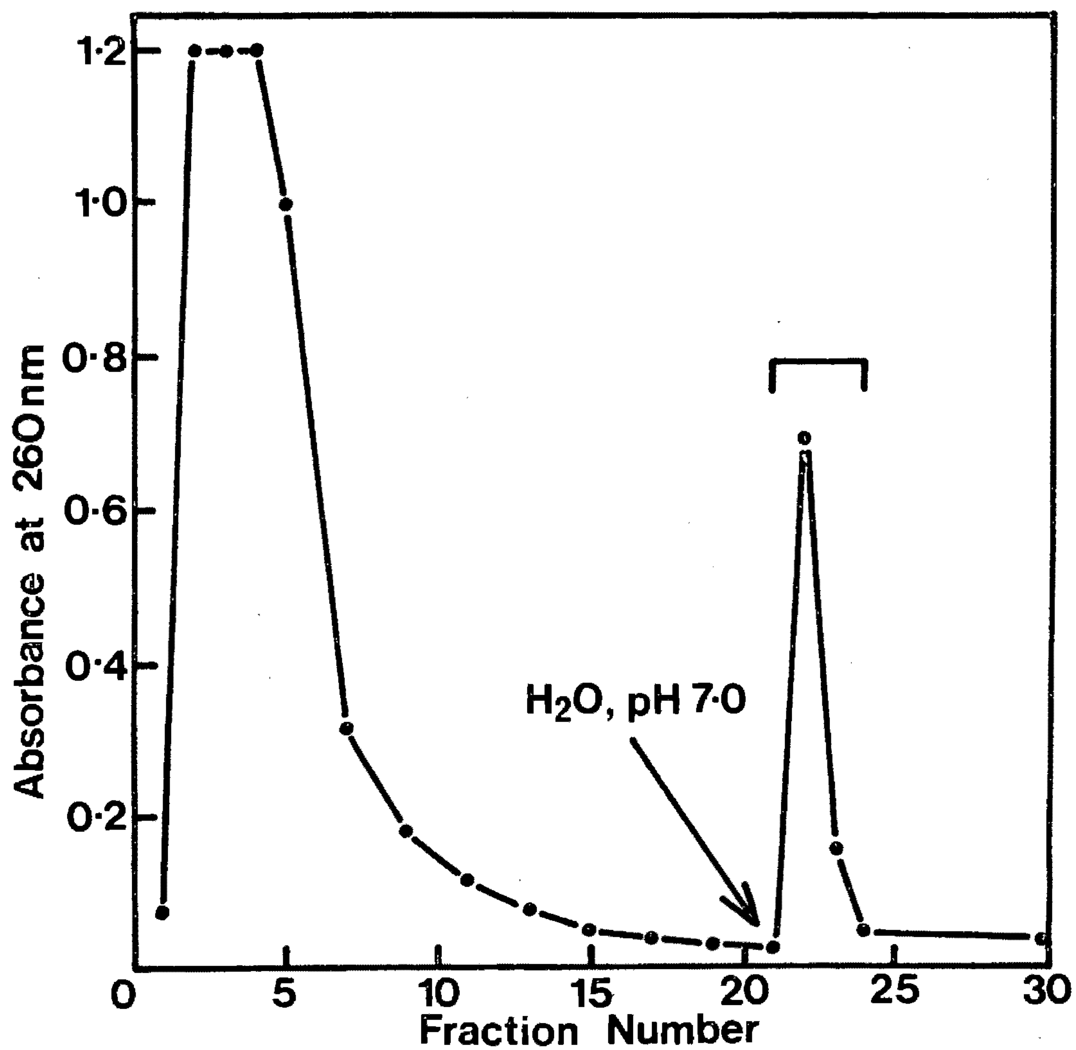
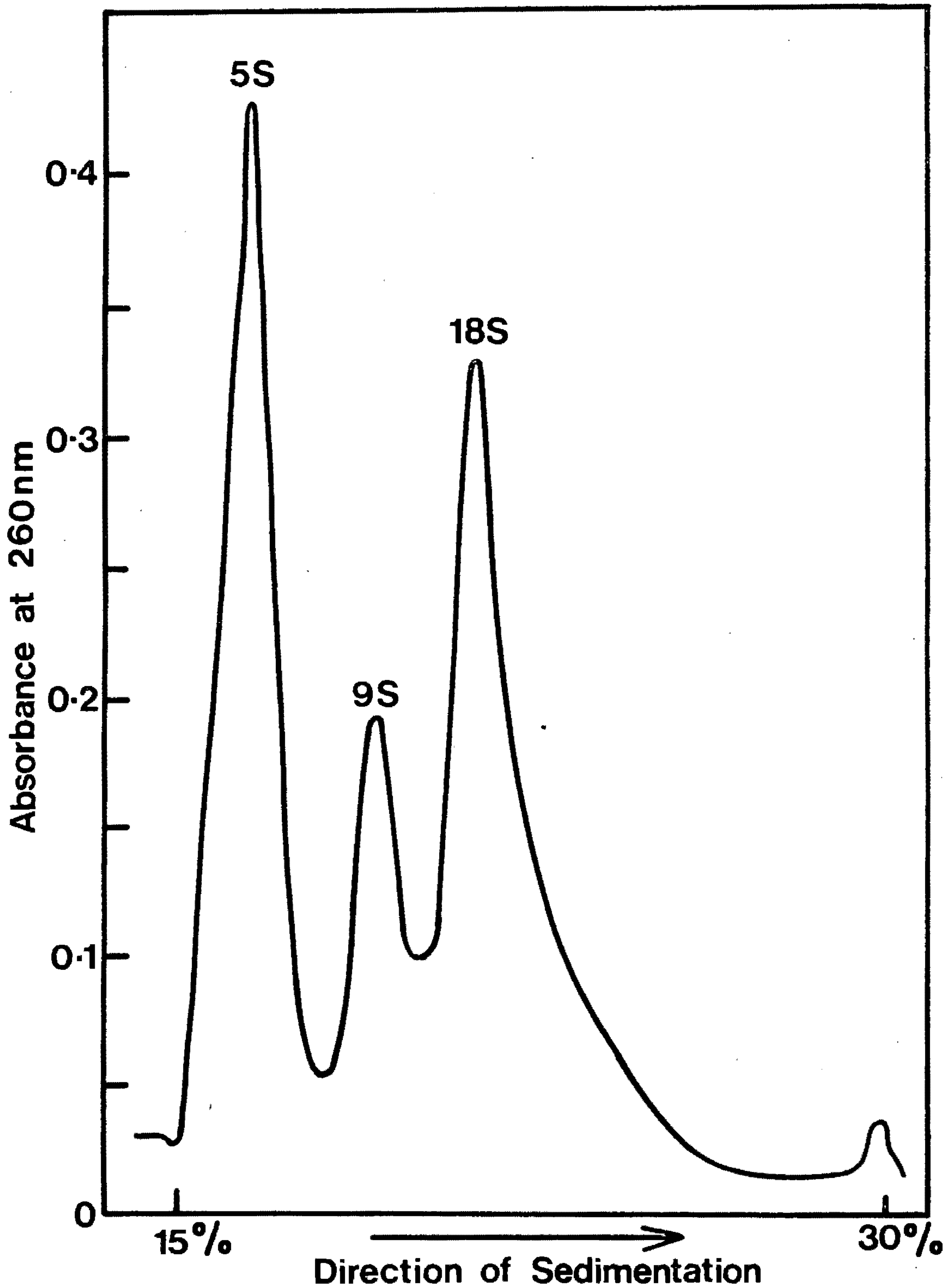


FIG. 4.10

Sucrose density gradient centrifugation analysis of RNA bound to unsubstituted cellulose and eluted with H_2O (fractions 21-24, Fig. 4.8). Centrifugation as in 7.3.4.2.



4.2.2 TRANSLATION OF mRNA

4.2.2.1 KREBS II ASCITES CELL FREE SYSTEM

Chicken 9S RNA added to an ascites cell free system, was shown to stimulate the incorporation of $[^3H]$-histidine into TCA-insoluble material to about the same extent as a rabbit globin mRNA preparation (Searle). 5S, 18S and 28S rRNA showed little or no messenger activity (Table 4.1).

The product synthesized in the presence of chicken 9S RNA was analyzed by acrylamide gel electrophoresis (Fig. 4.11). I was unable to identify the respective chains of chicken globin (see below) but have shown that on electrophoresis the 9S RNA-directed product synthesized _in vitro_ closely resembles the electrophoretic pattern of _in vivo_ labelled chicken globin obtained using the method described (Fig. 4.12).

Uncertainty exists as to the exact number of globin chains present in the two haemoglobins found in avian erythrocytes. Matsuda et al. (104,120) have concluded that 3 polypeptide chains make up the two haemoglobins AI and AII (two different α chains and a common β chain). This conclusion is supported by Imaizumi and co-workers (71). However, the work of Saha (121) and Moss and Thompson (122) suggest the presence of 4 different globin chains. The consensus of opinion seems to favour the presence of only 3 polypeptide chains but a final conclusion on this subject awaits further evidence.

As a control experiment, to confirm that the nature of the product synthesized by an ascites cell-free extract is dependent upon the type of mRNA added, the products synthesized in the presence of added rabbit

TABLE 4.1

Protein synthesis by an ascites cell-free system with different added RNAs. 5 μg of each RNA fraction were included in a reaction mixture prepared as described in Materials and methods, Section 7.4. Chicken 5S, 18S and 28S prepared as described in Section 7.6.6.

Added RNA	Incorporation (dpm)
Rabbit 9S	2454
Chicken 9S	2146
5S	620
18S	646
28S	676
No added RNA	511

FIG. 4.11

Acrylamide gel electrophoresis of the products obtained from a mRNA-directed cell free system. Chicken 9S RNA was included in an ascites cell-free reaction mixture as described in Section 7.4.1.3. After incubation, the products of synthesis were analyzed as described in Section 7.4.1.4.

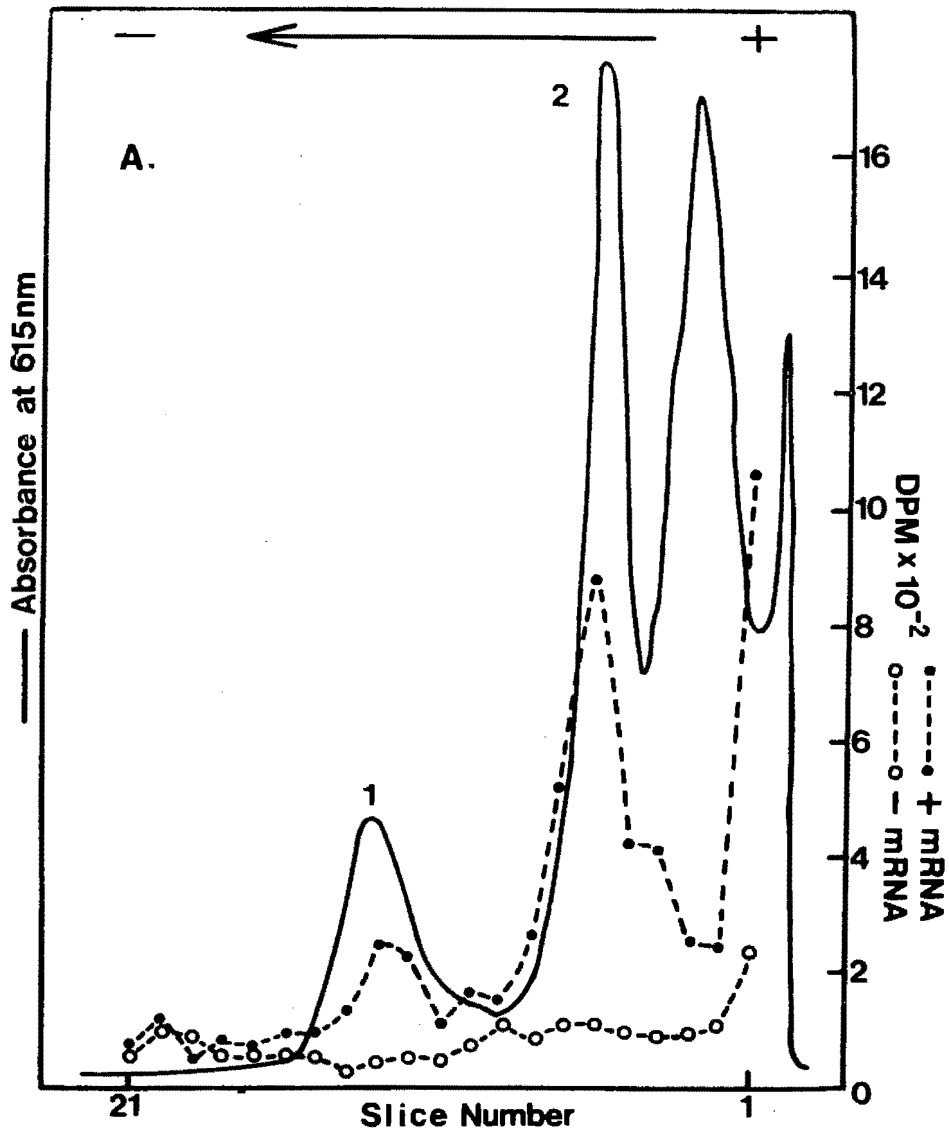
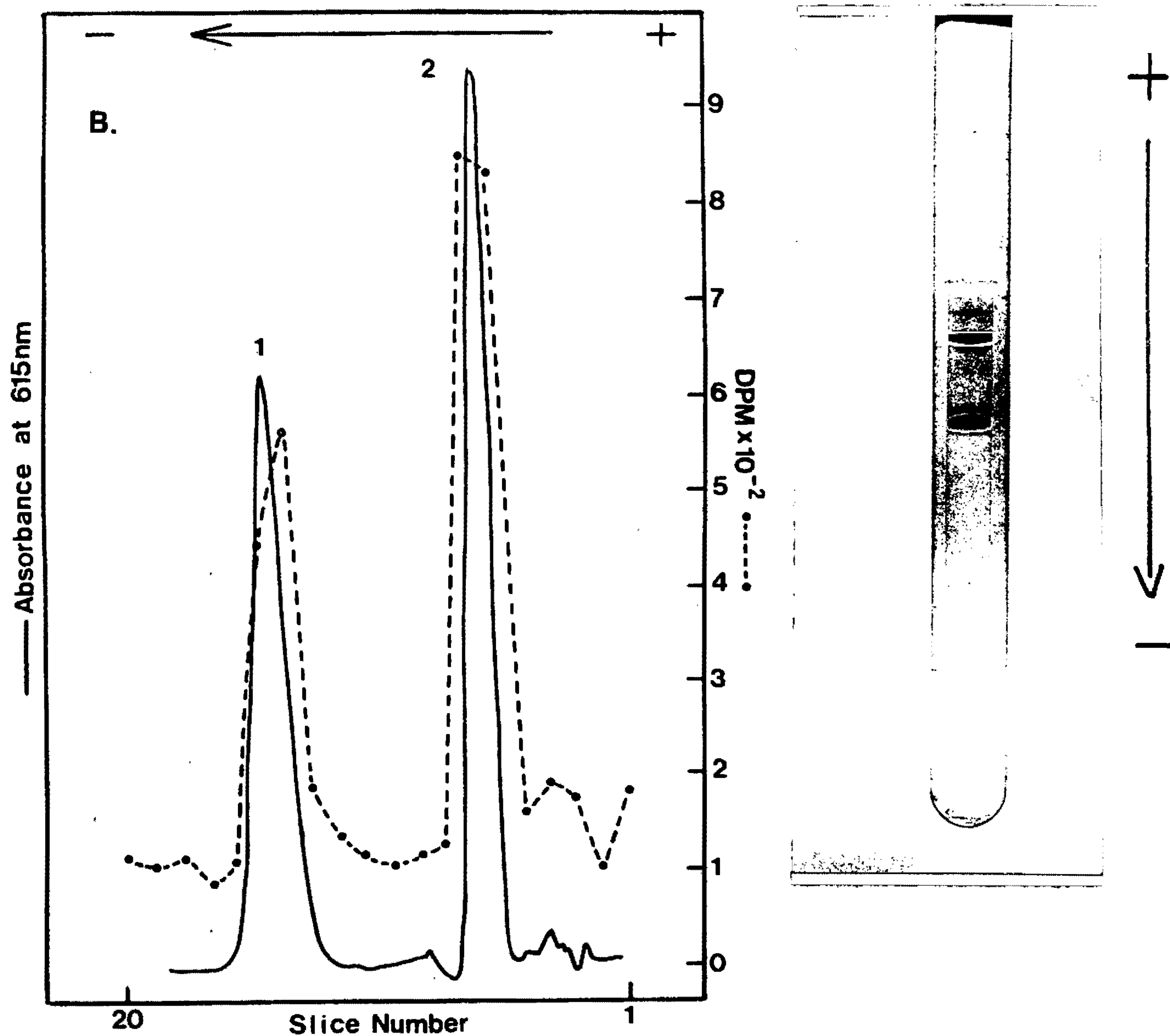


FIG. 4.12

Acrylamide gel electrophoresis of [^{3}H]-labelled globin obtained from immature chicken erythrocytes incubated in the presence of [^{3}H]-histidine. Aliquots of whole blood were incubated as described in Section 7.2.2.2.2 and globin prepared from the post mitochondrial supernatant and electrophoresed as outlined in Section 7.4.1.4.1. Absorbance peaks 1 and 2 in Figs. 4.10 and 4.11 are the same two components of total chicken globin.



9S RNA were examined. The results from Fig. 4.13 clearly show the synthesis of rabbit globin in the presence of added rabbit 9S RNA.

4.2.2.2 XENOPUS EGG SYSTEM

Aliquots of a chicken 9S RNA solution were injected into batches of anucleated Xenopus laevis eggs, as described by Gurdon and co-workers (123,124,125) (6-10 ng 9S RNA/egg). After incubation the products of synthesis were analyzed on Sephadex G-100 (Fig. 4.14). In the presence of chicken 9S RNA, a peak of radioactively labelled material was found co-incident with carrier chicken haemoglobin. Material from the haemoglobin region of the Sephadex elution profile (fractions 23-30 from the experiment in which 9S RNA was injected into eggs), was precipitated with acetone and analyzed by chromatography on carboxymethyl cellulose (Fig. 4.15). Most of the radioactively labelled material co-chromatographed with carrier chicken globin. Chicken globin elutes as a single peak under these conditions; the separation of α and β rabbit globin under the same conditions has been superimposed upon this chromatogram to illustrate the relative elution position of chicken globin.

These two translational systems, an ascites cell-free extract and anucleated Xenopus eggs have both clearly indicated that the 9S RNA preparation, isolated from polysomal RNA by repeated density gradient centrifugation, contains the mRNA for chicken globin.

4.2.3 SYNTHESIS OF COMPLEMENTARY DNA

A complementary DNA copy of the poly(A)-containing chicken 9S RNA fraction was synthesized using the commercially available RNA-dependent DNA polymerase from avian myeloblastosis virus (Table 4.2). The synthesized product had a specific activity of 15.98×10^6 dpm/μg. The product, when analyzed on alkaline sucrose gradients (Fig. 4.16) showed a heterogeneity in size with a predominant fraction sedimenting at 4-6S. Imaizumi et al. (71) have shown that the S-value of the product is dependent upon the concentration of the deoxyribonucleotide

FIG. 4.13

Acrylamide gel electrophoresis of the products obtained from a rabbit 9S RNA-directed ascites cell-free system. Refer to the legend of Fig. 4.11 for further details. Rabbit globin was prepared from a post mitochondrial supernatant of mature erythrocytes (normal blood) using the method described in Section 7.4.1.4.1. Rabbit globin migrates as a single band (Peak A) under the conditions of electrophoresis described.

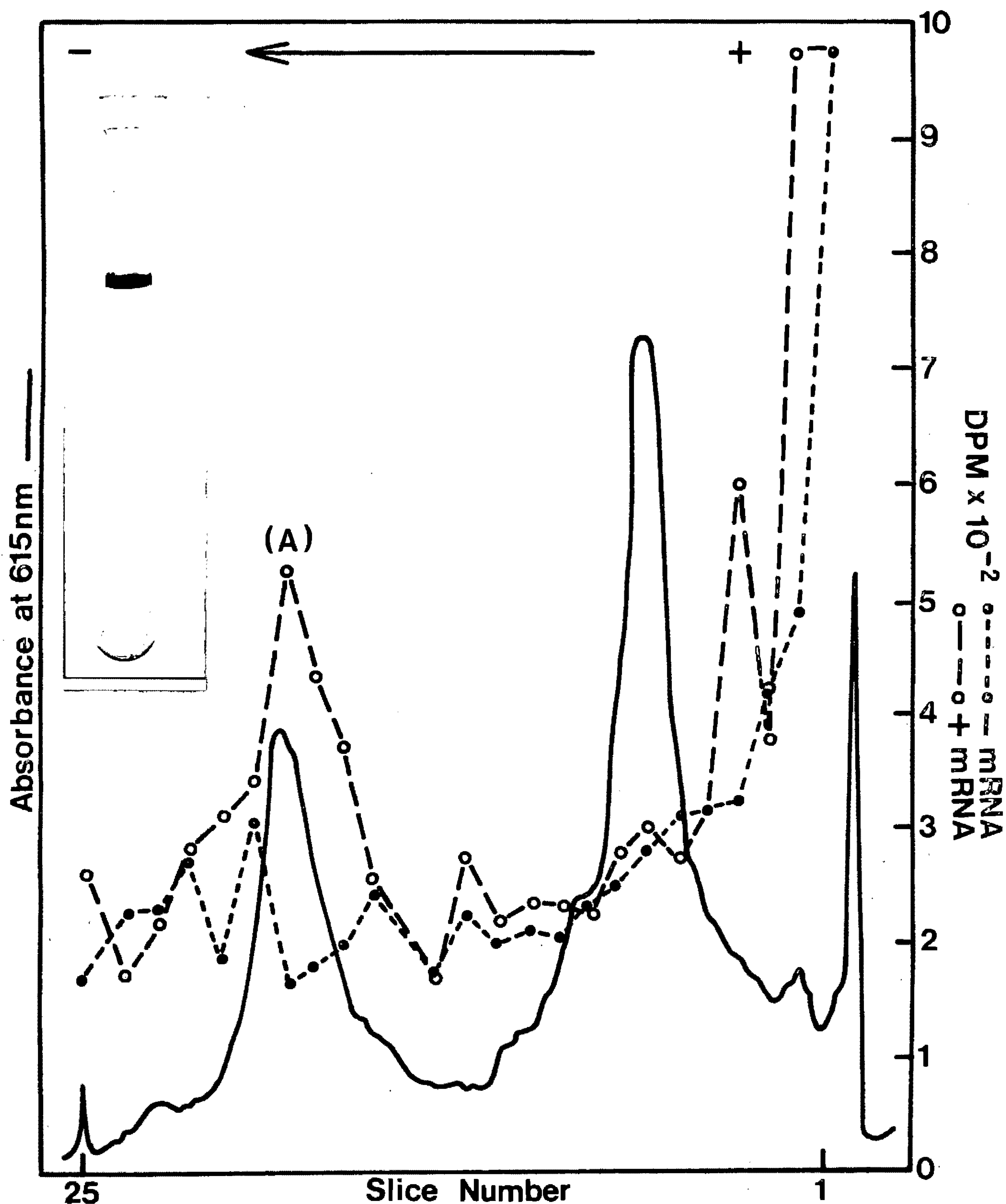


FIG. 4.14

Sephadex G-100 chromatography of the supernatant obtained after sonication of Xenopus laevis eggs injected with chicken 9S RNA (o----o) and saline (x——x). For further details see Materials and methods, Section 7.4.2. The carrier haemoglobin peak was identified by spectrophotometrically analyzing fractions at 415 nm.

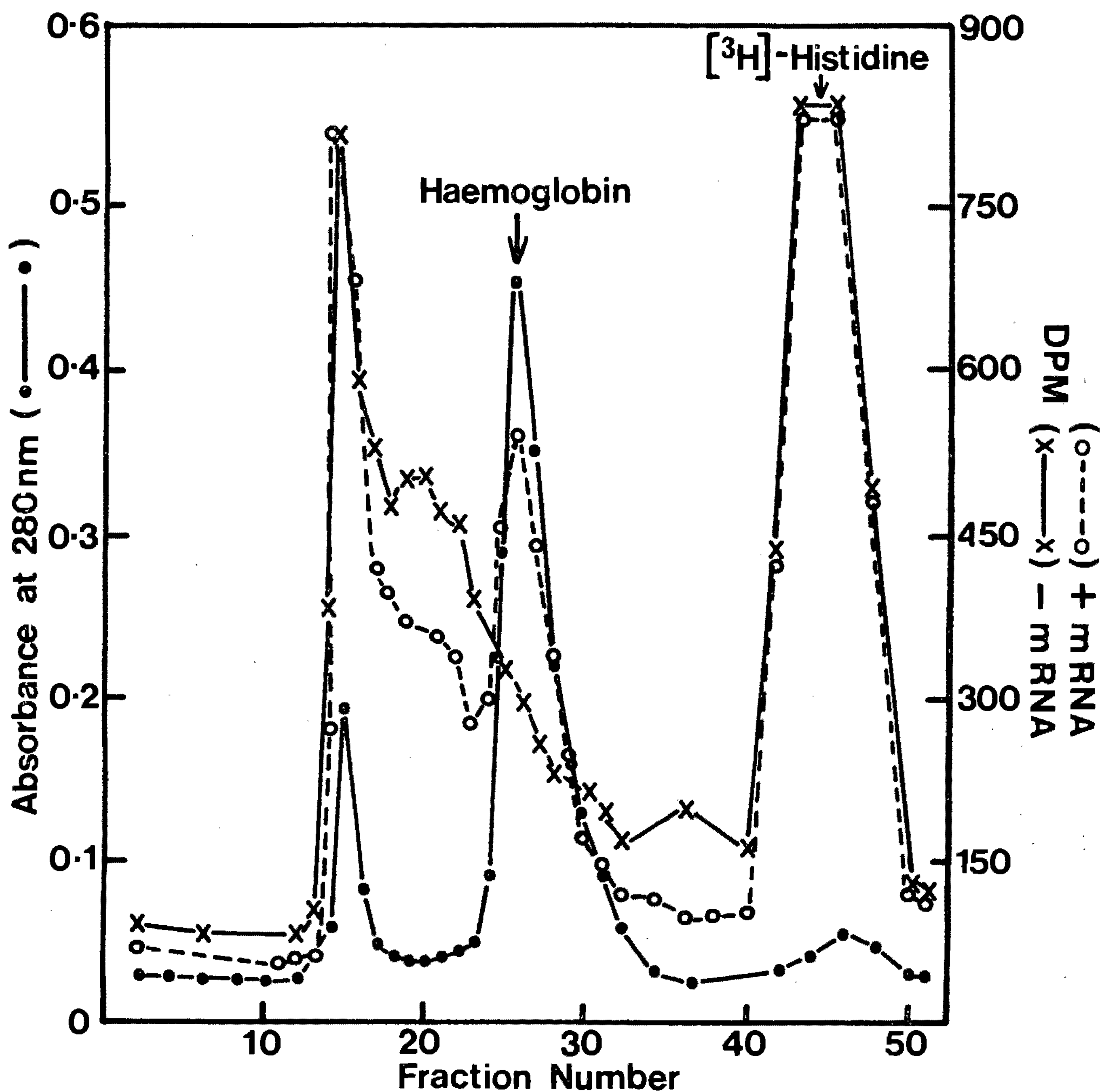


FIG. 4.15

Chromatography on CM-52 of fractions (23-30) after acetone precipitation. Protein was eluted with a 250 ml linear gradient of formic acid and pyridine as described in Section 7.4.2.3.2. 2.5 ml fractions were collected and analyzed as described. Rabbit globin, prepared from a post-mitochondrial supernatant using the method described in Section 7.4.1.4.1 was chromatographed as above.

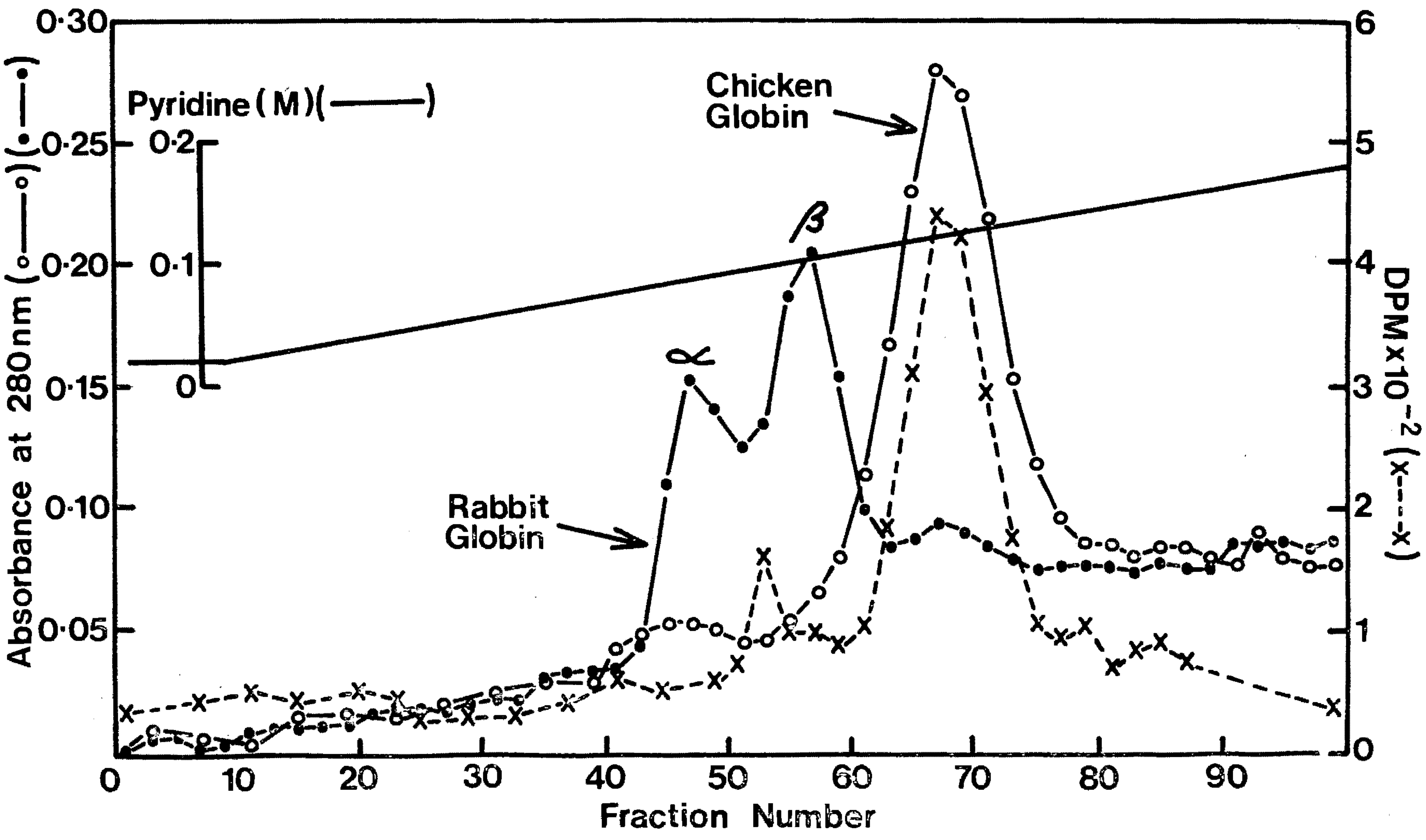


TABLE 4.2

The synthesis of the DNA complement of chicken 9S RNA. For details of the reaction mixture, see Materials and methods 7.5.1.

Reaction mixture	Incorporation (dpm x 10^{-3})
Complete	116
-Oligo(dT)	10
-9S RNA	0.3

present in the reaction mixture. The size of this DNA copy (Fig. 4.16) might be improved if higher concentrations of $[^3H]$-TTP are used (final incubation molarity : 0.01 mM). Furthermore, Williamson and co-workers (126) achieved a 9S DNA copy of human globin mRNA by including rat liver ribonuclease inhibitor in the reaction mixture; breakdown of 9S RNA during incubation would therefore also seem to be a factor in affecting the S-value of the final product.

To test the fidelity of the enzymatic transcription of the RNA template, $[^3H]$-cDNA was hybridized to chicken 9S RNA (Fig. 4.17), which significantly (65% nuclease resistance in RNA excess) and consistently hybridized to the labelled DNA product whereas 28S rRNA (as shown in Fig. 4.17) and 18S rRNA did not (3-9% nuclease resistance). A background (% nuclease resistance in the absence of added RNA) of 3% has not been subtracted from these figures; the percentage nuclease resistance observed in the presence of either 28S rRNA or 18S rRNA was considered therefore as variation in background values. Replotting the points that determined the hybridization curve for 9S RNA in Fig. 4.17 in the form of a C_ot plot (127) (Fig. 4.18), a $C_ot_{\frac{1}{2}}$ for the reaction between 9S RNA and cDNA

FIG. 4.16

Alkaline sucrose gradient of [^{3}H]-cDNA complementary to chicken 9S RNA. After incubation the sample was hydrolyzed in alkali (see Materials and methods 7.5.1) and then layered on a linear 15-30% (w/v) sucrose gradient in 0.9 M NaCl, 0.1 M NaOH, 5 mM EDTA. Centrifugation was for 8 h at 420 000 g, 2°C. 0.2 ml fractions were collected and acid precipitable radioactivity determined (7.2.2.2.2(a)).

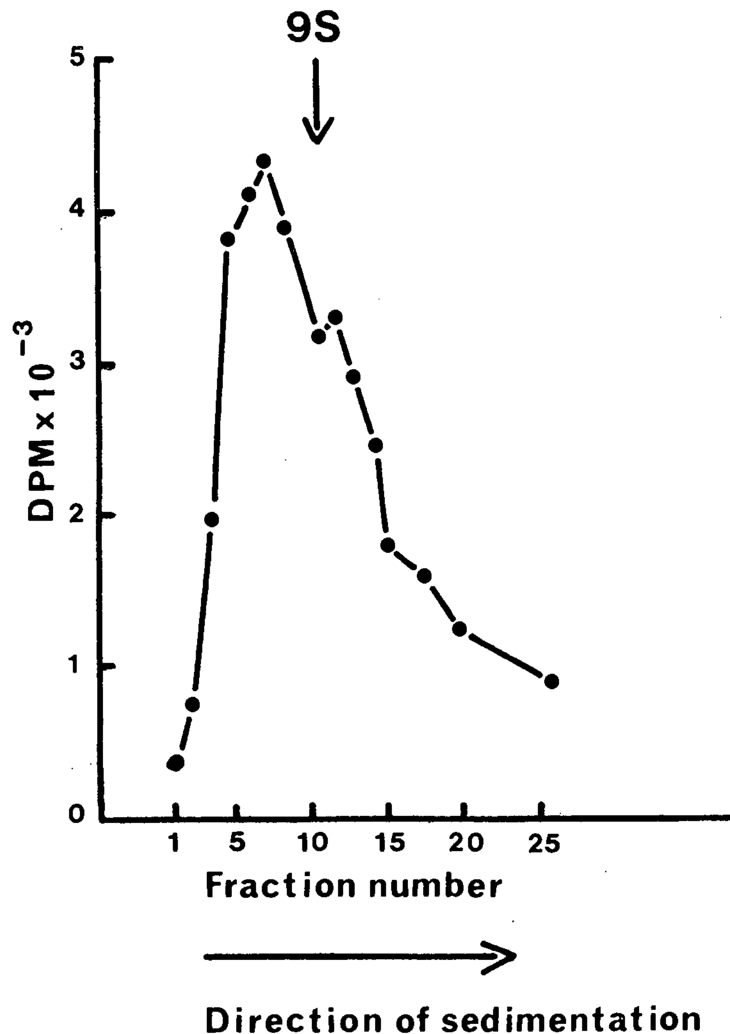


FIG. 4.17

Hybridization of [^{3}H]-cDNA to chicken 9S RNA and 28S RNA. The conditions used for annealing were as described in Materials and methods 7.5.2.

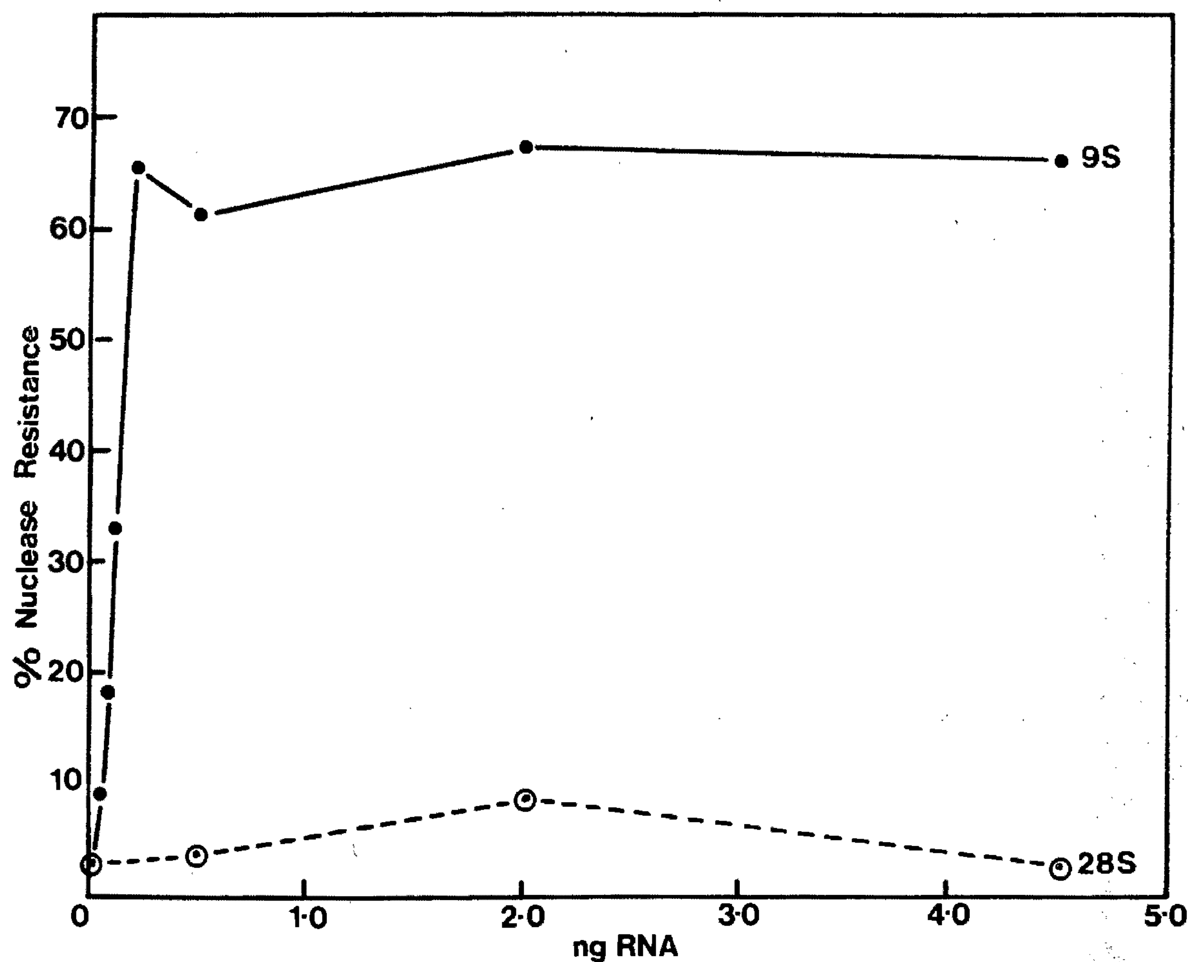
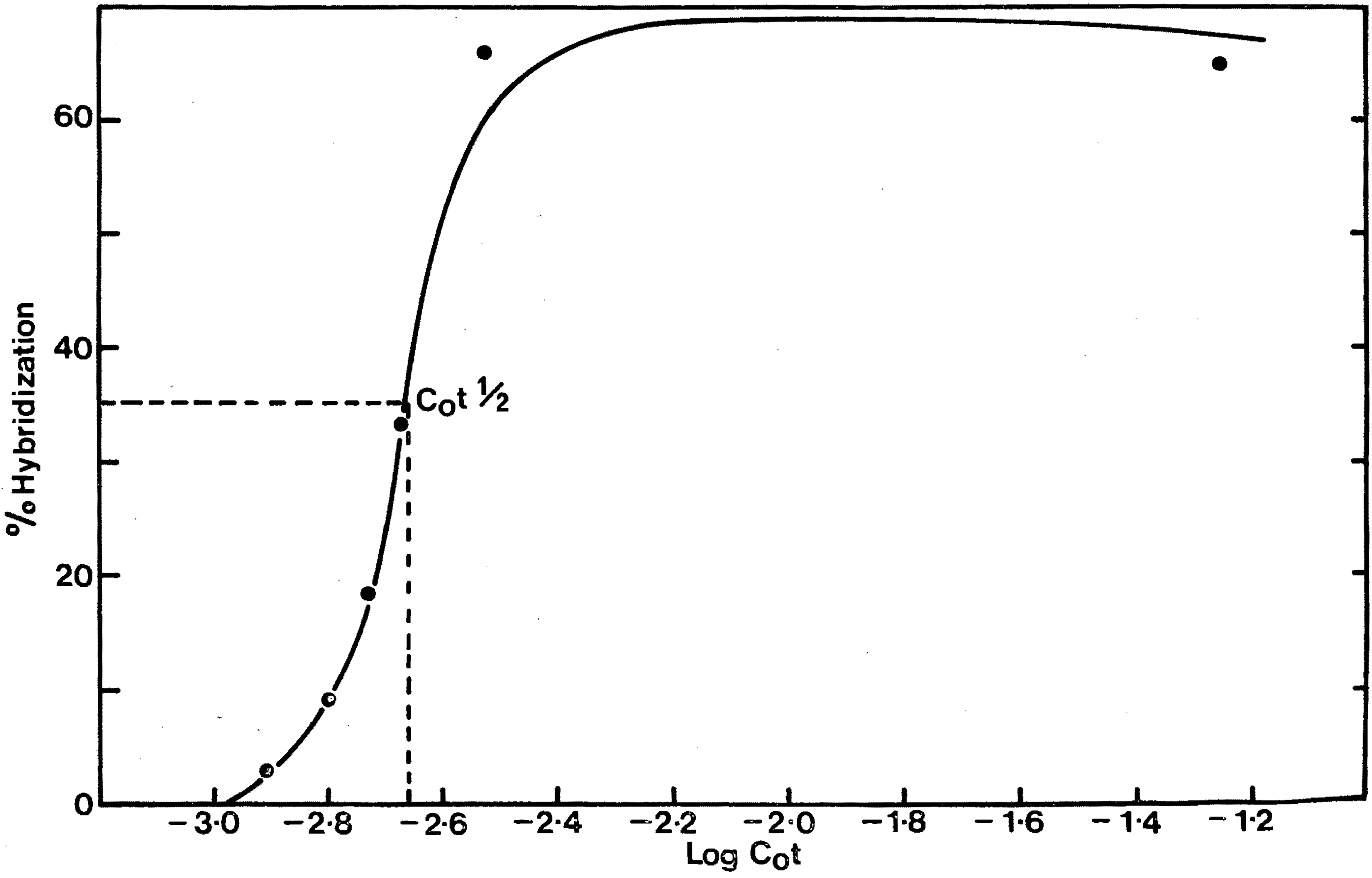


FIG. 4.18

Kinetics of annealing of chicken 9S RNA to [^{3}H]-cDNA.



of 2.18 x 10^{-3} mol. sec. $liter^{-1}$ was obtained. This value is in agreement with that of Barrett et al. (96) for chicken 9S RNA*

Nuclease resistance in hybridization studies where 9S RNA was added in excess, never exceeded 70%. As the synthesis of a complementary DNA copy of poly(A) containing mRNA depends upon the presence of primer oligonucleotide in the incubation mixture (94), the incorporation of [^{3}H]-TTP in the absence of oligo(dT) (Table 4.2) presumably represents the synthesis of polynucleotide material which is non-complementary to sequences of chicken globin mRNA. Gilmour et al. (117) have indicated that 10-20% of DNA complementary to mouse globin mRNA did not show any homology with globin mRNA. Moreover these authors have found that the fraction of labelled cDNA which did not hybridize to globin mRNA is less when the radioactively labelled deoxyribonucleotide used for the synthesis of complementary DNA was one other than TTP. I have used [^{3}H]-TTP for the synthesis of labelled DNA, complementary to chicken poly(A)-containing 9S RNA. The percentage of total synthesized DNA represented by DNA complementary to poly(A)-containing 9S RNA (most of which is globin mRNA) would be higher (and thus nuclease resistance greater, in RNA excess) if [^{3}H]-dCTP for example had been used as the radioactively labelled DNA precursor (see also below). Other factors which may affect hybrid formation include the temperature of hybridization and the size of the cDNA. However, in spite of the low S-value of the cDNA product and a low nuclease resistance in 9S RNA excess, of less than 80-90%, the specificity of cDNA for 9S RNA is without doubt. This DNA complement could now be used to test for the presence of ribosome bound replicated globin mRNA.

In earlier studies, before the commercial availability of reverse transcriptase, attempts were made to synthesize RNA complementary to chicken

*Note : Considerations of the kinetics of hybridization of cDNA to 9S RNA and the theoretical background to RNA:DNA hybridization are given in the following publications : Young et al. (128), McCarthy and Church (129), Kennell (130), Reiner (131), Bishop et al. (132) and Thomou and Katsanos (133).

9S RNA using the DNA-dependent RNA polymerase from *M.lysodeikticus* as outlined by Melli and Pemberton (134). Although the synthesis of a 9S RNA-dependent product was achieved (Table 4.3) subsequent hybridization studies demonstrated no 9S RNA specific reaction. The reasons for this are unknown.

TABLE 4.3

RNA synthesis by DNA-dependent RNA polymerase from *M.lysodeikticus* in the presence of calf thymus DNA or chicken 9S RNA. Reaction mixture was as described in Section 7.6.5 except 25 units of DNA-dependent RNA polymerase from *M.lysodeikticus* were added. 10 μg calf thymus DNA and 10 μg chicken 9S RNA were added where indicated. After incubation, acid precipitable radioactivity was determined as described.

Reaction conditions	Incorporation (dpm x 10^{-3})
+DNA	98.0
+9S RNA	7.0
no added nucleic acid	0.9

4.2.4 ASSAY FOR REPLICATED GLOBIN mRNA SEQUENCES IN [^{3}H]-POLYSOMAL RNA

Polysomal RNA, isolated from ribosomes incubated with [^{3}H]-UTP ([^{3}H]-polysomal RNA) as described in Materials and methods (7.7.1) was included in a reaction mixture with an aliquot of unlabelled cDNA (Materials and methods 7.5.1) and incubated at 65°C for 24 hours. The incubate was analyzed for nuclease resistant hybrids and the results, together with the appropriate control experiments, are shown in Table 4.4. Labelled cDNA annealed to the endogenous 9S mRNA present in total, unlabelled polysomal RNA (expts. 1,2). A low nuclease resistance of 4% (this represents a background value) was obtained after incubation of [^{3}H]-cDNA in the absence of any added RNA (expt. 3). A similar result was found using [^{3}H]-polysomal RNA (expt. 4). An equally low percentage nuclease resistance was demonstrated after incubation of [^{3}H]-polysomal RNA with unlabelled cDNA (expt. 5). Hybridization of unlabelled cDNA with limiting amounts of 9S RNA, in the presence of [^{3}H]-cDNA, resulted in a lower recorded nuclease resistance than labelled cDNA on its own (expts. 6,7). This offers a positive control as to the fidelity of the unlabelled cDNA fraction. It is important to emphasize, especially in the context of experiments five and seven, that percentage nuclease resistance refers to that percentage of total <u>radioactively labelled</u>, TCA-precipitable material that was nuclease S1-resistant.

Sufficient unlabelled cDNA (1.42 ng) was added to the incubate of experiment five to anneal to all the 9S globin mRNA present in the 31 ng of [^{3}H]-polysomal RNA (0.62 ng; equal to 2% of total polysomal RNA (135) and Section 4.2.1) plus whatever newly synthesized mRNA might have been made by a ribosome bound RNA replicase. If all the incorporated radioactivity in [^{3}H]-polysomal RNA were due to the activity of a globin mRNA replicase, a maximum nuclease resistance of about 65% would have been obtained. That no difference was found between nuclease resistance figures in the absence (expt. 4) and presence of unlabelled cDNA (expt. 5), demonstrates the absence of detectable radioactively labelled globin mRNA in [^{3}H]-polysomal RNA. Percentage nuclease resistance figures resulting from the incorporation of less than 10% of radioactivity from [^{3}H]-polysomal RNA into nuclease resistance hybrids are of doubtful significance. This represents the limit of sensitivity of this method. It is clear therefore that more than 90% of the newly synthesized ribosome

TABLE 4.4

Hybridization of cDNA to polysomal RNA labelled *in vitro*. Preparation of various fractions and hybridization procedure is as described in Materials and methods (Sections 7.5 and 7.7). Where indicated, ± figures are values for standard deviation. Amounts of reactants added to the various experiments are as follows :

1. 0.62 ng 9S RNA plus 0.142 ng [^{3}H]-cDNA (2276 dpm)
2. 31 ng polysomal RNA plus 0.142 ng [^{3}H]-cDNA
3. 0.142 ng [^{3}H]-cDNA
4. 31 ng [^{3}H]-polysomal RNA (1600 dpm)
5. 31 ng [^{3}H]-polysomal RNA plus 1.41 ng unlabelled cDNA
6. 0.102 ng 9S RNA plus 0.142 ng [^{3}H]-cDNA
7. 0.102 ng 9S RNA plus 0.142 ng [^{3}H]-cDNA and 0.142 ng unlabelled cDNA

Experiment	Hybridization mixture	% Nuclease Resistance	No. of experiments
1	9S RNA + [^{3}H]-cDNA	65 ± 4	4
2	Polysomal RNA + [^{3}H]-cDNA	64 ± 7	2
3	[^{3}H]-cDNA	4 ± 2	6
4	[^{3}H]-polysomal RNA	5 ± 3	4
5	[^{3}H]-polysomal RNA + unlabelled cDNA	4 ± 3	3
6	9S RNA + [^{3}H]-cDNA	42	1
7	9S RNA + [^{3}H]-cDNA + unlabelled cDNA	26	1

bound product is a polynucleotide other than globin mRNA.

The 9S RNA/cDNA ratio at the point of inflexion of the curve shown in Fig. 4.17 is 1.76. Although this is in agreement with the ratio of 1.4 given by Gilmour and Paul (135) (using mouse globin 9S RNA and its DNA complement), I expected a higher ratio due to the relatively small size of the cDNA. A factor that may influence the calculation of the amount of cDNA present in each incubation is the use of TTP as the radioactively labelled deoxyribonucleotide. The calculation of specific activity assumes that each of the four deoxyribonucleotides was incorporated equally during incubation. The short oligo(dT) primer (12-18 nucleotides) required to initiate the synthesis of complementary DNA can hybridize anywhere along the length of the poly(A) segment (which for most avian globin mRNAs is a stretch of polynucleotide composed of about 85 adenylic acid residues (105)). Therefore, should the oligo(dT) primer hybridize to the first 12-18 adenylic acid residues (from the 3' end) the RNA-dependent DNA polymerase will incorporate, until the globin message is reached, $[^3H]$-TMP only. The initial assumption concerning equal incorporation of all 4 deoxyribonucleotides cannot apply when using $[^3H]$-TTP. The specific activity of cDNA complementary to globin message and not poly(A) will be lower than that quoted (15.98×10^6 dpm/μg). The amount of true globin cDNA added per assay (Fig. 4.17) will consequently be lower, increasing the RNA/cDNA ratio.

Assuming an RNA/cDNA ratio of two (the higher this ratio, the less cDNA required to detect a fixed amount of RNA), the unlabelled cDNA, in order that the results in Table 4.4 remain valid, must be able to detect all the globin messenger sequences (and not just poly(A) which may or may not be replicated by a globin mRNA replicase) in the 0.62 ng 9S RNA present in experiment 5. This would require that 0.31 ng of the added cDNA be complementary to true globin messenger sequences (22% of total cDNA present in experiment 5). A complementary product of an adenylic acid sequence of 85 nucleotides only would, on alkaline sucrose density gradient centrifugation, result in a 4S product. The greater percentage of the product analyzed in Fig. 4.16 is larger than 4S (the 4S region of this gradient falls between fractions 4 and 5). However,

nuclear and 'cytoplasmic' DNA from primary cultures of mouse foetal livers to be similar. These authors have concluded therefore that cytoplasmic DNA arises as a result of nuclear chromatin cleavage and represents an artefact of isolation. This lends further support to the hypothesis that the globin gene undergoes no amplification by a process of cytoplasmic transcription.

PART 5

THE IDENTIFICATION OF THE PRODUCT OF RIBOSOME-BOUND RNA-DEPENDENT RNA POLYMERASE IN IMMATURE CHICKEN ERYTHROCYTES

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5.1 INTRODUCTION

Wilkie and Smellie (22) observed that : "They (the activities observed in the free ribosome fraction) may only be artefacts brought about by the conditions of assay or of isolation of the free ribosome fraction....." The question of whether a cytoplasmic enzyme activity (or its products), incorporating UTP into polynucleotide material, can be detected in intact cells, still remains an important consideration for an investigation of a ribosome-bound RNA-dependent RNA polymerase.

The insensitivity of RNA-dependent synthesis of RNA in the presence of actinomycin D is well known (137). I have shown that the incorporation of UTP into polynucleotide material by the ribosome-bound RNA-dependent RNA polymerase and the more purified enzyme preparation from immature chicken erythrocytes was insensitive to the presence of this antibiotic (Tables 3.10 and 3.11). High concentrations of actinomycin D (10 μg/ml) are known to completely suppress the DNA-dependent synthesis of both HnRNA and cytoplasmic mRNA in Hela cells and L-cells (138,139). Spohr and co-workers (67) using higher concentrations of actinomycin D (100 μg/ml) reported measurements of 9S and 12S polyribosome-bound RNA turnover in immature duck erythrocytes. These and other kinetic experiments (140) are consistent with the absence of nuclear DNA-dependent RNA synthesis occurring in the presence of high concentrations of this antibiotic.

What is the nature of the products synthesized in actinomycin D-treated cells (141,58)? If the actinomycin D-insensitive, ribosome-bound RNA-dependent RNA polymerase activity is present in immature erythrocytes, its product should be amongst those RNA species synthesized in the presence of this antibiotic. The detection of this product, in the absence of nuclear, DNA-dependent RNA synthesis, is considered in this chapter.

5.2 RESULTS AND DISCUSSION

5.2.1 RNA SYNTHESIS IN INTACT CELLS IN THE PRESENCE AND ABSENCE OF ACTINOMYCIN D

3 ml aliquots of whole blood (equal to 5.67 x 10^9 cells) collected from chickens rendered anaemic with phenylhydrazine hydrochloride (7.2.1) (reticulocyte count was usually greater than 50%) were incubated with [^{3}H]-uridine for 2 h at 37°C. Actinomycin D, where indicated, was present at a concentration of 80 μg/ml (for details see 7.2.2.1.2). At the end of the incubation, erythrocytes were lyzed and ribosomes isolated from the post-mitochondrial supernatant. Before these preparations were extracted with phenol, aliquots were removed and precipitated with TCA. Acid insoluble radioactivity was determined (Table 5.1). The presence of this antibiotic inhibited the incorporation of TCA-precipitable, radioactive material into ribosomes by 95%. This degree of inhibition is in confirmation of previous reports (48,141).

TABLE 5.1

Incorporation of [^{3}H]-uridine into polyribosomes in intact cells in the presence and absence of actinomycin D. Incorporation figures have been expressed as dpm per $OD_{260\ nm}$ units of ribosomes. A control value of 150 dpm (zero time of incubation) has been subtracted to obtain these figures.

Experiment	dpm/OD_{260}
Plus actinomycin D	2728
No actinomycin D	47990

5.2.1.1 ANALYSIS OF RNA PRODUCTS SYNTHESIZED IN INTACT CELLS IN THE ABSENCE OF ACTINOMYCIN D

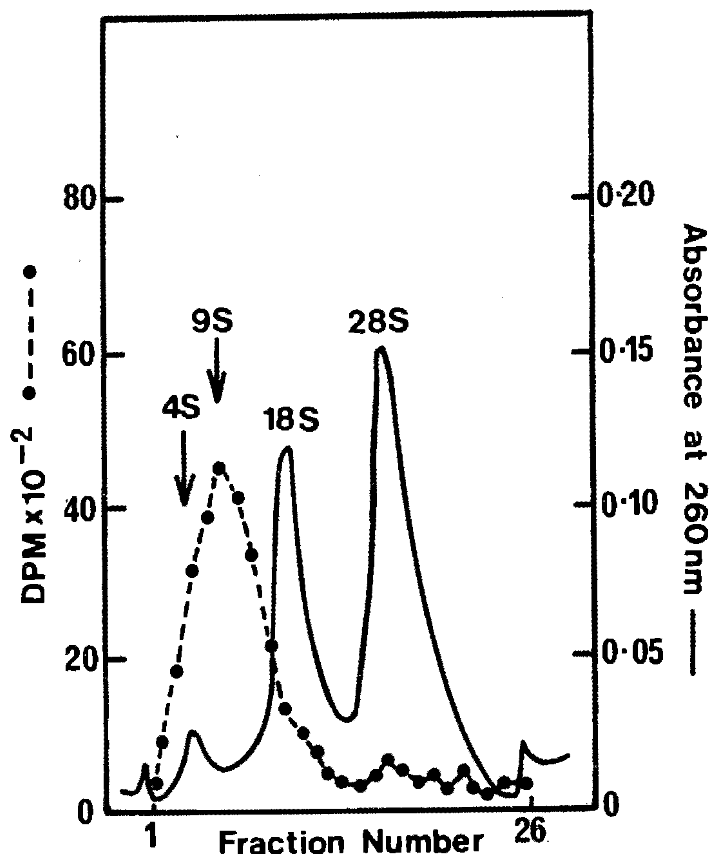
Polyribosomal RNA, extracted from ribosomes prepared from immature erythrocytes incubated with [^{3}H]-uridine, was analyzed by sucrose density gradient centrifugation (Materials and Methods 7.2.2.1.2). A typical result is shown in Fig. 5.1. The presence and proportions of 4, 18 and 28S RNA shown in this profile are well known. A peak of radioactive material in the 9S region of the sucrose gradient is evident. A number of workers (51,57) have shown that the labelling of 9S RNA in immature erythrocytes is due to the DNA-dependent synthesis of mRNA, predominantly globin mRNA (45). I have shown the presence of globin mRNA in the 9S RNA fraction derived from immature chicken erythrocyte polysomal RNA (see section 4.2.2).

The synthesis of 12S RNA has been reported by Spohr et al. (67) in immature duck erythrocytes. No peak of radioactively labelled 12S RNA was apparent in Fig. 5.1 but this could be due to insufficient resolution between 9 and 12S RNA on these gradients.

The resolution of this method of analysis revealed little or no labelling of 18 and 28S ribosomal RNA. Acid-insoluble radioactive material was found in the 18 and 28S regions of the sucrose gradient only when the time of cell lysis was increased from 1 min to three minutes; a 30 sec period of hypotonicity has been reported (142) as being specific for the lysis of erythrocytes only. An increase of time results in leucocyte lysis as well. The presence of labelled material in the 18 and 28S regions of the gradient could therefore be partially due to contamination by leucocyte polysomal RNA. This confirmed previous reports of this nature (143). In addition van der Westhuyzen et al. (63) have shown that the nuclei of immature circulating chicken erythrocytes have no detectable nucleolar DNA-dependent RNA polymerase. It would seem therefore that no labelling of these two rRNA species would be expected, if all the isolated polyribosomal RNA were extracted from immature erythrocyte ribosomes. This is not in agreement with the findings of Evans and Lingrel (142) who, working with immature mouse

FIG. 5.1

Sucrose gradient centrifugation analysis of polyribosomal RNA from ribosomes prepared from immature erythrocytes incubated with [^{3}H]-uridine in the absence of actinomycin D. 0.50 OD_{260} units polyribosomal RNA was applied to a 15-30% sucrose gradient (see Materials and methods 7.2.2.1.2) and centrifuged at 420 000 g for 3 h at 20°C. The gradient was analyzed at 260 nm (——); 0.2 ml fractions were collected and acid-precipitable radioactivity was determined (●----●).



Note : In practice, it was not possible to distinguish, unequivocally, the difference between a 4S and 5S RNA peak. The 4S RNA above therefore represents the 4-5S region of a sucrose gradient.

erythrocytes, showed no difference in the labelling pattern of 18 and 28S rRNA isolated from blood preparations where white cells had been removed and whole blood where white cells had not been removed. These authors provided data showing that rRNA was synthesized early in the erythroid cell development while only 9S RNA synthesis occurred in more mature cells (polychromatic erythrocytes; for nomenclature, see Lucas and Jamroz (46)). The cells of the anaemic blood used in my investigations were predominantly (79%) mid- and late polychromatic erythrocytes. Both these cell types lack nucleoli and are not able to synthesize rRNA (63,75). More immature erythrocytes (basophilic erythroblasts) in the blood preparations used by Evans and Lingrel (142) and, more recently, Spohr et al. (67) might account for the synthesis of rRNA observed by these workers.

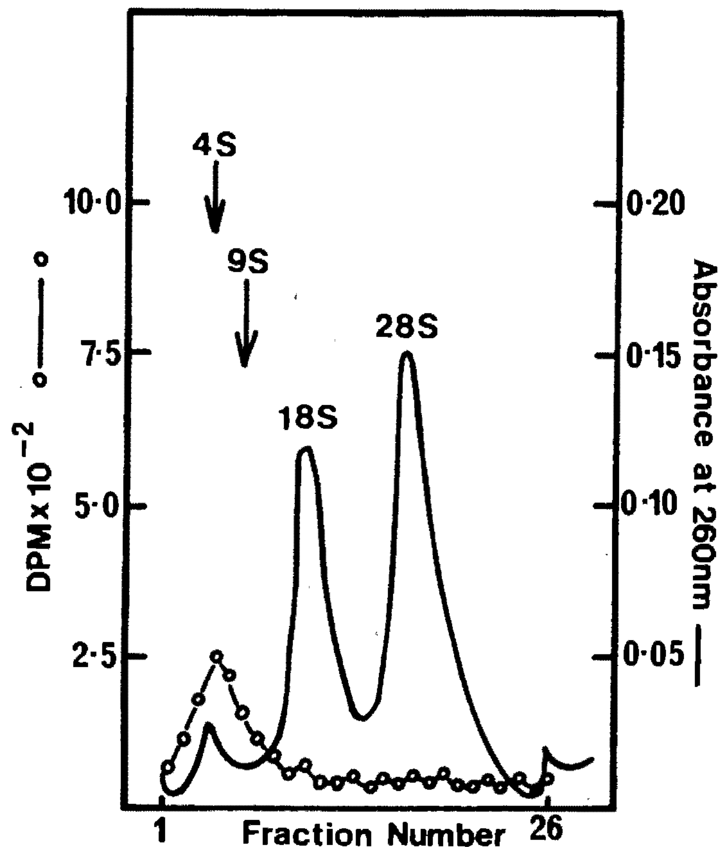
5.2.1.2 ANALYSIS OF RNA PRODUCTS SYNTHESIZED IN INTACT CELLS IN THE PRESENCE OF ACTINOMYCIN D

Polyribosomal RNA, extracted from ribosomes prepared from immature erythrocytes incubated with [^{3}H]-uridine in the presence of actinomycin D, was analyzed by sucrose density gradient centrifugation (Fig. 5.2). In the presence of this antibiotic, a single peak of radioactivity in the 4S-region of the sucrose gradient was present. Within the limitations of resolution of this method, no apparent 9S-labelling was ever noticed. It cannot be ascertained from these data whether less radioactively labelled material is present in the 4S region of the sucrose gradient in the presence of actinomycin D than in its absence.

Eason and co-workers (141) have shown, using Krebs II ascites tumour cells incubated with [^{3}H]-uridine, the appearance of radioactively labelled 4S material in the presence of actinomycin D. This radioactively labelled 4S RNA, obtained by extracting whole cells with phenol, was hydrolyzed with alkali and the resulting 3'(2')-monophosphates were separated by paper electrophoresis. The major portion of the radioactivity was found as [^{3}H]-CMP. The authors attribute most of this actinomycin-insensitive RNA synthesis to terminal addition of [^{3}H]-CMP

FIG. 5.2

Analysis by sucrose density gradient centrifugation of polyribosomal RNA from ribosomes prepared from immature erythrocytes incubated with [^{3}H]-uridine in the presence of actinomycin D (80 μg/ml). 0.50 OD_{260} units of polyribosomal RNA was applied to a 15-30% sucrose gradient and centrifuged at 42 000 g for 3 h at 20°C. The gradient was analyzed at 260 nm (——); 0.2 ml fractions were collected and acid-precipitable radioactivity was determined (o----o).



to tRNA. This type of actinomycin-insensitive RNA synthesis has been reported or presumed to occur by a number of workers (74,143).

In order to investigate the nature of the radioactively labelled 4S RNA (Fig. 5.2), total polysomal RNA was digested with alkali (Materials and methods, section 7.7.2.2) and the products were separated by thin layer electrophoresis. This method results in good separation of the 4 component nucleoside 2',3'-monophosphates and the nucleoside, uridine (Fig. 5.3). The appropriate parts of the cellulose plate were divided into rectangles. The cellulose was scraped off, and samples were solubilized and counted. The distribution of radioactivity in the 4 nucleotides and uridine is given in Table 5.2.

TABLE 5.2

Distribution of radioactivity in ribonculeosides and ribonucleotides obtained after alkaline hydrolysis of polysomal RNA from immature erythrocytes incubated in the presence of actinomycin D. 3515 dpm total polysomal RNA was precipitated and washed with ethanol and hydrolyzed as described in section 7.7.2.2. Electrophoresis and determination of radioactive distribution as described. Recovery of radioactivity after application to the thin layer plate was usually 90-95%.

Hydrolysis products	% of total radioactivity	$\frac{\text{nucleotide}}{\text{nucleoside}}$
UMP	85.08	5.50
Uridine	15.47	
GMP	0	
AMP	0	
CMP	0	

FIG. 5.3

Electrophoresis of nucleotides and nucleosides on thin layer cellulose plates. 50 μg (in 10 μl) of each of the indicated nucleotides and uridine were spotted onto thin layer cellulose glass plates. To spot a mixture, equal volumes of the 4 component nucleotides and uridine were mixed and 50 μl was applied to the cellulose plate. Electrophoresis was performed as described in Section 7.7.2.2. The plates were dried and analyzed under U.V.

+

I

AMP GMP CMP UMP Uridine UTP Mixture

In three such experiments all the radioactivity was found, quite clearly, in the UMP and uridine spots. No conversion therefore of [^{3}H]-uridine into [^{3}H]-CMP was detectable. To ensure that the presence of radioactively labelled UMP and uridine did not arise through adsorption of these acid-soluble compounds to polysomal RNA, all samples, after repeated ethanol precipitation and extensive washing with ethanol (Materials and Methods 7.7.2.2), and prior to alkaline digestion, were checked to confirm that TCA-precipitable radioactivity was equal to total radioactivity present in the sample. This control was carried out on all samples prior to thin layer electrophoresis.

A UMP/uridine ratio of 5.50 was obtained. The presence of radioactively-labelled uridine indicates the presence of this nucleoside in a terminal position of a polynucleotide chain. As the synthesis of homo- and hetero-polyribonucleotide material by cytoplasmic RNA-dependent RNA polymerases is possible (Sections 1.1.2 and 3.2.2), it cannot be ascertained from these data whether in fact the ratio of 5.5 means a poly(U) tail of 5-6 residues long; heteropolymer formation would result in a longer oligonucleotide product.

Why has the terminal and non-terminal addition of UMP to a 4S polysomal RNA in intact cells incubated with [^{3}H]-uridine in the presence of actinomycin D never been reported ? Eason et al. (141) isolated total cellular RNA from Hela cells incubated with [^{3}H]-uridine in the presence of actinomycin D. The large amount of tRNA (and the associated [^{3}H]-CMP at the 3' end) could possibly mask the presence of a 4S-labelled RNA species bound to ribosomes. (These authors do not discuss the significance of the 9% radioactively labelled UMP present in alkaline digests of 4S RNA - Table 1 of ref (141)). Marks and co-workers (143), working with rabbit reticulocytes concluded that the TCA-precipitable radioactivity present in the 4S region of the sucrose gradient would "appear to reflect only counts incorporated into sRNA since the ribosomes ...... had no detectable counts." The low specific activity of the label ([^{14}C]-guanine) (40 µC/mg ≡ 5.7 mCi/mmole; I used [^{3}H]-uridine with a specific activity of 44.5 Ci/mmole) might have prevented the detection of any ribosome bound radioactively labelled 4S RNA.

I found it surprising that no detectable [^{3}H]-CMP could be found in alkaline digests of polysomal RNA obtained from immature erythrocytes in the presence of actinomycin D. [^{3}H]-CMP was observed in alkaline digests of RNA isolated from the post-ribosomal supernatant of immature erythrocytes incubated in the presence of this antibiotic. This labelled nucleotide was also found in digests of polysomal RNA from immature erythrocytes incubated in the absence of actinomycin. The antibiotic therefore does not inhibit CTP synthetase but might possibly affect attachment of tRNA to ribosomes.

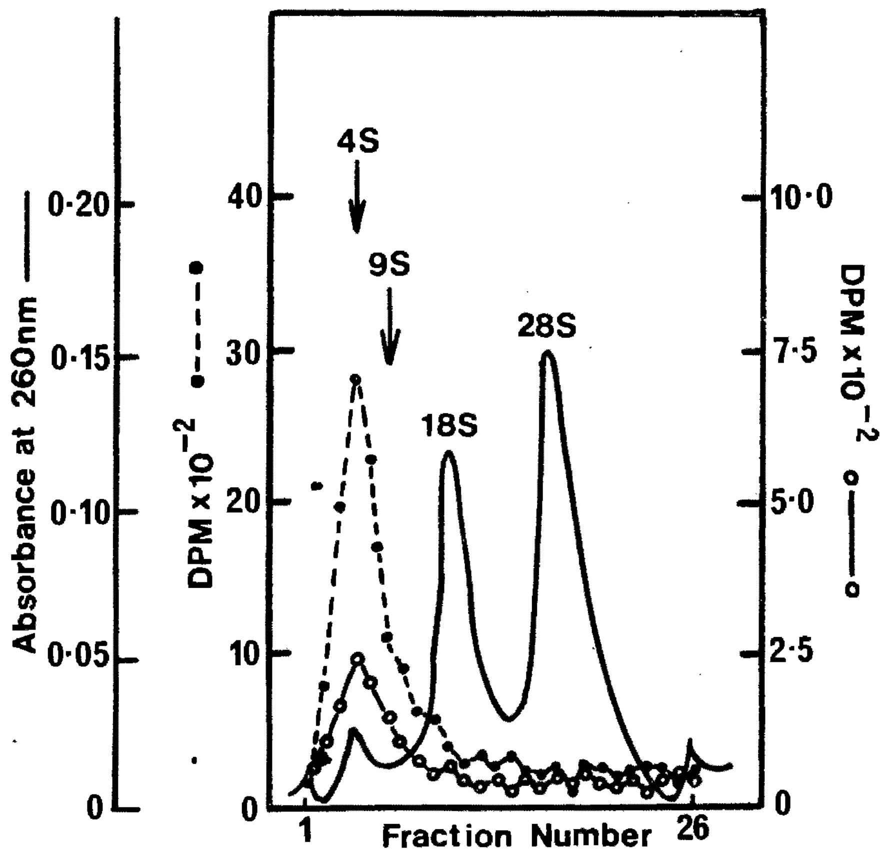
McReynolds and Penman (144) have demonstrated the synthesis of low molecular weight RNA in Hela cell nuclei incubated in the presence of actinomycin D. These authors, in addition, have found this RNA to have a UMP/uridine ratio of less than ten. They attribute the presence of this RNA to the low levels of synthesis of polynucleotide by a nuclear DNA-dependent RNA polymerase between blocked guanylic acid residues (the site of inhibition by actinomycin D (83)). Although the possibility has not been excluded it seems unlikely that this low molecular weight RNA, a product of 'abortive' nuclear RNA synthesis, could account for the presence of radioactively labelled 4S RNA, after cell lysis and centrifugation of the post-mitochondrial supernatant, in the ribosome fraction.

5.2.2 ANALYSIS OF RNA AFTER INCUBATION OF RIBOSOMES WITH [^{3}H]-UTP *IN VITRO*

Ribosomes, isolated from immature chicken erythrocytes, were incubated with [^{3}H]-UTP (Materials and methods, 7.7.2.1). Total polyribosomal RNA was extracted with phenol and analyzed by density gradient centrifugation (Fig. 5.4). All of the incorporated TCA-precipitable radioactive material was found in the 4S region of the sucrose gradient. This is in agreement with the labelling pattern shown by previous workers (22).

FIG. 5.4

Sucrose density gradient centrifugation analysis of polyribosomal RNA from ribosomes incubated with [^{3}H]-UTP *in vitro*. 18 000 dpm polyribosomal RNA was applied to a 15-30% sucrose gradient and centrifuged at 420 000 g for 3 h at 20°C. The gradient was fractionated into 0.2 ml fractions and TCA-precipitable radioactivity was determined in each fraction (Materials and methods 7.7.2.1). The pattern of radioactive incorporation (----) has been superimposed upon the radioactive and absorbance profiles from Fig. 5.2.



The profile of radioactive incorporation by ribosomes incubated with [^{3}H]-UTP has been superimposed, for purposes of comparison, on the radioactive and absorbanceprofiles of polysomal RNA isolated from immature erythrocytes incubated with [^{3}H]-uridine in the presence of actinomycin D. The position of the single peak of radioactivity from the profile of polysomal RNA isolated from intact cells and ribosomes incubated in vitro is exactly the same (4S).

Total polysomal RNA isolated from ribosomes incubated with [^{3}H]-UTP (in vitro), was treated with alkali and the digestion products were separated and analyzed as described in Materials and methods (7.7.2.2). Fig. 5.5 illustrates the typical radioactive distribution obtained on these electrophoretograms. The quantitative distribution of radioactivity in the 4 nucleotides and uridine is given in Table 5.3.

Radioactivity was found associated with UMP and uridine only. The nucleotide/nucleoside ratio was 6.09 in close agreement with the UMP/uridine ratio obtained from the hydrolysis of total polysomal RNA isolated from immature erythrocytes incubated with [^{3}H]-uridine in the presence of actinomycin D (Table 5.2).

The close similarity of sedimentation coefficient and UMP/uridine ratios between total polysomal RNA from intact cells and ribosomes incubated in vitro supports the hypothesis that this radioactively labelled 4S RNA species, present in polysomal RNA isolated from immature erythrocytes incubated in the presence of actinomycin D, might be the product of the ribosome bound RNA-dependent RNA polymerase activity observed in vitro. It was mentioned earlier (5.2.3 and Fig. 5.2) that no apparent radioactively labelled 9S RNA was present in polysomal RNA from immature erythrocytes incubated in the presence of actinomycin D. The contribution of labelled 9S RNA to alkaline hydrolysates would be radioactively labelled UMP only. The addition of uridine to terminal

FIG. 5.5

Separation by thin layer electrophoresis of products obtained after alkaline hydrolysis of polyribosomal RNA isolated from ribosomes incubated with [^{3}H]-UTP.
14 000 dpm total polyribosomal RNA was precipitated and washed with ethanol and hydrolyzed as described in section 7.7.2.2. Electrophoresis and determination of radioactive distribution as described.

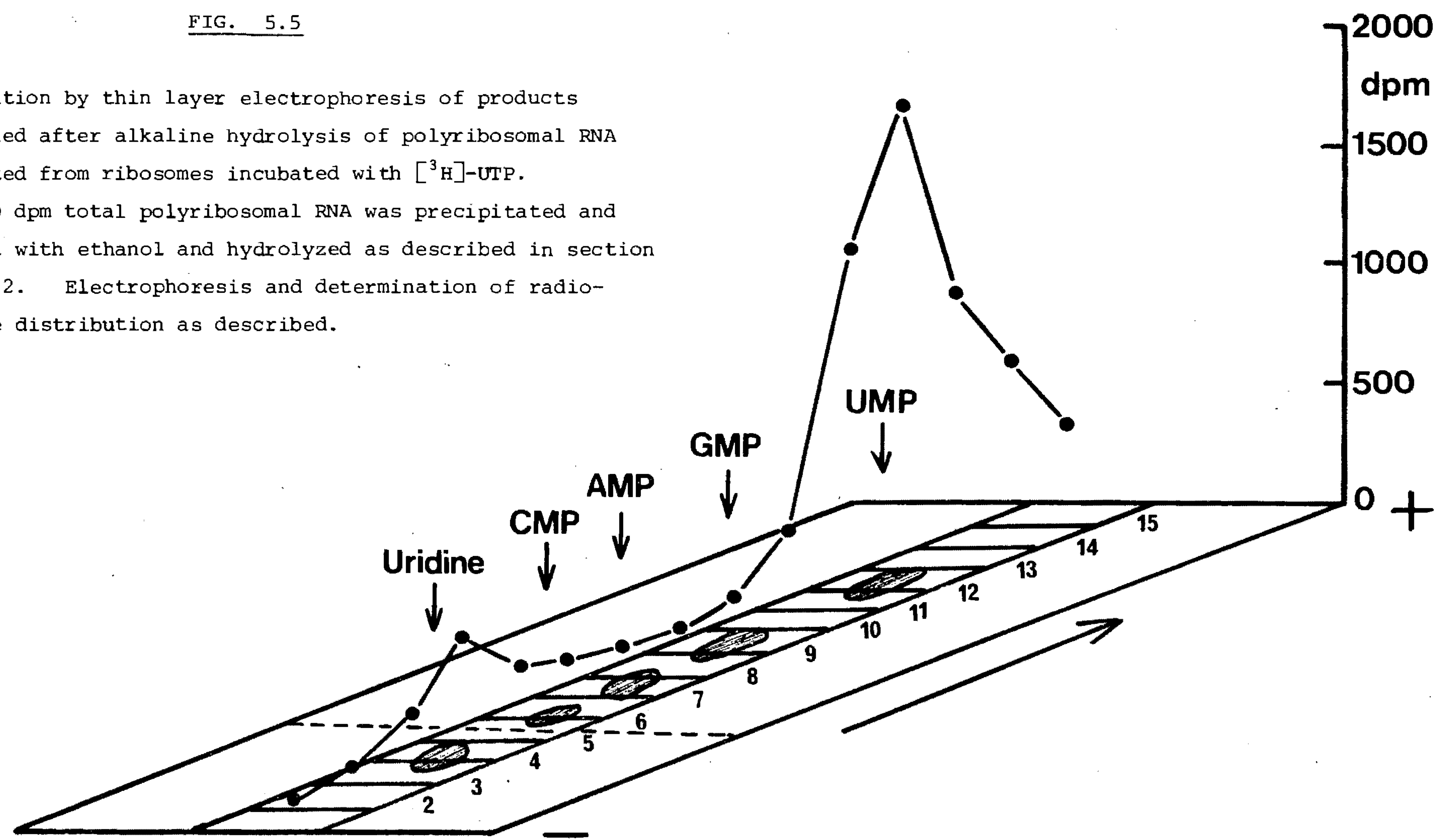


TABLE 5.3

Distribution of radioactivity in ribonucleosides and ribonucleotides obtained after alkaline hydrolysis of polysomal RNA from ribosomes incubated with [^{3}H]-UTP *in vitro*. For details see legend to Fig. 5.5.

Hydrolysis products	% of total radioactivity	nucleotide / nucleoside
UMP	85.90	6.09
Uridine	14.11	
GMP	0	
AMP	0	
CMP	0	

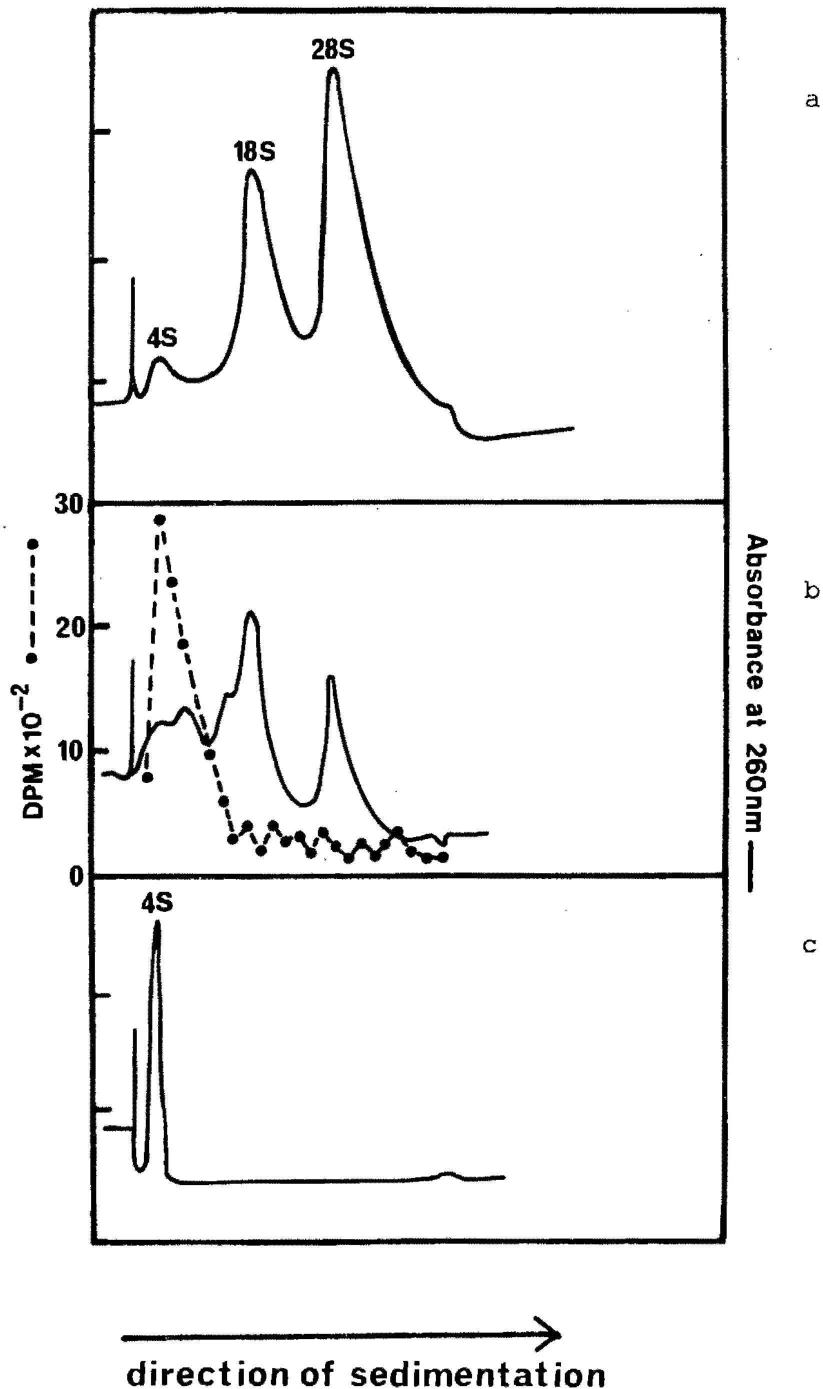
positions of 9S RNA is not known to occur (34). It is unlikely therefore that such contamination would significantly affect the UMP/uridine ratio reported in Table 5.2. The similarity therefore of nucleotide/nucleoside ratios between total polysomal RNA from intact cells and ribosomes incubated in vitro should not be affected.

The absorbance profile of total polysomal RNA from ribosomes incubated in a medium containing [^{3}H]-UTP (see Materials and methods 7.7.2.1) differs from the conventional profile (Figs. 5.1 and 5.2) and deserves mention. A typical absorbance profile of polysomal RNA from incubated ribosomes is shown in Fig. 5.6(b). The proportions of 18 and 28S rRNA have changed; the relative amounts of 9 and 12S RNA have increased markedly. To test the possibility that these changes in relative amounts of polyribosomal RNA might be due to breakdown of RNA by contaminating ribonucleases, a control incubation was prepared in which total polyribosomal RNA, prepared by extraction of unincubated ribosomes with phenol, replaced the ribosome suspension in an otherwise identical incubation medium (polysomal RNA suspended in the same buffer that the ribosomes were suspended in). After incubation at 37°C for 30 min, RNA was re-extracted with phenol and analyzed on a sucrose gradient (Fig. 5.6(a)). No difference was found between this polysomal RNA and unincubated polysomal RNA (Fig. 5.2). The shift in relative proportions of polyribosomal RNA must either be due, therefore, to contaminating ribonucleases present in the ribosome pellet prior to its suspension in buffer or to a ribosome-bound endonuclease (section 3.2.2.4) 'activated' by one of the incubation constituents during incubation, as the incubation at 37°C itself has no effect on the proportions of total polysomal RNA extracted from ribosomes incubated in 0.05 M Tris-HCl pH 7.8 alone.

The unusual proportions of RNA species in total polyribosomal RNA from ribosomes incubated in vitro were never found in polyribosomal RNA from intact cells. It would seem to result therefore from the conditions used in vitro.

FIG. 5.6

Analysis by sucrose gradient centrifugation of polyribosomal RNA incubated in a medium containing [^{3}H]-UTP (a), and polyribosomal RNA from ribosomes incubated in a medium containing [^{3}H]-UTP (b). Polyribosomal RNA and ribosomes were incubated (for details of reaction mixture see Materials and methods 7.7.2.1) at 37°C for 30 min. RNA was extracted directly from the incubation mixture with phenol (7.3.3.1). The final RNA pellets were dissolved in 10 mM Tris-HCl pH 7.8 and applied to 15-30% sucrose gradients in 10 mM Tris-HCl pH 7.8 at 2°C. Centrifugation was for 4 h at 420 000 g at 2°C. Spectrophotometric analysis (and distribution of radioactivity in (b)) was as described (7.2.2.1.2). tRNA was centrifuged as a 4S marker (c).



It is unlikely that this breakdown in polyribosomal RNA from ribosomes incubated *in vitro* would detract from the central conclusions of the experiments described in this chapter viz. the possibility that the radioactively labelled 4S RNA observed in intact cells is the product of the ribosome bound RNA-dependent RNA polymerase activity present *in vitro*.

5.3 CONCLUSION

Ribosome-bound RNA-dependent RNA polymerase has been shown to incorporate [^{3}H]-UTP, in vitro, into terminal and non-terminal positions in polynucleotide material with a sedimentation coefficient of 4S. The ratio of non-terminal (UMP) to terminal (uridine) incorporation is 6.08. Immature erythrocytes incubated with [^{3}H]-uridine in the presence of high concentrations of actinomycin D (80 µg/ml) effect the incorporation into polyribosomal RNA of radioactive material sedimenting in the 4S region of a sucrose gradient. Alkaline digestion revealed that UMP and uridine were the only hydrolysis products that were radioactive. No radioactively labelled CMP was ever detected in polysomal RNA from cells incubated in the presence of actinomycin D. The UMP/uridine ratio was 5.50.

Within the limitations of using actinomycin D as an inhibitor of DNA-dependent RNA synthesis (side effects of this antibiotic have been reported (146,147)) and in the knowledge that no definitive proof has been provided that total suppression of nuclear DNA-dependent RNA synthesis has occurred in immature chicken erythrocytes incubated in the presence of actinomycin D, the close similarity between sedimentation coefficients and nucleotide/nucleoside ratios suggests the possibility that this radioactively labelled 4S RNA species present in polysomal RNA isolated from immature erythrocytes incubated in the presence of actinomycin D might be the product of the ribosome-bound RNA dependent RNA polymerase activity observed *in vitro*.

PART 6

CONCLUSION

A ribosome bound RNA-dependent RNA polymerase was shown to be present in maturing chicken erythrocytes. The change in total and specific activity, concurrent with changes in globin and RNA synthesis during erythrocyte maturation (figures 2.11 and 2.12) implicate the involvement of this enzyme in the synthesis of globin. The possibility that a more specific function might be in the replication of globin mRNA (as proposed by Downey et al. (12)) has been excluded by the following observations :

1. Using unlabelled complementary DNA, no radioactively labelled globin mRNA (or its complement) was detected in the product obtained after incubation of ribosomes with [^{3}H]-UTP (Table 4.4).

2. No template dependent synthesis of RNA could be demonstrated using a purified RNA-dependent RNA polymerase preparation from immature chicken erythrocytes (Table 3.3).

3. The product of the ribosome bound RNA-dependent RNA polymerase reaction was found in the 4S region of a sucrose gradient (Fig. 5.4); no apparent 9S radioactively labelled material was evident.

4. The purified enzyme preparation exhibited no selective preference for globin mRNA containing fractions (Table 3.4).

The evidence from this thesis has in fact shown the ribosome bound RNA-dependent RNA polymerase from immature chicken erythrocytes to be a predominantly primer dependent enzyme activity, incorporating UTP, CTP and to a lesser extent ATP and GTP into polynucleotide material. Due to the different patterns of nucleotide incorporation by the purified and ribosome-bound RNA-dependent RNA polymerase preparations it is unclear whether homo- or heteropolyribonucleotide material (or a mixture of both) is the product(s) synthesized *in vivo*.

Although it was not ascertained to what extent the ribosomal fraction from immature chicken erythrocyte contains membrane-bound ribosomes, it may be possible, by analogy to the work of Wilkie and Smellie (section 1.1.2 and reference (22)), that the homopolymer synthesis by the purified enzyme fraction from ribosomes of immature chicken erythrocytes may represent the activity of the free ribosome fraction; the synthesis of heteropolyribonucleotides may be a characteristic of membrane bound ribosomes (the possible contamination by postribosomal RNA dependent RNA polymerase activities however cannot be excluded). Further studies utilising [α-^{32}P]-labelled ribonucleotides should resolve many of these questions.

Incubating ribosomes with [^{3}H]-UTP, the product was shown to be a short chain polymer terminating in uridine and containing five to six non-terminally situated UMP residues (Table 5.4). As the radioactivity was found in the 4S region of the sucrose gradient, it would seem likely that this short chain polymer was synthesized on the end of a low molecular weight RNA primer (about 80-90 nucleotides in length; molecular weight about 25 000 daltons). The 3' end has always been shown to be the site for primer dependent addition of nucleotides (11,15,21); this will most likely prove to be the end to which addition is effected by the ribosome bound RNA-dependent RNA polymerase from immature chicken erythrocytes.

Assured by the results of chapter 5 which suggest that the product of ribosome bound RNA-dependent RNA polymerase exists in immature erythrocytes, one is faced with the following question : what function could homo- or hetero-oligoribonucleotide material rich in UMP and CMP and attached to the 3' end of a low molecular weight RNA primer, fulfil in the synthesis (or control of synthesis) of proteins?

Heywood and co-workers (148,149,150,151,152) have demonstrated the existence of a low molecular weight RNA in the initiation factor (IF-3) fraction from embryonic chick red muscle which these authors have called translational control RNA (tcRNA). This RNA species was shown to be rich in UMP and CMP and could be considered therefore as a possible

primer-product of a ribosome bound RNA polymerase incorporating UTP and CTP into polynucleotide material. However, the low molecular weight of this RNA (6 500 daltons (148)) argues against such a possibility; in addition, the decreased amount of UMP in polysome bound tcRNA relative to cytoplasmic mRNP-tcRNA favours the presence of a ribosome bound ribonuclease rather than a nucleotidyl transferase in the role of this RNA species in the control of protein synthesis.

An RNA species of molecular weight more comparable with the primer-product of ribosome bound RNA-dependent RNA polymerase is pre-4S RNA (precursor to tRNA) (153,154). This RNA has a mobility between 4S and 5S RNA on acrylamide gels and a high UMP content at the 3' end (154). However, the synthesis of the CUUU sequences at the 3' end of pre-4S RNA seems to have been coded for in the DNA of prokaryotic cells (155).Therefore, although the additional cytoplasmic incorporation of UMP and/or CMP into pre-4S RNA is not impossible, evidence to date does not favour such an idea.

Shine and Dalgarno (156) and Steitz and Jakes (157) have demonstrated a nucleotide sequence complementarity between polypyrimidine rich regions at the 3' end of 16S rRNA from E.coli and polypurine rich regions adjacent to the initiator codon in bacterial mRNAs. These authors have suggested the involvement of these (U,C)-rich regions at the 3' end of 16S rRNA, by base pairing to the complementary regions of mRNA, in the initiation of protein synthesis. Kabat (69), working with globin mRNA from immature rabbit erythrocytes, has shown that such potentiation mechanisms may exist in eukaryotic systems. He suggests that the specific base pairing of translationally inactive 9S globin mRNA with 18S rRNA results in a potentiated messenger which, on interaction with initiation factors, commences the synthesis of protein. It could be suggested that the synthesis of polypyrimidine-rich regions of rRNA are effected by a ribosome bound RNA-dependent RNA polymerase. The absence of any incorporation of [^{3}H]-UTP by ribosome bound RNA-dependent RNA polymerase into the 18S or 28S regions of the sucrose gradient does not support such a proposal (figures 6.2 and 6.4). The addition of UMP and CMP to the 3' end of low molecular weight, 4-5S RNA would imply, in the context of RNA potentiation, that poly-

pyrimidine-rich regions of low molecular weight RNA may play a role in specific RNA-RNA interactions of importance to ribosome function. Evidence suggesting the interaction of sequences in 5S RNA and the GTψC loop of tRNA (158), important to the fixing of incoming tRNA to the ribosome acceptor site, has been demonstrated. However, polypyrimidine rich regions are not implicated in such hybrid formation (the relevant sequence in 5S RNA is thought to be the pentanucleotide UGAAC (158)). Available evidence therefore would not support the proposal that the ribosome bound synthesis of (U,C) heteropolymers is of importance in the known potentiation mechanisms related to the control of protein synthesis.

Other small molecular weight RNA molecules rich in pyrimidines at the 3' end are the small molecular weight nuclear RNAs (SnRNA), thought to exist exclusively in the nucleus (34). As the functions of this class of RNA are completely obscure, no proposal, based on any existing evidence can therefore be put forward, relating SnRNA with cytoplasmic RNA synthesis.

A number of RNA species therefore, rich in pyrimidine at the 3' end, are known to exist. Although to date it is not possible to relate the ribosome bound synthesis of polymers rich in UMP and CMP, with the proposed functions of these polypyrimidine rich regions of cellular RNAs, it is not unlikely that, once more is known about the functions of these (and other) RNA species, the requirement for such ribosome bound RNA-dependent RNA polymerases will be evident.

PART 7

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7.1 MATERIALS

All radioactive materials were obtained from the Radiochemical Centre, Amersham except [^{3}H]-poly(U) which was purchased from Schwarz-Mann. Calf thymus DNA and heparin were purchased from Sigma Chemical Co.; ribonuclease-free sucrose, yeast RNA, M.lysodeikticus DNA, poly(A,G), poly(U,C), poly(A), poly(U), oligo(dT)$_{12-18}$, dithiothreitol, unlabelled ribonucleosides and ribonucleotides, deoxyribonucleoside triphosphates, bovine serum albumin (fraction V), pepsin, horse haemoglobin and nuclease S_1 (A. oryzae; one unit is that amount of enzyme which produces 1 µg acid soluble deoxypentose phosphate in 30 min at 37°C) were obtained from Miles Laboratories Inc. Diethylpyrocarbonate, double distilled glycerol and sodium dodecyl sulphate (SDS) were supplied by Merck (Germany); α-amanitin from Boehringer (Germany); actinomycin D from P.L. Biochemicals; reverse transcriptase (AMV; 1 unit of this RNA-dependent DNA polymerase is defined as the enzymatic activity which incorporates 1 pmole of dTMP into acid precipitable product with poly rA. dT as primer in one min at 37°C) from Schwarz-Mann. CM-52 was purchased from Whatman; PPO scintillator and BBS-3 solubilizer from Beckman Ikstruments; Oligo(dT)-cellulose, DNA-dependent RNA polymerase (M. lysodeikticus) and rabbit haemoglobin mRNA from Searle (High Wycombe, England); Imferon from Fisons (England); Pancreatic ribonuclease from Sigma. Chicken erythrocyte DNA was prepared according to Packman et al. (91) and haemin as described by Labbe and Nishida (81). All chemicals not further described were of Analar (or equivalent) grade.

Chickens (Plymouth Rock/Cornish Game) were obtained from County Fair Foods (Pty) Ltd.; rabbits (New Zealand/Commercial hybrid) from Coney Protein (Pty) Ltd.; Whatman glass fibre filters (GF/C) were supplied by Reeve Angel (London). Different grades of Sephadex and Sephadex ion exchangers were obtained from Pharmacia (Uppsala, Sweden).

Sucrose gradients were made up using a Beckman density gradient former; centrifugation was performed in a Beckman L2-75B ultracentrifuge using SW25.1, SW65LTi or SW40Ti rotors with cellulose nitrate tubes. Sucrose gradients were analysed using an Isco density gradient fractionator (model 640) and UA-5 U.V. absorbance monitor. Other centrifugation work was performed using the Beckman L2-75B or Sorvall RC2-B centrifuges with the appropriate rotors.

Thin layer electrophoresis was conducted using CAMAG HVE equipment and TLC-glass plates (cellulose F), supplied by Merck (Germany).

Polyacrylamide gel electrophoresis was carried out with a Shandon disc electrophoresis apparatus (Shandon Scientific, London) at room temperature and destaining of gels with a Shandon transverse electrophoretic destainer. A Vitatron TLD-100 universal densitometer (Vitatron, Dieren, Holland) was used with a Vitatron UR 400 recorder for densitometric tracings of polyacrylamide gels. Dried polyacrylamide gel slices were oxidized using a Packard (model 306) sample oxidizer.

Where indicated, solutions were stored frozen in an LD-40 liquid nitrogen container (Union Carbide, USA) either above (-100°C) or below (-196°C) the liquid nitrogen.

Counting of radioactive samples was performed in a Beckman model LS-250 liquid scintillation counter to 2% error; dpm were calculated using suitable quench correction curves.

Glass, double-distilled water was used for all solutions.

All operations were carried out at 0-4°C unless otherwise stated.

7.2 ISOLATION AND CHARACTERIZATION OF MATURE AND IMMATURE ERYTHROCYTE CELL POPULATIONS

7.2.1 INDUCTION OF ANAEMIA AND COLLECTION OF BLOOD

8 week old cocks weighing 1.5 - 2.0 Kg were injected, subcutaneously, with neutralized, isotonic, phenylhydrazine hydrochloride (5 mg/Kg body weight) for 4 consecutive days; the phenylhydrazine solution was freshly prepared each day. Concurrent with the last injection of phenylhydrazine, an intramuscular injection of 'Imferon' (equivalent to 25 mg Fe^{++}) was administered. Blood was collected from the jugular vein on the day after the last injection (anaemic blood) in an equal volume of a solution containing 2% (w/v) sodium citrate, 2% (w/v) glucose. Smaller aliquots of blood were obtained from the wing vein with a hypodermic syringe previously rinsed with 0.1% (w/v) heparin as the anticoagulant.

Normal blood was obtained from uninjected animals.

Blood analysis : Cell counts were determined using the improved Neubauer haemocytometer. Haemoglobin concentration was determined by the reaction with potassium hexacyanoferrate (III) and potassium cyanide using human haemoglobin as standard (159). Blood cells were stained with Leishman stain in dried blood films or by vital staining of reticulocytes with brilliant cresyl blue. Stained preparations were examined with a Zeiss RA microscope. The nomenclature and identification of the erythrocyte maturation stages was according to Lucas and Jamroz (46).

7.2.2 MEASUREMENT OF RNA AND PROTEIN SYNTHESIS IN MATURING CHICKEN ERYTHROCYTES

7.2.2.1 RNA SYNTHESIS

7.2.2.1.1 ANALYSIS DURING MATURATION

0.1 ml aliquots of a (3:2) suspension of blood in a solution containing 2% (w/v) sodium citrate, 2% (w/v) glucose were incubated with 1 μC [^{3}H]-uridine (3.33 Ci/mmol) for 1 h at 37°C. 1 ml ice-cold 10% (w/v) trichloro-acetic acid (TCA) containing 1 mg/ml uridine was added and precipitates were allowed to develop by standing the tubes in ice for 30 min. Acid-insoluble material was collected by centrifugation in a bench centrifuge (Roto Uni II) and washed 5 times by repeated suspension in 5 ml ice-cold 5% (w/v) TCA followed by centrifugation. Suspension was facilitated by placing a glass rod in the incubation test tube and shaking by means of a mechanical test tube shaker (Whirlimixer, Fisons). The pellet was finally washed once with 5 ml ice-cold 96% (w/v) ethanol, hydrolyzed (0.5 ml 0.25 M NaOH for 45 min at 100°C), cooled and decolourized (0.1 ml H_2O_2 overnight). 0.1 ml saturated ascorbic acid followed by 0.2 ml 5% (v/v) glacial acetic acid was added to the slightly yellow hydrolysate which was then solubilized (10 ml 0.5 % (w/v) PPO, 10% (v/v) Biosolv BBS-3 in toluene) and counted. Incubations were carried out in duplicate and the values of controls having a zero time of incubation were subtracted from each test value.

7.2.2.1.2 ANALYSIS OF LABELLED RNA SYNTHESIZED IN IMMATURE ERYTHROCYTES

3 ml aliquots of whole blood, obtained from the wing vein of anaemic chickens as described in section 7.2.1.1 were incubated at 37°C for 2 hours in 2.0 ml 2% (w/v) glucose, 2% (w/v) sodium citrate containing

500 μCi $[^3H]$-uridine (44.5 Ci/mmol) in the presence or absence of actinomycin D (80 μg/ml). After incubation, the cells were spun down by centrifugation at 4 500 g for 10 min at $20^{o}C$. The cell pellet was washed once by centrifugation as above in 10 vol. 0.14 M NaCl, 5 mM KCl, 1.5 mM $MgCl_2$. The packed cells were then lyzed as described (7.3.2). Ribosomes were prepared from the clear post-mitochondrial supernatant by centrifugation through 1.0 ml 36% (w/w) sucrose in 30 mM Tris-HCl, pH 7.8, 30 mM KCl, 1.5 mM $MgCl_2$ (420 000 g for 1 h in Beckman SW65LTi rotor) and extracted with phenol as described in section 7.3.3.1. The washed RNA pellet was dried in vacuo, dissolved in 10 mM Tris-HCl pH 7.8, 0.5% (w/v) sodium dodecyl sulphate (SDS), layered on 15-30% sucrose gradients in 10 mM Tris-HCl pH 7.8, 0.5% SDS, and centrifuged at 420 000 g for 3 h at $20^{o}C$ (SW65LTi rotor). The gradients were analyzed spectrophotometrically, 0.2 ml fractions were collected and RNA precipitated with 0.5 ml ice-cold 10% (w/v) TCA; 0.1 ml (2 mg/ml) total yeast RNA was added as carrier. Acid precipitable radioactivity was determined (7.6.5).

7.2.2.1.3 RNA SYNTHESIS IN ERYTHROCYTES SEPARATED ON DISCONTINUOUS BSA GRADIENTS

Blood cells collected from discontinuous BSA gradients, washed and finally suspended in 0.15 M NaCl (see section 7.2.3) were included in a reaction mixture containing : 0.025 M Tris-HCl, pH 7.5, 0.005 M glucose, 0.036 M $FeSO_4$, 1.19 μg/ml Folic acid, 0.119 μg/ml vitamin B_{12}, 0.13 M NaCl, 4.8 mM KCl, 7.4 mM $MgCl_2$, 0.1 mM guanosine, cytidine and adenosine, 0.005 mM $[^3H]$-uridine (200 mCi/mmole) and 0.2 ml cell suspension (2-4 x 10^9 cells/ml) in a final volume of 1.0 ml. The mixture was incubated at $37^{o}C$ for 20 min and then 3 ml ice-cold 10% (w/v) TCA containing 1 mg/ml uridine was added. Determination of acid insoluble radioactivity was as described in 7.2.2.1.1.

7.2.2.2 PROTEIN SYNTHESIS

7.2.2.2.1 ANALYSIS DURING MATURATION

For maturation studies, 0.1 ml aliquots of a blood suspension (7.2.2.1.1) were incubated with 1 μC [^{3}H]-histidine (55 Ci/mmol) for 1 h at 37°C. TCA precipitation and determination of acid-insoluble radioactivity was performed as described previously (7.2.2.1.1).

7.2.2.2.2 ANALYSIS OF LABELLED PROTEIN SYNTHESIZED IN IMMATURE ERYTHROCYTES

3 ml aliquots of whole blood were incubated as described in section 7.2.2.1.2 with 200 μC [^{3}H]-histidine (55 Ci/mmol) as at labelled precursor (in the absence of actinomycin D). Cells were washed and lyzed as described.

a. CMC chromatography of chicken haemoglobin

An aliquot of the post-mitochondrial supernatant (29 $OD_{280\ nm}$) was applied to a 1.3 cm x 11 cm CM-52 column previously equilibrated with 0.01 M phosphate buffer, pH 6.3. Protein was eluted with a 250 ml 0.01 M - 0.20 M linear gradient of phosphate buffer, pH 6.3 (flow rate: 64 ml/h). 80,3 ml fractions were collected; these fractions were analyzed at 280 nm and 415 nm. 0.5 ml aliquots of each fraction were then precipitated with 3 ml 10% (w/v) TCA. The precipitates, after standing for 30 min, were collected on Whatman GF/C glass fibre filters, washed (100 ml 5% (w/v) TCA, followed by 20 ml 96% (w/v) ethanol), dried, placed in 10 ml scintillator (0.5% (w/v) PPO in toluene) and counted.

b. Polyacrylamide gel electrophoresis of chicken haemoglobin

Polyacrylamide gel electrophoresis of chicken haemoglobin was performed on 10% acrylamide gels essentially as described by Smithies (160). The following solutions were prepared :

Bath buffer : 0.09 M Tris-HCl pH 8.3, 1.6 mM Na_2 EDTA, 50 mM boric acid

Gel solutions : A. 10 g acrylamide, 0.2 g N,N'-methylene bisacrylamide, 0.12 ml N,N,N',N'-tetramethyl-ethylenediamine (TEMED) made up to 96 ml with bath buffer.

B. 0.35 g ammonium persulphate in 10 ml bath buffer.

All solutions were stored at $4^{o}C$. The persulphate solution (B) was prepared fresh each week.

Gels were prepared by mixing cold solutions A and B in the volume ratio 24:1. This solution was degassed with a water vacuum pump for 2 min with shaking, poured into 10 cm x 0.5 cm glass tubes sealed at the bottom with Parafilm (Gallenkamp), to a height of 8 cm. Bath buffer was layered on top of the solution. Polymerization of the gel was allowed to occur for 30 min at room temperature.

The gels were pre-electrophoresed in a disc gel electrophoresis apparatus (Shandon) at 4 mA/gel for two hours with bath buffer as the electrophoresis buffer.

The post-mitochondrial supernatant prepared as described in 7.2.2.2.2 was dialyzed against water overnight and then freeze-dried. The dried powder was made up to a concentration of 4 mg/ml in 10% (w/v) sucrose, 10 mM 2-mercaptoethanol. 5μl volumes were applied to the gel surface; electrophoresis was performed at 2 mA/gel for 2 h.

Gels were removed from the tubes by cooling the tubes in ice and then loosening the gels by forcing destaining solution between gel and tube with the aid of a syringe and 20 gauge needle.

Gels were stained in 0.5% (w/v) amido black, 25% (v/v) ethanol, 7% (v/v) glacial acetic acid for 30 min, and destained in a transverse

electrophoretic destainer (Shandon) filled with destaining solution (25% (v/v) ethanol, 7% (v/v) glacial acetic acid). Gels were stored at 4°C in a destaining solution and could be kept for many months without any apparent loss of stain.

Densitometric tracings of gels were obtained with a Vitatron, TLD 100 densitometer using a 0.25 mM diameter diaphragm and 615 nm filter.

7.2.3 FRACTIONATION OF MATURING ERYTHROCYTE POPULATIONS

Buoyant density gradient centrifugation was performed using bovine serum albumin (BSA) as described by Leif and Vinograd (64) with the following modifications :

Stock solutions of BSA were prepared by dissolving BSA (fraction V) in twice distilled water in the ratio 3.5 g BSA to 6.5 g water (35% (w/w)). This solution contained 0.82 g NaCl per 100 ml and was diluted with 0.15 M NaCl to prepare solutions with lower percentage concentrations of BSA.

Discontinuous gradients of BSA were prepared in 14 ml glass centrifuge tubes (Sorvall). Each layer of BSA was 2.0 ml. 2.0 ml of whole blood, removed from the wing vein as described (7.2.1.1), was layered on top of the gradient and tubes were centrifuged at 4 541 g for 1 h (Sorvall HS-4 swinging bucket rotor). Cells were collected from the discontinuous gradient using a Pasteur pipette, washed by suspension in 10 vol. 0.15 M NaCl followed by centrifugation at 10 000 g for 2 min. This procedure was repeated twice. The final pellet was suspended in 2 vol. 0.15 M NaCl.

7.3 ISOLATION OF CHICKEN 9S RNA

7.3.1 CONDITIONS OF ISOLATION

All solutions used during the course of isolation of 9S RNA (from the point of cell lysis) were rendered ribonuclease free by autoclaving

(120°C, 15 min; Almor steam autoclave) or, when sucrose was present, diethylpyrocarbonate (Baycovin) was added to a weighed solution with stirring, to a final concentration of 0.05% (v/v). This sterilizing agent was then removed by boiling the solution for 20-30 minutes (Baycovin has a very characteristic smell; 20-30 minutes of boiling was sufficient to completely remove all trace of this smell; for details of this compound as an RNase inhibitor see (161,162)). The sterile, ribonuclease free solution was then made up to weight with twice distilled autoclaved water. The mouth to the glass container (generally a narrow-necked conical flask) was then sealed with a plug of sterile non-absorbent cotton wool and stored, at room temperature or 4°C, until required (never longer than 12 h).

All glassware was autoclaved, dried and stored (never longer than a week) wrapped in aluminium foil (Alcan) and plugged, where appropriate, with non-absorbent cotton wool.

Sterilized rubber gloves were worn during all stages of the isolation.

7.3.2 ISOLATION OF RIBOSOMES

Blood was collected from the jugular vein of anaemic chickens, prepared as described in section 7.2.1.1, in an equal volume of a solution containing 2% (w/v) sodium citrate, 2% (w/v) glucose, at room temperature. The blood suspension filtered through two layers of cheese cloth and centrifuged at 4 500 g for 10 min at 20°C. The packed cells were suspended in 10 vol. (packed cell volume) 0.14 M NaCl, 5 mM KCl, 1.5 mM $MgCl_2$ and recentrifuged as above. All further procedures were carried out at 0-4°C. The packed cells were lyzed in 5 vol. 5 mM $MgCl_2$ 1 mg/ml bentonite. After 1.0 min of vigorous shaking, 1 vol. 1.5 M sucrose, 0.15 M KCl was added and the lysate centrifuged at 10 000 g for 20 min.

Ribosomes were prepared from the clear post-mitochondrial supernatant by centrifugation through 36% (w/w) sucrose in 30 mM Tris-HCl pH 7.8, 30 mM KCl, 1.5 mM $MgCl_2$ (360 000 g for 1 h in Beckman 60Ti rotor).

The post-ribosomal supernatant and sucrose were decanted; the tubes were placed upside down in the cold room for 10 min to allow liquid adhering to the sides to drain; this was wiped off with tissue paper and the pellets were then suspended in the appropriate buffer using a glass rod and 5 ml Dounce homogenizer with a loose fitting pestle, to a concentration of at least 200 $OD_{260\ nm}$/ml. Yield of ribosomes was about 25-50 mg per 100 ml anaemic blood (yield was evaluated from the relationship 1.0 mg ribosomes/ml ⟶ $A^{1\ cm}_{260\ nm} = 10$).

The buffer used to suspend the ribosome pellets was one of three :

a. 10 mM Tris-HCl, pH 7.8; for SDS treatment of ribosomes.
b. 30 mM Tris-HCl, pH 7.8, 30 mM KCl, 1.5 mM $MgCl_2$; for analysis of ribosomes on sucrose gradients.
c. 0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 1 mM EDTA; for phenol extraction of RNA.

Ribosome suspensions (in any of the 3 buffers) were stored at -100°C until required.

7.3.3 ISOLATION OF 9S RNA BY REPEATED SUCROSE DENSITY GRADIENT CENTRIFUGATION

7.3.3.1 EXTRACTION WITH PHENOL

RNA was extracted with phenol from immature chicken erythrocyte ribosomes, using the method of Aviv and Leder (113). The ribosomes were suspended in 0.1 M Tris-HCl pH 9.0, 0.1 M NaCl, 1 mM EDTA at a concentration of 20 $A_{260\ nm}$ units/ml and then made 1% with respect to SDS. An equal volume of phenol-chloroform-isoamylalcohol (50:50:1) was added (phenol freshly distilled before use), the mixture shaken vigorously for 10 min at room temperature, chilled to 5°C and the phases separated by centrifugation at 12 000 g for 10 min. The clear aqueous phase was removed, extracted again as above, and finally made 2% in potassium acetate (pH 5.5). Crude polysomal RNA was precipitated overnight. RNA was collected by centrifugation at 12 000 g at -20°C for 20 min.

The RNA pellet was washed twice with ethanol, 0.2 M NaCl (2:1) and dried in vacuo. The dry, white powder was dissolved in 10 mM Tris-HCl pH 7.8, 0.5% (w/v) SDS, heated to 65°C for 10 min, cooled rapidly to room temperature, applied to 15-30% sucrose gradients in 10 mM Tris-HCl pH 7.8, 0.5% SDS and centrifuged at 284 000 g for 18 hours at 20°C (Beckman SW40Ti rotor; 150 $A_{260\ nm}$ units/gradient). Fractions of the gradient corresponding to 9-10S RNA were pooled and made 0.1 M with respect to NaCl. RNA was precipitated overnight with 2 vol. ethanol at -20°C. Pure 9S RNA was isolated from this crude preparation by one further centrifugation step, as described above. The final, dried RNA pellet was dissolved in 10 mM Tris-HCl pH 7.8 at a concentration of 1 mg/ml and stored in 0.1 ml aliquots, at -100°C.

7.3.3.1.1 MICROGEL ELECTROPHORESIS OF RNA

Purified chicken 9S RNA was analyzed on gradient micropolyacrylamide gels using the method of Neuhoff (163).

The following solutions were prepared :

A. 0.86 g Tris
   8 ml H_2O
   0.063 ml TEMED
   3.6 N H_2SO_4, to pH 8.8
   H_2O to 10 ml

B. 20 g acrylamide
   0.4 g bis acrylamide
   3.75 mg $K_3Fe(CN)_6$
   H_2O to 37.5 ml

C. 35 mg ammonium persulphate to 50 ml in 2% (v/v)
   Triton X-100 in H_2O

Bath buffer.

3.0 g Tris, 14.4 g glycine to 500 ml in H_2O (pH 8.4)

D. 1. 60% (w/v) sucrose

2. 24 mg ammonium persulphate to 10 ml in 2% (v/v) Triton X-100 in H_2O

3. 0.86 g Tris
8 ml H_2O
3.6 N H_2SO_4, to pH 8.8
H_2O to 10 ml

To prepare solution D, mix 7 parts (1) to 2 parts (2) to 1 part (3).

All stock solutions were stored for prolonged periods at $4^{o}C$ except for solution (C) which was prepared fresh each week.

Sulphuric acid-washed 5 µl capillary pipettes (Drummond Scientific Co.) were used for gradient microgel electrophoresis. Before filling, the middle of the capillary was marked with a felt-tip pen. The capillary was then dipped into solution C, and filled up to this mark by capillary attraction. Immediately afterwards it was held in a stock solution mixture A/B (one part A to 3 parts B) and filled to the tip of the capillary by capillary attraction. The capillaries were then placed vertically in a small beaker containing 2-4 mm of solution D. The gels were allowed to polymerize overnight at room temperature in a moist chamber. The gradient gels were removed from the solution D, un-polymerized solution was removed using a finely drawn out glass tube and replaced by the RNA sample (1 mg/ml; about 4-8 mm of capillary available for sample (0.625 - 1.25 µl)).

Electrophoresis was performed for 60 min starting at 90 µA. Sample migration was towards the anode.

The gels were extruded from the capillary tube using a tight-fitting steel wire and stained in 0.2% (w/v) toluidene blue, 10% (v/v) glacial acetic acid for 15 min. They were then destained in 7.5% (v/v)

glacial acetic acid for 10 min and examined immediately using a Zeiss stereomicroscope.

Gels were stored in staining solution, at $4^{\circ}C$, for several weeks without any apparent loss of stained bands.

7.3.3.2 EXTRACTION WITH SDS

Ribosomes, suspended in 10 mM Tris-HCl pH 7.8 at a concentration of 64 $A_{260\ nm}$ units/ml were made 0.5% with respect to SDS. This suspension was incubated at $37^{\circ}C$ for 10 min, 1.0 ml aliquots were applied to 5-20% (w/v) sucrose gradients in 10 mM Tris-HCl pH 7.8, 0.5% SDS and centrifuged at 90 000 g for 24 hours at $20^{\circ}C$ (Beckman SW25.1 rotor). Fractions of the gradient corresponding to 9-10S RNA were pooled, precipitated with ethanol (as described in section 7.3.3.1) and re-centrifuged as above.

7.3.4 ISOLATION OF 9S RNA BY AFFINITY CHROMATOGRAPHY

7.3.4.1 OLIGO(dT)-CELLULOSE

An aliquot (100 $A_{260\ nm}$ units) of total polysomal RNA, obtained from ribosomes by extraction with phenol as described (7.3.3.1) and dissolved in 3.0 ml 0.01 M Tris-HCl pH 7.8, 0.5 M KCl (application buffer), was mixed with 2.0 ml (0.5 g dry weight) Oligo(dT) cellulose, previously equilibrated with application buffer. The mixture was stirred for 15 minutes at room temperature and then centrifuged for 5 minutes at 5 000 g at $20^{\circ}C$. The supernatant was decanted and the cellulose rewashed with 3.0 ml aliquots of application buffer until no further RNA could be detected in the supernatant wash fraction ($A_{260\ nm} < 0.05$). Material retained by the cellulose was eluted with 0.01 M Tris-HCl pH 7.8, precipitated with ethanol, redissolved in 10 mM Tris-HCl pH 7.8 and analyzed on 15-30% sucrose gradients in 10 mM Tris-HCl pH 7.8 (420 000 g in Beckman SW65LTi rotor for 5 hours at $2^{\circ}C$).

7.3.4.2 UNSUBSTITUTED CELLULOSE

153 $A_{260\ nm}$ units polysomal RNA extracted with phenol (7.3.3.1), in application buffer, was applied, at room temperature to a column of unsubstituted cellulose (7.0 ml bed volume) previously equilibrated with application buffer. Unretained material was eluted with application buffer and fractions collected until no further RNA could be detected in the eluant ($A_{260\ nm} < 0.05$). Retained material was eluted using neutralized water. The water eluted peak was concentrated by ethanol precipitation, redissolved in 10 mM Tris-HCl pH 7.5 and analyzed on sucrose gradients as described (7.3.4.1).

7.4 TRANSLATION OF CHICKEN 9S RNA

7.4.1 KREBS II ASCITES CELL FREE SYSTEM

A cell free system prepared from Krebs ascites mouse cells essentially as described by Jacobs-Lorena and Baglioni (164) was used for the translation of chicken and commercially available rabbit mRNA.

7.4.1.1 GROWTH OF ASCITES CELLS

Krebs II ascites cells were obtained from the Department of Biophysics, Groote Schuur Hospital, Cape Town, and maintained by intraperitoneal injection every 14 days as follows : 10 ml ascitic fluid (rejecting 'bloody' tumours) obtained from 2-5 mice, was centrifuged in graduated centrifuge tubes for 5 min at 2 500 g at room temperature. The cell pellet was washed 3 times by repeated suspension and centrifugation in 10 vol. sterile saline; the final, washed cell pellet was suspended in 2 volumes sterile saline and 1 ml aliquots were injected, intraperitoneally into uninfected albino mice.

7.4.1.2 PREPARATION OF CELL FREE ASCITES EXTRACT

Ascitic fluid from 2-5 mice was collected and centrifuged at 2 500 g for 5 min at 4°C. The packed cells were washed 4 times with 10 vol. 35 mM Tris-HCl pH 7.6, 0.146 M NaCl. To the washed packed cells, 2 vol. of a hypotonic buffer was added (10 mM KCl, 1.5 mM magnesium acetate, 10 mM Tris-HCl pH 7.6) and the cells were allowed to swell for 5 min on ice. They were then lyzed by 20 strokes of a tight fitting Dounce homogenizer. A tenfold concentrated solution was immediately added to the homogenate to obtain the final salt concentration required (30 mM Tris-HCl pH 7.6, 0.125 M KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol). This isotonic homogenate was centrifuged at 31 000 g for 10 min at 4°C.

The supernatant was removed and incubated at 37°C for 40 min after addition of 2 mg/ml creatine phosphate, 0.2 mg/ml creatine kinase, GTP to 0.2 mM and ATP to 1 mM. The incubation was centrifuged for 5 min at 30 000 g (to remove a colourless gel that forms during incubation) and the supernatant was applied to a Sephadex G-25 column (2.5 cm x 21 cm) equilibrated with 30 mM Tris-HCl pH 7.6, 0.125 M KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol. The fractions eluted in the excluded volume were pooled (ascites extract), placed on ice and used for cell-free protein synthesis within 16 h of preparation. Protein concentration was about 4 mg/ml.

7.4.1.3 COMPONENTS OF CELL-FREE SYSTEM

0.12 ml aliquots of the ascites extract were included in 0.2 ml reaction mixtures containing, in addition, 24 mM KCl, 0.06 mM 2-mercaptoethanol, 8 mM Tris-HCl, pH 7.6, 1.0 mM ATP, 0.2 mM GTP, 2.0 mg/ml creatine phosphate, 0.2 mg/ml creatine kinase, 0.5 mM unlabelled amino acids (no histidine), 0.091 µM [^{3}H]-histidine (55 Ci/mmol), RNA sample (25 µg/ml). After incubation at 30°C for 1 h, 0.5 ml 10% (w/v) TCA + histidine (2 mg/ml) was added and the tubes were left to stand on ice for 1 h. The TCA precipitates were heated to 90°C for

10 min, cooled and collected on glass fibre filters. TCA-precipitable radioactivity was determined as described in section 7.2.2.2.2.

7.4.1.4 ANALYSIS OF LABELLED PRODUCTS

After incubation, the reaction was stopped by addition of 20 μg/ml pancreatic ribonuclease and EDTA up to 10 mM; incubation was then continued for 15 min at 37°C. Carrier chicken haemoglobin was added and total globin precipitated in 20 vol. acid-acetone as described in section 7.4.1.4.1. The dried precipitate was dissolved in 0.2 ml 8 M urea, 0.05 M 2-mercaptoethanol and 0.1 ml aliquots were applied to 10% polyacrylamide gels. Electrophoresis and determination of radioactivity were as described (7.4.1.4.1).

7.4.1.4.1 PREPARATION AND ELECTROPHORESIS OF CHICKEN GLOBIN

Chicken or rabbit globin were prepared essentially as described by Anson and Mirsky (165).

Globin from a post-ribosomal supernatant (7.3.2) was precipitated, at -20°C, by the addition of 40 vol. cold (-20°C) acetone containing 10% (v/v) 1 N HCl. The precipitate was centrifuged at 5 000 g for 5 min at -20°C. The pellet was washed in cold acetone, 1% (v/v) 1 N HCl, finally in cold acetone alone and dried in vacuo. The dried pellet was dissolved in 8 M urea, 0.05 M 2-mercaptoethanol and 10 μg (5-100 μl) samples were applied to 10% acrylamide gels. Preparation of gels and electrophoresis essentially as described in section 7.2.2.2.2(b) except that 50 mM Tris-HCl pH 7.5 was used in preparing solutions A and B and the bath buffer was 0.7 M formic acid pH 1.7. Sample migration was towards the cathode. Staining and destaining as previously described (7.2.2.2.2(b)). For determination of radioactive incorporation, the destained gels were sliced into 1.0 mm slices; the slices were dried, oxidized (Packard model 306 sample oxidizer) and counted.

7.4.2 XENOPUS EGG SYSTEM

7.4.2.1 PREPARATION OF SOLUTION TO BE INJECTED

Haemin (10 mg/ml), prepared by the method of Labbe and Nishida (81) was mixed with an aliquot of chicken 9S RNA (350 μg/ml) in the ratio of 1:9 (v/v). To this haemin : 9S RNA mixture, an equal volume of [^{3}H]-histidine (55 Ci/mmol) was added. Individual eggs were injected with approximately 50 nl of this radioactive mixture.

7.4.2.2 PREPARATION AND INJECTION OF EGGS

Eggs, obtained from X. laevis females were irradiated, in Medium 199 (166) approximately 17 cm away from a U.V. light source 130 W General Electric germicidal U.V. lamp, G30T8) for 4 min. 15 eggs were then injected with about 50 nl aliquots of the haemin : 9S RNA : [^{3}H]-histidine mixture. The control batch of 15 eggs were injected with a haemin : saline : [^{3}H]-histidine mixture. Incubation, in 0.5 ml Medium 199/5 eggs, was for 17 h at 20°C.

7.4.2.3 PRODUCT ANALYSIS

7.4.2.3.1 IDENTIFICATION OF LABELLED HAEMOLGOBIN

After incubation, the eggs were transfered to 2.0 ml 0.05 M glycine, 0.052 M Tris-HCl pH 8.9, 0.1% (w/v) histidine and sonicated (Biosonik III; micro tip, 60 seconds at 30% setting). The sonicate was then centrifuged at 7 500 g for 30 min at 4°C. The supernatant was decanted, 7 mg carrier chicken haemoglobin (isolated as in 7.2.2.2.2(b)) was added and applied to a Sephadex G-100 column (1.5 cm x 90 cm) equilibrated in 0.052 M glycine, 0.052 M Tris-HCl pH 8.9. 60 3ml fractions were collected. These fractions were analyzed at 280 nm and 415 nm; 0.5 ml aliquots of each fraction were then removed, solubilized in 10 ml 0.5% PPO, 10% (v/v) BBS-3 in toluene and counted.

7.4.2.3.2 IDENTIFICATION OF LABELLED GLOBIN

The peak of haemoglobin, eluted in the inner volume of the Sephadex G-100 column described above was pooled, dialyzed, freeze-dried and globin prepared by acid-acetone precipitation as described (7.4.1.4.1).

The labelled product was applied to a CMC column (1.3 cm x 16 cm) previously equilibrated with 0.02 M pyridine, 0.2 M Formic acid pH 2.56. Protein was eluted with a 250 ml linear gradient of 0.02 M pyridine, 0.2 M formic acid - 0.2 M pyridine, 2 M formic acid pH 2.56. Fractions were collected and analyzed at 280 nm and for radioactivity as described (7.2.2.2.2).

7.5 SYNTHESIS AND CHARACTERIZATION OF DNA COMPLEMENTARY TO CHICKEN 9S RNA (cDNA)

7.5.1 SYNTHESIS OF cDNA

A DNA copy of chicken 9S RNA was synthesized in a 0.10 ml reaction mixture containing 0.1 M Tris-HCl pH 7.5, 0.04 M KCl, 6 mM magnesium acetate, 2 mM dithiothreitol, 0.2 mM each dATP, dCTP and dGTP, 0.01 mM [^{3}H]-TTP (9.3 Ci/mmol), actinomycin D (20 µg/ml), oligo(dT)$_{12-18}$ (25 µg/ml), chicken 9S RNA (5 µg/ml) and 30 units reverse transcriptase (7.1). After incubation at 37°C for 60 min, EDTA was added to a final concentration of 2 mM. Sonicated _M. lysodeikticus_ DNA (200 µg) was added as carrier and the incubate was placed on a Sephadex G-50 column (1.5 x 30 cm) equilibrated with 0.1 M NaCl. The excluded fraction was collected, precipitated with 2 vol. ethanol, dissolved in 0.9 M NaCl, 0.1 M NaOH 5 mM EDTA and heated at 60°C for 5 min. After neutralization to pH 7 the DNA was precipitated with ethanol, dried in vacuo and finally dissolved in 0.1 M NaCl. The specific activity of the synthesized product was 15.98 x 10^6 dpm/µg and 2 000 - 2 500 dpm were used in each hybridization assay. Unlabelled cDNA was synthesized in parallel in the identical manner except 0.01 mM TTP was used.

7.5.2 RNA, DNA HYBRIDIZATION

RNA and DNA were denatured (4 min, $98^{\circ}C$) in a 30 µl hybridization mixture containing 0.3 M NaCl, 0.05 M Tris-HCl pH 7.5, 0.1% (w/v) SDS, 2 mM EDTA and then annealed at $65^{\circ}C$ for 24 h (using melting point capillary tubing). The incubation was rinsed into 0.8 ml 30 mM sodium acetate, pH 4.5, 0.15 M NaCl, 1 mM $ZnSO_4$. Four 0.2 ml aliquots were taken, two of which were digested for 90 min at $37^{\circ}C$ with 2×10^3 units nuclease S_1. All the samples were precipitated with 0.5 ml 10% TCA; 0.1 ml (2 mg/ml) total yeast RNA was added as carrier. Acid precipitable radioactivity was determined as described in 7.2.2.2.2. All results are expressed as a percentage of total counts resistant to nuclease digestion.

7.6 PURIFICATION OF RIBOSOME BOUND RNA-DEPENDENT RNA POLYMERASE

7.6.1 CONDITIONS OF ISOLATION

During the preparation of ribosomes and subsequent isolation of RNA-dependent RNA polymerase, many of the precautions used for the prevention of RNase activity (7.3.1) could not be employed due to the presence of either 2-mercaptoethanol or dithiothreitol. Autoclaved water was used throughout and sucrose used was ribonuclease free (Miles Laboratories Inc.). No solutions were autoclaved or treated with diethylpyrocarbonate. All other previously mentioned conditions were applicable (7.3.1).

A stock solution of 2 x TM-30 was prepared and stored at $-20^{\circ}C$. TM-30 was prepared from this stock by dilution with double-distilled, cold, autoclaved water and used immediately. A stock solution of 3.0 M ammonium sulphate, adjusted to pH 8.0 with ammonium hydroxide, was used to prepare TM-30 buffers which contained various concentrations of ammonium sulphate. 50 mM Tris-HCl pH 7.8, 1.0 mM dithiothreitol, 1.0 mM EDTA, 0.25 M sucrose, 0.5 M KCl was prepared fresh each time it was required.

The ratio of oxidized to reduced dithiothreitol (DTT) could be checked spectrophotometrically (reduced DTT : molar extinction coefficient,

E(283 nm) = O; oxidized DTT : E(283 nm) = 273.

Protein was measured spectrophotometrically by the method of Lowry et al. (167) using crystalline bovine serum albumin as a standard. For protein determination of RNA-dependent RNA polymerase fractions samples were precipitated with 15% (w/v) TCA. RNA was extracted by heating the precipitate in 5% TCA at $90^{o}C$ for 20 min. The remaining precipitate was collected by centrifugation, dissolved in 1.0 M NaOH and protein was determined.

Ammonium sulphate concentration was determined with Nessler's reagent.

7.6.2 ISOLATION OF RIBOSOMES

Blood was collected from anaemic chickens and the cell suspension was washed as described in section 7.3.2. The washed, packed cells were lyzed in 5 vol. 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol (no bentonite). After 1.0 min of vigorous shaking, 1 vol. 1.5 M sucrose, 0.15 M KCl was added and the lysate centrifuged at 10 000 g for 20 min. Ribosomes were prepared from the post-mitochondrial supernatant by centrifugation as described (7.3.2). The ribosomal pellets were suspended in 50 mM Tris-HCl pH 7.8, 1.0 mM DTT, 1.0 mM EDTA, 0.25 M sucrose, 0.5 M KCl using a glass rod and 5 ml Dounce homogenizer, with a loose fitting pestle to a concentration of 40 $A_{260\ nm}$ units/ml (see footnote following section 7.7.2.2).

7.6.3 EXTRACTION OF RNA-DEPENDENT RNA POLYMERASE ACTIVITY

The ribosome suspension was stirred for 30 min at $0^{o}C$ and then centrifuged at 152 000 g for 60 min at $2^{o}C$. The supernatant was decanted and brought to 60% saturation with ammonium sulphate (see ref. (168) for tables) and left overnight at $0^{o}C$. This ammonium sulphate precipitate was collected by centrifugation at 30 000 g for 15 min and dissolved, with stirring, in 50 mM Tris-HCl pH 7.8, 0.1 mM EDTA, 1.0 mM DTT, 30% (v/v) glycerol, 5.0 mM $MgCl_2$ (TM-30) at a concentration of 40 A_{260} units/ml. 5 ml aliquots of this extract were then dialyzed

against 500 ml TM-30 for 3 h at $4^{O}C$ with one change of buffer. This dialyzed extract, when not required was stored at $-100^{O}C$.

7.6.4 DEAE-SEPHADEX CHROMATOGRAPHY

185 $A_{260\ nm}$ units of dialyzed extract (7.6.3) were applied to a 1.3 cm + 11 cm column of DEAE-Sephadex A-25 previously equilibrated with TM-30 buffer and washed with 20 ml of the same buffer. Protein was eluted with a 60 ml linear gradient of 0 - 1.0 M ammonium sulphate in TM-30. Fractions were collected and analyzed at 280 nm and 260 nm. 0.05 ml aliquots of fractions were analyzed for RNA-dependent RNA polymerase activity as described in section 7.6.5. Fractions corresponding to the peak of activity were pooled and stored in aliquots at $-100^{O}C$.

7.6.5 RNA-DEPENDENT RNA POLYMERASE ASSAY

The standard assay mixture for RNA-dependent RNA polymerase activity contained 80 mM Tris-HCl pH 7.8, 1.6 mM $MnCl_2$, 1.0 mM EDTA, 1.0 mM DTT, 80 mM $(NH_4)_2SO_4$, 0.16 mM each ATP, GTP, CTP, 8 μM [^{3}H]-UTP (250 μCi/μmol), 30 μg 18S RNA (prepared as described in section 7.6.6), enzyme fraction, in a final volume of 0.25 ml. After incubation for 30 min at $37^{O}C$, the reaction was stopped with 2 ml cold 10% (w/v) TCA (containing 1 mg/ml carrier UTP); 0.1 ml total yeast RNA (2 mg/ml) was added as a carrier. Acid precipitable radioactivity was collected on Whatman GF/C glass fibre filters, washed (100 ml 5% TCA, followed by 20 ml 96% (w/v) ethanol), hydrolyzed (0.7 ml 0.25 M NaOH for 45 min at $98^{O}C$), buffered (0.2 ml 5% (v/v) glacial acetic acid) solubilized in scintillator (0.5% PPO, 10% Biosolv BBS-3 in toluene) and counted.

1 unit of RNA-dependent RNA polymerase activity is defined as that amount of enzyme which incorporates 1 pmole of UMP under the assay conditions mentioned above.

Incubations were always carried out in duplicate and values of controls having a zero time of incubation were subtracted from each test value.

7.6.6 PREPARATION OF RNA PRIMERS

Chicken 9S RNA used in the primer dependency studies of RNA-dependent RNA polymerase was prepared as described (7.3). Crude 5S RNA, 12S RNA, 18S RNA and 28S RNA were isolated by pooling the relevant fractions obtained after analysis and fractionation of sucrose gradients to which total polysomal RNA had been applied (7.3.3.1). These RNA fractions, after ethanol precipitation and suspension in 10 mM Tris-HCl pH 7.8, 0.5% (w/v) SDS were purified by one further centrifugation step, as described (7.3.3.1). Each RNA fraction was homogeneous as judged by sucrose gradient centrifugation.

7.6.7 SDS-POLYACRYLAMIDE ELECTROPHORESIS OF RNA-DEPENDENT RNA POLYMERASE-CONTAINING FRACTIONS

SDS-polyacrylamide gel electrophoresis of chicken RNA-dependent RNA polymerase was performed on 5% acrylamide gels. The following solutions were prepared.

Bath buffer : 0.1% (w/v) SDS, 0.1 M sodium phosphate buffer, pH 7.8.

Gel solution: A. 5 g acrylamide, 0.25 g bis-acrylamide, 0.12 ml TEMED made up to 96 ml with bath buffer.

B. 0.35 g ammonium persulphate in 10 ml bath buffer.

All solutions were stored at room temperature to prevent precipitation of SDS. Solution B was prepared fresh each week.

Gels were prepared and pre-electrophoresed as described (7.2.2.2.2b).

Sodium phosphate buffer pH 7.8, SDS and 2-mercaptoethanol were added to an aliquot of an RNA-dependent RNA polymerase sample (fractions eluted off DEAE-Sephadex, 7.6.4) to a final concentration of 0.005 M, 1% (w/v) and 0.05 M respectively. 0.04 ml aliquots (30 - 40 μg protein) were applied to gels; electrophoresis was performed at 10 mA/gel for 1 h. Staining and destaining were as described (7.2.2.2.2b) except that gels were not placed on ice prior to extrusion from glass tubes.

Pepsin and horse haemoglobin were run as molecular weight standards.

Polyacrylamide gels of RNA-dependent RNA polymerase in the absence of SDS were prepared and electrophoresis was conducted exactly as described above except no SDS was present. Sample migration, in the presence of SDS, was towards the anode; in the absence of SDS, in separate chambers, sample migration was towards the anode and cathode.

7.7 PREPARATION AND ANALYSIS OF POLYSOMAL RNA ([^{3}H]-POLYSOMAL RNA)

7.7.1 PREPARATION AND ANALYSIS OF [^{3}H]-POLYSOMAL RNA USING cDNA

0.58 mg chicken ribosomes, prepared as described in section 7.6.2 were incubated in a standard RNA-dependent RNA polymerase assay mixture (7.6.5) except that the specific activity of the [^{3}H]-UTP was increased to 49 Ci/mmol and no 18S RNA was added. Incubation was for 2 h at 37°C. Total RNA, which included polysomal RNA and the incorporated, TCA precipitable radioactivity was prepared by extraction with phenol as described in section 7.3.3.1. The dried RNA pellet was dissolved in 10 mM Tris-HCl pH 7.8, 0.5% (w/v) SDS. The specific activity of this RNA preparation was 5.16 x 10^4 dpm/µg total polysomal RNA and 1 600 dpm were used in each hybridization assay. The procedure for incubation of [^{3}H]-polysomal RNA with unlabelled cDNA was as described in section 7.5.2.

7.7.2 ANALYSIS OF [^{3}H]-POLYSOMAL RNA BY THIN LAYER ELECTROPHORESIS AND SUCROSE GRADIENT CENTRIFUGATION

7.7.2.1 SUCROSE GRADIENT CENTRIFUGATION

Ribosomes were incubated in a standard RNA-dependent RNA polymerase assay mixture (7.6.5) except that no 18S RNA was added. RNA was extracted from ribosome pellets as described above (7.7.1). The dried RNA pellet,

dissolved in 10 mM Tris-HCl pH 7.8, 0.5% (w/v) SDS was layered on 15-30% sucrose gradients and centrifuged and analyzed as described (7.2.2.1.2).

7.7.2.2 THIN LAYER ELECTROPHORESIS

RNA samples, in 10 mM Tris-HCl pH 7.8, 0.5% SDS were precipitated with 2 vol. ethanol. The precipitates were washed 5 times with ethanol : 0.2 M NaCl (2:1), dried in vacuo, dissolved in 0.05 M KOH and hydrolyzed, in sealed capillary tubes, at 97°C for 40 min. The hydrolysate was neutralized with HCl and spotted, with the appropriate standards, onto 20 cm x 20 cm TLC-cellulose plates. Electrophoresis in pyridine : acetic acid : water (1:10:292) pH 3.6 was performed for 1 hour at 2 000 V (7 mA). Sample migration was towards the cathode. The plates were dried and analyzed under U.V. (Fluotest U.V. lamp, set at 254 nm); for determination of radioactivity, the appropriate sections of the plate were divided into 1 cm x 2 cm rectangles; the cellulose was scraped off and collected in a scintillation vial. These scrapings were suspended in 0.75 ml, 0.25 NaOH which was then buffered by the addition of 0.2 ml 5% (v/v) glacial acetic acid. This buffered suspension was then solubilized in scintillator and counted (7.2.2.2.2).

Footnote

Ribosomes isolated from immature erythrocytes that had been washed at 0-4°C and exposed to a hypotonic medium for 30 min (82) were used as starting material for many enzyme preparations. Whilst giving identical results in all aspects of RNA-dependent RNA polymerase activity, the method described in section 7.6.2 was adopted in later studies as it reduced leucocyte lysis and improved polyribosome content.

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DEFINITIONS

1. RNA synthesis

Throughout this thesis I have referred, for the sake of simplicity, to all cytoplasmic activities incorporating ribonucleotides into TCA-insoluble material as RNA polymerases (E.C. 2.7.7), although many of them should be more strictly referred to as terminal transferases. In addition, as has been shown, some cytoplasmic RNA biosynthetic systems will incorporate only a limited number of nucleotide residues to the end of existing polynucleotides; while these additions cannot be regarded as true biosynthesis of RNA, they are nevertheless related reactions and will be referred to as RNA synthesis.

1.1 Primer-dependent

Primer-dependent RNA synthesis is defined as the addition of ribonucleotides to the end of an existing RNA molecule (the primer) by a particular enzyme.

1.2 Template-dependent

Template-dependent RNA synthesis is defined as the incorporation, by an enzyme preparation, of ribonucleotides into a polynucleotide product with a nucleotide sequence complementary to an existing RNA molecule (template).

1.3 Homopolyribonucleotide

Poly- or oligoribonucleotide material composed of only one ribonucleotide. In the context of primer-dependent synthesis, homopolyribonucleotide refers to the synthesized product only.

1.4 Heteropolyribonucleotide

Poly- or oligoribonucleotide material composed of more than one ribonucleotide. Once again, in the context of primer-dependent synthesis the term refers only to the synthesized product.

1.5 Incorporation

Ribonucleoside 5'-triphosphates are referred to in this thesis as being incorporated into polynucleotide material by an enzyme preparation; polymers of ribonucleoside 5'-monophosphates are the products of this synthesis.

2. Ribosome

The ribosome fraction from immature chicken erythrocytes includes single ribosomes or monosomes and polyribosomes. Total RNA, extracted with phenol, will therefore include ribosomal RNA and various mRNAs, collectively referred to as polysomal RNA.

2.1 Ribosome-bound

The RNA-dependent RNA polymerase preparation found in the ribosome fraction of immature chicken erythrocytes is referred to as a bound enzyme as it sediments with ribosomes during centrifugation of the post-mitochondrial supernatant. It has not been conclusively shown however that the activity is indeed bound to ribosomes and not derived from a non-ribosome fraction (a 70S non-ribosome, ribonucleoprotein particle containing the enzyme activity, for example).

3. Erythroblast

Scherrer et al. (67,58) and Zentgraf and co-workers (48) use the term erythroblast to refer to immature erythrocytes. Erythroblast, in this thesis, and consistent with Lucas and Jamroz (46) refers to those cells in the earliest stages of erythrocyte maturation that can be found in the circulating blood.

4. 9S RNA and globin mRNA

The 9S RNA fraction, isolated from immature chicken erythrocyte ribosomes, contains globin mRNA in addition to other minor 9S RNA species.

5. TM-30

0.05 M Tris-HCl, pH 7.9, 30% (v/v) glycerol, 0.1 mM EDTA, 1.0 mM dithiothreitol, 5.0 mM $MgCl_2$.

6. Standard deviation (S.D.)

All $\pm$ figures refer to standard deviation and were calculated using the relationship : $S.D. = [\Sigma(\bar{x} - x)^2 / n-1]^{\frac{1}{2}}$

7. Calibration of sucrose gradients

All S-values were determined from calibration curves established using the relationship $d = St\omega^2$ where d = distance travelled by RNA in sucrose gradient; S = sedimentation coefficient; t = time of centrifugation; ω^2 = square of the angular velocity of rotation of the rotor (145). A standard preparation of total polysomal RNA, isolated from rat liver as described by Kirby (61) was used (S-values determined by centrifugation in Beckman model E analytical ultracentrifuge) for each calibration curve (plots of d vs. S x t). A calibration curve was required for each type of sucrose gradient used; where no calibration curve was available, the computer program for determination of approximate S-values, described by Dingman (171), was used.