THE REGULATION OF PROTEIN BIOSYNTHESIS DURING

PLAICE (PLEURONECTUS PLATESSA) EMBRYOGENESIS

A Thesis submitted by

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ABSTRACT

· The regulation of protein biosynthesis at fertilisation was studied in North Sea Plaice (Pleuronectus platessa). RNA and protein synthesis were studied in fertilised and unfertilised plaice eggs. There was no detectable RNA synthesis in the egg in early embryonic stages, but protein synthesis at all stages of development. However, using RNA synthesis inhibitors (actinomycin D and ethidium bromide), it was demonstrated that certain groups of proteins were synthesised independently of any newly synthesised messenger RNA. Different rates of protein synthesis in various subcellular fractions from both fertilised and unfertilised eggs were demonstrated. Proteins precipitated by vinblastine were shown to exhibit the most changes between the egg and fertilised egg. These proteins were further characterized on several polyacrylamide-gel electrophoresis systems.

RNA was isolated from the polysomes and the supernatant fractions of eggs and fertilised eggs. This RNA was tested for messenger RNA activity in a rabbit reticulocyte cell-free system and also in a wheat germ in vitro system. Messenger RNA activity was demonstrated in both the polysomal and supernatant fractions from the egg and also from the fertilised egg, and was purified by affinity chromatography and sucrose gradient centrifugation.

Regulatory mechanisms have been demonstrated at the translational level of protein synthesis in early plaice embryogenesis.

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A B B R E V I A T I O N S

A.B.M.F. Sea Water	Anti-biotic treated, millipore filtered sea water.
cpm	Counts per minute
EDTA	Ethylenediamimetetra-acetate
m.w.	Molecular Weight
m-RNA	Messenger RNA
HnRNA	Heterogenous RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
Ribosomal DNA	Ribosomal RNA sequences on DNA
RNP	Ribonucleoprotein
S	Svedberg unit
SDS	Sodium dodecyl sulphate
TCA	Tricholoroacetic acid
Tris	Tris (hydroxymethyl) methylamine

I N T R O D U C T I O N

Embryogenesis, the orderly development of an organism from a comparatively simple fertilised egg to a complex multicellular organism, has been studied in a wide variety of different animals. Embryos of phylogenetically distant animals exhibit remarkably similar developmental characteristics. Morphological development of the fertilised egg is characterised by transformation of this single cell into many smaller cells, usually with very short intermitotic intervals. In most cases this process of cleavage results in the formation of a single-layered hollow ball of cells, the blastula. This is followed by extensive differentiation (i.e. gastrulation), and eventually leads to the formation of primitive organs and organ systems. Biochemical studies have shown that these ordered morphological changes are accompanied by the synthesis of specific proteins. This implies precisely timed sequential changes in gene expression during embryogenesis.

It is well known that DNA molecules are not the direct templates for protein synthesis. The genetic information of DNA is first transferred to messenger RNA (m-RNA) molecules (i.e. transcription), which, in turn, act as the primary templates that order amino acid sequences into proteins (i.e. translation). In addition, the initial m-RNA transcripts undergo considerable modification (post-transcriptional processing) before translation occurs (la). Therefore studies which attempt to unravel the regulatory mechanisms controlling the synthesis of specific proteins are generally concentrated on the molecular events related to transcription, post-transcriptional processing and translation.

In the past two decades, the fertilised egg and its subsequent development (particularly in sea urchin and frog) has been studied extensively as a possible model system for understanding the mechanisms involved in regulating gene expression in eukaryotic systems. This subject has been reported in several excellent reviews (1 - 5). Of particular interest is the important role played by maternal m-RNAs in directing early development. That the proteins synthesised immediately after fertilisation are independent of any newly synthesised RNA strongly suggests the involvement of m-RNA already present in the unfertilised egg. Recent developments in techniques for the isolation and identification of specific m-RNAs have helped provide evidence for the regulatory functions of maternal m-RNA.

1.   **RNA and protein synthesis during development.**

Macromolecular synthesis is routinely estimated by measuring the incorporation of radioactively-labelled precursors into the macromolecule under consideration. Experiments with intact cells, however, are difficult to interpret in precise quantitative terms since the specific activity of the precursor at the actual site of synthesis is extremely difficult to determine. This is due to the permeability barrier presented by the cell membrane as well as the various precursor pools present within the cell. Developing systems provide further problems in that both the permeability properties of the cell membrane and the relationship between the different pools may be undergoing change.

Most of the results on the levels of RNA and protein synthesis in maturing oocytes and developing embryos have been obtained using this technique and are therefore subject to criticisms, already outlined. Indeed in sea urchin and amphibian, the mature egg is virtually impermeable to all RNA and protein precursors. This problem has been circumvented by injecting the gravid females with appropriate labelled precursors before the eggs have developed this permeability barrier, by micro-injecting label into the isolated eggs directly, and by incubating the egg with Na_2 $^{14}CO_3$ dissolved in solution (1). However, once fertilised, the eggs quickly regain their ability for nucleotide and amino acid uptake.

1.1 · **RNA synthesis before fertilisation**

RNA is actively synthesised by immature amphibian and sea urchin eggs. Synthesis decreases during maturation and is virtually absent in the mature egg (1). Most of the RNA synthesised is ribosomal and is conserved throughout oogenesis into early embryogenesis (1, 6, 7). In amphibian oocytes, ribosomal RNA synthesis occurs predominantly in the several hundred extra chromosomal nucleoli, and to a lesser extent in the chromosomal apparatus (8, 9, 10). Nucleolar DNA has been isolated and shown to have similar physical and chemical properties to ribosomal DNA. Direct evidence that the nucleoli contain DNA complementary to ribosomal RNA has been obtained by RNA-DNA hybridisation experiments (1). Differential multiplication of the ribosomal RNA cistrons occurs early in oogenesis at the time of nucleolar formation (11, 12). Gene amplification is known to occur in other systems (1, 8, 13, 14) and may also account for the high levels of ribosomal RNA synthesis in the sea urchin egg (3). The marine worm, Urechis capo, differs from amphibian and sea urchin in that all stages of oogenesis and embryogenesis are permeable to RNA precursors and synthesise ribosomal RNA (21, 22).

DNA-RNA hybridisation studies have shown that smaller amounts of the non-ribosomal RNAs are also synthesised in the amphibian oocyte. Under experimental conditions that favour association with repetitive DNA, saturating amounts of P^{32} labelled oocyte RNA hybridise with 1.6% of the total somatic DNA. Therefore, about 3% of the DNA is being transcribed at this time (ribosomal DNA represents only 0.1% of the total DNA). Many of these types of RNA (about 65%) are still present in the mature egg, since RNA isolated from the mature egg greatly reduced the formation of P^{32} labelled oocyte RNA-DNA hybrids (15, 16). Hybridisation studies with purified, labelled, non repetitive DNA and mature egg RNA support the conclusion that m-RNA is present in the mature egg and is transcribed from about 1.2% of the DNAs. As an approximation, this amount of DNA has the coding potential for about 30,000 different proteins. Competition-hybridisation experiments with RNA isolated from various embryonic stages have shown that RNA sequences present in the mature egg are also present in early blastular and, to a lesser extent, gastrular stages (17). Similar experiments with 9s m-RNA isolated from polysomes of sea urchin embryos (containing histone m-RNA) indicate that similar RNA sequences are present in the mature egg (18, 19). There is now good evidence that the mature sea urchin egg contains histone m-RNA. RNA isolated from a post mitochondrial, 20s RNP particle has been shown to direct the synthesis of histones in a mouse ascites cell-free system. The finding that RNA from other RNP particles directed the synthesis of numerous proteins suggests that other m-RNAs are also present (20). Therefore, m-RNA synthesised earlier in oogenesis appears to be present in the mature egg prior to fertilisation.

As stated earlier, there is virtually no RNA synthesised in the mature egg (1, 3, 7). Xenopus differs from the sea urchin in that the mature egg undergoes ovulation (i.e. the hormonal stimulation of the egg and its subsequent release prior to fertilisation). Low levels of RNA synthesis have been reported in ovulated eggs (2, 23). However, these results may be explained by the presence of contaminating immature eggs, an unstable RNA fraction or end sequence turnover (1, 2, 3).

1.2   RNA synthesis after fertilisation

After fertilisation, the first class of RNA to be synthesised is the low molecular weight (5-25s) RNA, followed later by the synthesis of larger molecular weight RNA (up to 50s). The pattern of RNA synthesis during early embryonic development is similar in amphibians (1, 2),

teleosts (5,24), echinoderms (3), marine worms (25) insects (26)
acidians (27), snails (28), coot clams (29, 30) and ascaries (31, 32).
At gastrulation, with the decrease in rapid cell division and the
beginning of differentiation, ribosomal and transfer RNA become the
principal RNAs synthesised.

In sea urchins, the first detectable RNA synthesis occurs
near the first cleavage (43), with a significant increase about the
eight cell stage (44). This RNA can be divided into two broad peaks,
21s and 10s (45), some of which becomes associated with the polysomes
immediately (46). Therefore, it is assumed that there is no necessity
for simultaneously synthesised proteins to serve in the transport of
at least this portion of RNA. The function of this newly synthesised
RNA may be in the synthesis of nuclear proteins required for rapid cell
division during cleavage (48, 49, 50). The RNA in the 10s peak has been
characterised as at least several species (45) which are synthesised on
reiterated nuclear genes (51) and therefore tentatively identified as
histone m-RNAs. The precise function of the remainder of this HnRNA must
be further clarified. Studies indicate that a significant amount of this
RNA hybridises with mitochondrialDNA (52). Therefore, the newly
synthesised m-RNA in sea urchin embryos makes a very small or negligible
contribution to protein synthesis during the first cleavage. Their con-
tribution may be considered significant only by the 16 cell stage. However
by the 250 cell stage about one third of the proteins synthesised are
dependant on m-RNAs synthesised in the embryo (49).

Therefore, there are two main classes of m-RNA that code for
proteins in early development; "egg" and "embryo". However, the role of
the newly synthesised RNA is not apparent until the cells begin to differ-
entiate and specific proteins for differentiation are required (i.e.structural
proteins, enzymes, etc.) The m-RNA stored in the egg as a ribonucleo-
protein plays a major role in the increase in the rate of protein
synthesis initially after fertilisation. These preformed messengers
provide an interesting system because their expression is regulated at
the translational level. Recently Slater and co-workers (54-57) detected
poly A sequences on the m-RNAs in fertilised sea urchin eggs. By the two
cell stage there was a doubling of m-RNA with poly A sequences. Similar
results have been found with Xenopus (64) and the marine worm, Urechis capo
(65). At first, it was thought that these poly A sequences play a role in
the regulation of m-RNA (58, 59), but experiments by Humphreys and Mescher
(60) showed that the formation of poly A after fertilisation was not
necessary for the activation of maternal m-RNA. More recent studies seem
to show that the poly A sequence functions to stabilise m-RNA and is not
a prerequisite for translation (61, 62, 63).

1.2.1 Mitochondrial RNA synthesis.

The size of a fertilised egg of echinoderms, fish or amphibians, is very large in relation to the size of the cells in the final individual. This zygote contains all the essentials to carry it through the initial stages of embryogenesis. In the eggs studied, the majority of the DNA present was found in the mitochondria, e.g. in sea urchins 80% of the DNA is mitochondrial (33) and in amphibians the percentage is also very high (34). Selvig and co-workers found that in sea urchin the RNA synthesised immediately after fertilisation was not inhibited by actinomycin D (35). Actinomycin D is known to inhibit RNA synthesis by binding to the DNA and preventing transcription by RNA polymerase. At low concentrations, DNA polymerase activity is unaffected (39). This unexpected result with sea urchin could be explained by the fact that transcription was actually occurring in the mitochondria, which may be impermeable to actinomycin D. Chamberlin also suggested that the increase in RNA synthesis at fertilisation in sea urchin embryos may be due to mitochondrial synthesis because it was found that RNA precursors were being incorporated into the mitochondrial RNA (36, 37). Craig confirmed this by showing that the newly synthesised RNA hybridised with mitochondrial DNA. The RNA synthesised in the mitochondria appeared to have predominantly a molecular weight range of 15 - 17s (38). Further, Craig and Piatigasky showed that when enucleated sea urchin fertilised eggs were treated with ethidium bromide, a compound which severely curtails mitochondrial and cytoplasmic RNA synthesis, no RNA synthesis occurred. Low concentration of ethidium bromide may selectively inhibit mitochondrial RNA synthesis by altering the tertiary structure of the mitochondrial circular DNA molecules (39). The addition of both actinomycin D and ethidium bromide also blocked RNA synthesis (39). Amphibians and loach, a fresh water fish, also contain large amounts of mitochondrial DNA, but it appears that here mitochondrial RNA synthesis does not begin until gastrulation (40, 41, 42).

1.3 RNA synthesis during blastulation and gastrulation

There is a sharp increase in nuclear RNA synthesis in sea urchin embryos at late blastulation. This consists mostly of rapidly decaying RNA but m-RNA synthesis increases at this time. At gastrulation nuclear and m-RNA synthesis decreases and ribosomal and transfer RNA become the major RNA classes synthesised. Ribosomal RNA synthesis increases for a time, but then remains constant through the later stages of development (73, 79-82). Sconzo et al (83) claimed that the beginning of ribosomal RNA synthesis is shortly after fertilisation, but this is not thought to be due to end

labelling of CCA sequences on transfer RNA (84, 85, 86)

In amphibians, low molecular weight nuclear RNA and transfer RNA synthesis begins in blastulation (23, 53). There have been reports of labelling RNA at earlier stages of development but this appears to be due to end labelling of transfer RNA also and does not actually represent true RNA synthesis (75). Bachvarova and Davidson have shown that in Xenopus there is a sharp increase in nuclear RNA synthesis just before gastrulation in the presumptive ectoderm and mesoderm cells and the same process occurs somewhat later in mid gastrula in presumptive ectoderm (76, 77). Thus it appears that the commencement of differentiation in Xenopus is accompanied by a corresponding activation of the embryonic genome. This increase in ribosomal RNA synthesis is also associated with the appearance of the nucleoli at gastrulation in both sea urchins and amphibians (87, 88, 89). Several studies of RNA synthesis in the mero-blastic embryos of loach, Misgurnus fossilis, have been carried out by Soviet researchers. Transfer and m-RNA synthesis was demonstrated in the blastula stage (5, 24). In addition, nucleoli appeared during this period (78, 90). Therefore, it appears that the pattern of RNA synthesis in teleosts may be similar to other organisms studied.

1.4   RNA synthesis in mammalian embryos.

This group is discussed separately because the pattern of nucleic acid metabolism differs from other systems. Mammalian embryos exhibit a number of different characteristics which may be related to their dependence on the maternal organism. Cleavage in most of these organisms is relatively slow. Of all the mammalian organisms, the mouse has been the most frequently studied. Mouse embryos are permeable to radioactive precursors and RNA synthesis can be detected almost immediately after fertilisation (91, 92). Ribosomal and transfer RNA synthesis can be detected as early as the fourth cleavage (93, 94). Nuclear RNA is synthesised at a very low rate until blastulation, whereupon a sharp increase occurs (95). Rabbits and Syrian hamsters have also been found to have similar patterns of RNA synthesis (99, 100). Thus, it appears that mammalian embryogenesis has a far greater control over RNA metabolism and very little dependence on the information from the stored egg.

1.5   Protein synthesis at fertilisation.

In sea urchin there is a sharp increase in the rate of
protein synthesis as measured by the uptake of labelled amino acids
into protein immediately after fertilisation (101). This is also
confirmed by the conversion of monosomes ‑to polysomes (46). This
increase in protein synthesis has been demonstrated to be independent
of RNA synthesis, since protein synthesis continued in the presence
of actinomycin D (102) or after physically removing the nucleus (103).
The precise quantitative nature of the increase in the rate of protein
synthesis has been disputed by many authors because of the known
increased permeability of these cells to exogenous labelled amino
acids after fertilisation. If protein synthesis is inhibited, cleavage
ceases (47, 115); this may imply that the inhibited proteins are
necessary for DNA synthesis (116).

A similar increase in protein synthesis at fertilisation has
been found in most other invertebrates studied, such as surf clam,
Spisula solidissima (104); and the marine worm, Urechis capo (25).

It appears that in Xenopus ovulation results in the increase
in protein synthesis while fertilisation itself does not (105).
However, analysis of the proteins on polyacrylamide gel electrophoresis
suggests that there may be qualitative changes in the proteins synthesised
immediately before and after fertilisation. Therefore it appears that at
least two control mechanisms are operating in the Xenopus system; that
of hormonal control and the other related to fertilisation (106, 107).
It would appear that the activation of protein synthesis at this time
is hormonal due to the fact that it begins after ovulation (1), and
can be considered the developmental equivalent to the activation of
protein synthesis at fertilisation in sea urchins. Xenopus and sea
urchins are therefore similar during the early stages of embryo genesis
in their lack of a requirement for direct genomic control over protein
synthesis.

In the star fish, Paterea runcata, unlike the sea urchin, the
mature eggs undergo ovulation and the pattern of protein synthesis is
similar to Xenopus rather than sea urchins (97).

Fertilisation of the mammalian embryos does not result in any
changes in the rate of protein synthesis (90, 91). However, they differ
from the other embryos described in that when RNA synthesis is blocked both
protein synthesis and cell division cease (96, 97, 98).

1.6   Protein synthesis dependent on maternal m-RNA.

As described for sea urchin in newly fertilised egg, protein
synthesis occurs even in the absence of concommitant RNA synthesis.
Many radioactively labelled proteins have been identified on polyacrylamide
gel electrophoresis (108-113) or by column chromatography (114). The
absence of protein synthesis in the mature egg prior to fertilisation
suggests that m-RNAs for many different proteins are, at least
temporarily, not being translated and that fertilisation is required for
their expression. This is further confirmed by Davidson and Hough's
hybridisation studies (16) described earlier which suggest that the
egg contains enough m-RNA for thousands of different proteins. The
precise amount of RNA that is translated, however, is not known since
the majority of these proteins have not been identified. To date, the
only specific m-RNA which has been identified in the egg and shown stored
as a ribonucleoprotein is the messenger for histone (20). There is also
some indirect evidence for other proteins with stored m-RNA in the egg.

That microtubulin-like proteins are synthesised in newly
fertilised eggs in the presence of actinomycin D also suggests that
microtubulin m-RNA are stored in the egg (117, 118). Microtubulin proteins
are found exclusively in eukaryotic cells. Recent evidence indicates
that native tubulins are globular molecules of approximately 110,000 m.w.,
composed of two distinct subunits (α and β tubulins). These subunits
have a m.w. of 52,000 each, as determined by sodium dodecyl sulphate
(SDS) polyacrylamide gel electrophoresis. The tubulin molecules self-
aggregate to form a complex fibrous system. Colchicine and the vinca
alkaloids (vinblastine and vincristine) are known to form complexes with
individual tubulin subunits and prevent their polymerization by a
mechanism not yet elucidated. Microtubulins are known to be involved
in a variety of cellular processes. In this context, it is known that vinblastine
can prevent spindle formation required for cell division (119, 119a)

1.7   Protein synthesis dependent on nuclear RNA synthesis.

As described earlier, RNA synthesis commences at a low level
after fertilisation and gradually increases during later stages of
development. Therefore, although most, if not all, of the proteins
synthesised in the earliest stages are coded for by pre-existing m-RNA,
as development proceeds the embryo increasingly utilises newly synthesised
m-RNA. The relative contribution of the newly synthesised m-RNA is measured
by following the amount of labelled RNA associated with polysomes isolated
at different stages of development, immediately after fertilisation and

during the first cleavage. Only a small fraction of the polysomes
is associated with the newly synthesised RNA (48, 117). By mid-
cleavage this fraction increases to about 30% and the new RNA is
detected in a special class of small polysomes (49, 120, 121). That
these small polysomes were responsible for the synthesis of histones
was suggested by their size (122, 123). This was confirmed by Moav
and Nemer (124), who characterised the proteins synthesised on these
small polysomes as histones by acid extraction, gel filtration, cation
exchange chromatography and acrylamide gel electrophoresis.

However, the newly synthesised histone m-RNA does not account
for total histone synthesis in the early stages and the bulk is programmed
by m-RNA stored in the mature egg. It appears that the majority of
proteins synthesised in early development are programmed in the egg.
Not until the embryo begins to differentiate, does newly synthesised
RNA play a major role in directing protein synthesis.

1.8   Regulation of protein synthesis in the mature and fertilised egg.
Fertilisation (in the sea urchin) or ovulation (in Xenopus)
initiates protein synthesis and there is now reasonable evidence that
this synthesis is dependent on the utilisation of pre-formed m-RNA -
present in the mature egg. This raises an interesting question as to
the nature of the regulatory mechanisms involved in controlling both
the storage and reutilisation of "maternal" m-RNAs. Studies on sea
urchin have concentrated on examining the different components of the
protein synthesising machinery from mature to fertilised eggs for possible
defects or inhibitors. Suggestions have included the inadequate supply
of aminoacyl-transfer RNA (84, 125, 126), inhibited ribosomes (127, 128)
inactive polysomes or m-RNA ribosome complexes (129, 134), and inactive
m-RNA protein particles called "informosomes" (130, 131). Cell free
preparations prepared from fertilised and unfertilised eggs showed no
difference in their ability to translate synthetic polynucleotides
suggesting that neither the ribosomes nor the supply of transfer RNA was
limiting (132).

An inhibitor of protein synthesis has been identified in the
salt washes of the ribosomes of unfertilised but not of fertilised sea
urchin eggs. This inhibitor reduces the rate of elongation measured
in the rabbit reticulocyte cell free system. However, its physiological
role is doubtful since a concentration of fifty times that found in the
unfertilised egg ribosomes is necessary to obtain a 50% inhibition of
poly (U)-primed phenylalanine incorporation by an equivalent amount of

ribosomes from fertilised eggs (133, 134, 135).

Humphreys observed no difference in the rates of initiation or elongation of nascent proteins chains in either unfertilised or fertilised eggs and concluded that the increased protein synthesis was due to the increased availability of m-RNA (136, 137). There is now direct evidence for a cytoplasmic histone m-RNA - protein complex present in the egg (an informosome) (20). Whether or not informosomes are the only means for storing m-RNA in mature eggs is still unresolved. In addition, the nature of the mechanisms by which such m-RNAs are made available at fertilisation is also unknown. Some authors (1, 42, 112) even suggested the possibility that different stored m-RNAs are translated at different times after fertilisation. To date no detailed studies have been made of the protein patterns at successive stages of early embryonic development. Comparable studies carried out in amphibians (138) and ascidians (139) support the concept of stored m-RNA.

2.   Isolation and translation   of eukaryote m-RNA in cell-
      free systems.

    Eukaryote m-RNA was shown to be a metabolically unstable
species of RNA with a base composition similar to that of DNA and a
heterogeneous size distribution. It also represented a small percentage
of the total cellular RNA (about 1%). None of these known characteristics
were particularly useful as a basis for the isolation of m-RNA. However,
the later discovery that most eukaryotic m-RNA contain a long poly (A)
sequence has now provided means for bulk separation of m-RNAs for other
RNA components. In addition, cell-free systems capable of responding to
exogenous m-RNA have only recently been developed and methods are now
available for assaying m-RNA activity. As yet, there are no adequate
techniques for the isolation of specific m-RNAs from bulk RNA or m-RNAs
lacking poly (A). Methods for the isolation and detection of m-RNA have
been extensively reviewed (66, 69, 140-143, 151, 152).

2.1   Isolation of m-RNA.

    All RNAs, by virtue of their large size and chemical composition,
are susceptible to mechanical breakage and nuclease attack. The technique
X for cell disruption, (i.e. hypotonic lysis, homogenisation, etc.) must be
gentle and is determined by the tissue under study. Rigorous precautions
must also be taken to eliminate or reduce nuclease activity. Normally,
all operations are carried out at $0^{\circ}C$ using heat-sterilised glassware and
diethylpyrocarbonate-treated solutions. Ribonuclease inhibitors, such as
heparin, bentonite or rat liver RNase inhibitor are often added to the
solutions (144).

    Various procedures (e.g. differential centrifugation, density gradient
centrifugation) are available for the isolation of specific cell fractions
(14, 147, 148, 153). The m-RNA is usually isolated from the polysome
fraction, although other cell fractions have been used in particular
studies. Both ionic strength and monovalent/divalent ion composition of
the isolating medium are important in that they affect the stability and
aggregation properties of the polysomes (145). The polysomes are normally
X deproteinised by phenol extraction, the RNA remains in the aqueous phase.
Various chemicals have been added to the aqueous phase to improve deprotein-
isation or inhibit RNase such as EDTA (154), SDS (140), isoamyl alcohol (45),·
m-cresol (157) and 4 - amino salicylate (157). Phenol extraction results in
the loss of poly (A) - containing m-RNA unless an alkaline or low ionic strength buffer
was used in the aqueous phase (156). The inclusion of chloroform also
favoured extraction of poly (A) m-RNA (155).

Until the discovery of poly (A), m-RNAs were separated from
other RNA species by techniques based on the differences in molecular
weight (141, 145, 158, 159, 160). This approach, however, is limited to
those m-RNAs having molecular weights widely different from ribosomal
and transfer RNAs. The observation that m-RNA appears to be the sole
cytoplasmic species that contains poly (A) has provided the theoretical
basis for several procedures for the separation of poly (A) containing
RNAs from other RNA components. For example, poly (A) containing RNA
molecules bind to poly (U) Sepharose or oligo(dT) cellulose at high
strength
ionic conditions (69-70a). These poly A sequences are also very resistant to
RNase degradation (71, 72). These techniques are obviously not applicable
to m-RNAs lacking poly (A) (67, 68) and in themselves do not necessarily
provide the means for the isolation of specific m-RNAs.

Specific classes of polysomes have been isolated according to
the type of polypeptide synthesised either by immuno precipitation with
antisera to the polypeptide of interest (149, 150) or by selecting an
expected size distribution (146, 147, 148). The RNA is then isolated
by the usual techniques and provides a preparation enriched in specific
m-RNA. In the same instance it is possible to use specific m-RNA protein
complexes released from EDTA treated polysomes for m-RNA isolation (153).
Although there have been tremendous advances in the techniques for the
isolation and purification of eukaryote m-RNAs in the last five years,
many interesting m-RNAs by virtue of their minute amounts and association
with many other m-RNAs, remain unobtainable.

2.2   Translation of m-RNA in cell free systems.

Since 1970, several cell free systems for assaying m-RNA activity
have been developed and are now routinely used. The systems, inclusive
of their respective advantages and disadvantages, have been reviewed (141a).

2.2.1 Reticulocyte cells.

Large numbers of reticulocytes (immature red blood cells) may
be isolated from anaemic animals, usually induced experimentally by
administration of the hemolytic agent, phenylhydrazine. The reticulocytes
are broken by hypotonic lysis and the crude 15,000 g supernatant is used
directly for assaying m-RNA. The lysate contains little RNase and
maintains a high rate of protein synthesis for up to one hour (161).
In the presence of hemin, the rate is linear during this time period (162).
Hemin blocks the formation of an inhibitor which renders the methionine-
transfer RNA/40S subunit complex inactive. The lysate retains its

activity for up to six months when stored in liquid nitrogen.

The chief advantage of this system is its high protein synthetic efficiency due to its rapid reutilisation of m-RNA. However, the high levels of endogenous globin-m-RNA result in massive synthesis of globin chains. Therefore, in the assay for exogenous m-RNAs, methods for quantitating the specific proteins synthesised are essential.

2.2.2 Wheat germ.

The wheat germ in vitro system is potentially the easiest obtainable and cheapest cell-free system to prepare. The homogenate of the wheat germ is centrifuged at 30,000 g, preincubated for a short period at 30°C and then passed through a Sephadex G25 column. The turbid fractions are collected and used for assaying m-RNA activities (163). The extremely low level of endogenous protein synthesising activity permits the estimation of total m-RNA activity directly by measuring incorporation of labelled amino acids into proteins. In addition, product analysis is not complicated by the presence of high levels of endogenously synthesised proteins. However, compared to the reticulocyte system, the translational efficiency of the wheat germ is considerably less. Several laboratories have observed the phenomenon of "premature termination", and also the exogenous m-RNA may be translated only a limited number of times. Heywood claims to have overcome this problem with myosin m-RNA by adding initiation factors from chick embryo fibroblasts (164). Translational efficiency also depends on the source of wheat germ (164a).

2.2.3 Krebs ascite cells

Krebs II ascites cells are grown by injecting ascities cells into the peritoneal cavities of mice. These cell samples are derived from the original Krebs II mouse tumor, a solid carcinoma found in the inguinal region. The crude supernatent (30,000g) is preincubated and fractionated by Sephadex chromatography as in the preparation of the wheat germ system (165). The ascities cell free system is also characterised as being a low background, less efficient protein synthesising system, and therefore has the same benefits and limitations as the wheat germ system.

2.2.4 Xenopus oocytes.

Gurdon and co-workers have worked out a system for assaying

m-RNA activity using Xenopus eggs and oocytes. The m-RNA solutions
are micro injected directly into the eggs and oocytes. Although there
is usually only a small increase in the rate of total protein synthesis,
the system continues to incorporate labelled amino acids for long
periods of time. This system is by far the most efficient in that nanogram
quantities of m-RNA may be analysed and each m-RNA is translated several
hundred times. As with the reticulocyte system, effective product
analysis is required because of the endogenous proteins synthesised.
The other limitation is the expertise related to the maintenance and
handling of the oocytes themselves (166).

The systems described are routinely used for the assay of
heterologus m-RNA preparations. The fact that the same m-RNA preparation
is translated with different efficiencies, depending on the type of cell
free systems used, emphasises the problems related to measuring m-RNA
activity in precise quantitative terms. Further, in dealing with a
mixed population of m-RNAs, in vitro analysis may bear little relation-
ship to the true activities of the different m-RNAs in vivo. An
additional interpretative problem would be the case of a changing
population of m-RNAs as in the case of a developmental system (e.g.
embryogenesis). One obvious answer would be to study m-RNAs in a homologous
cell free system. However, this raises the difficulty of initially
purifying the system free from the RNAs to be studied, without altering
other components in the system.

3.   The aim of the investigation

Plaice, (Pleuronectus platessa) is commonly found on the
sandy parts of the European continental slope between the western
Mediterranean and the North Sea. It is also caught around Iceland
and the Faroes. The principal spawning grounds -are the North Sea
and spawning occurs once a year between December and late March.
Fish collected at this time can be maintained in large tanks supplied
with running sea water for eventual mechanical stripping of eggs and
sperm (169). Up to a million eggs may be released from a single mature
female. It has been possible to carry out controlled fertilisation
experiments and to follow the embryological development under well
defined conditions. The rate of development is extremely temperature
dependent. For example the developmental sequence of plaice at $7^{o} - 8^{o}C$
is as follows:

Stage	Time
Fertilisation	0 min.
First polar body forms and breaks down	approx. 30 min.
Second polar body forms and breaks down	40 min.
First cleavage	4.5 - 5h
Last cleavage	18h
Gastrulation	24h
Hatching	3 weeks

The yolk provides all the nutritional requirements during
development, but there are several essential amino acids which must be
derived from the environment, one of which is leucine (167, 168, 170,
171).

The majority of the work on plaice culture has been carried out
at the Ministry of Agriculture, Fisheries and Food Fisheries Laboratory
at Lowestoft. On the basis of their experience, it seemed that plaice
offered a very favourable alternative to sea urchin and frog for
studies on the regulation of gene expression at fertilisation. Dr. C.
Purdom of the Fisheries Laboratory, Lowestoft kindly offered to supply the
eggs and sperm and also to teach our laboratory the techniques for
fertilisation and growing the embryos in culture. Under the correct
conditions, it is possible to obtain 100% fertilisability and synchronous
development. The major disadvantage of the plaice system is that in spite
of the enormous quantities of eggs and sperm obtainable their availability

is restricted to a very limited breeding season each year.

Initially, it was necessary to investigate the permeability properties of eggs, before and after fertilisation, in order to ascertain the possibility of using the incorporation of labelled precursors as a measure of RNA and protein synthesis. Next,any changes in the rates of RNA and protein synthesis, or in the pattern of protein synthesized as a consequence of fertilisation were studied. Finally, the m-RNA profiles of the different developmental stages were investigated. Special emphasis throughout the study was placed on those proteins precipitated by vinblastine sulphate.

RESULTS AND DISCUSSION

4.   RNA and protein biosynthesis during early plaice embryogenesis.

4.1   Uptake of RNA and protein precursors by eggs and embryos.

    In order to test the feasibility of using incorporation of radioactively labelled precursors as a measure of net RNA and protein synthesis, it was first of all necessary to examine the permeability characteristics of eggs at different stages after fertilisation. If, for example, any stage was shown to be impermeable to precursor uptake, the lack of incorporation would not necessarily reflect lack of synthesis. Batches of eggs were incubated for one hour with either $[^3H]$-uridine or $[^3H]$-leucine, washed and then counted directly for uptake of labelled precursors. Six separate experiments were carried out over a three year period. The results of the permeability experiments are shown in Table 1 and 2. Eggs and fertilised eggs were permeable to uridine and leucine at all times examined. Leucine uptake was greater than that for uridine, although the uptake of both precursors was reduced immediately after fertilisation. It would appear that fertilisation altered the permeability properties of the egg membrane without making it completely impermeable. Doubling the external concentration of the precursors resulted in increased uptake. Variations between the experiments were believed to be due to the different stocks of fish used, the different times during the breeding season when the eggs were collected and the fluctuation in the incubator temperatures. The main conclusion is that incorporation can be used as a measure of synthesis in plaice eggs at the stages studies, providing the precursor uptake is not limiting and the pools are freely accessible for macromolecular biosynthesis. This system is very similar to Urechis capo (27) but differs from amphibians and sea urchin where the prefertilised eggs are impermeable to precursors (1-5). Evidence that leucine is not readily catabolised in eggs was obtained by demonstrating that all the radioactivity in post mitochondrial supernatants co-chromatographed with leucine standards.

4.2   RNA and protein synthesis in eggs and embryos.

    The next experiments were designed to determine the levels of RNA and protein synthesis in eggs and early embryonic stages. As shown in Table 3, virtually no RNA synthesis occurred in the unfertilised egg or in eggs up to five hours after fertilisation. Measurable RNA synthesis did not occur until late cleavage (about 3% of the uridine uptake). This was confirmed by processing larger samples of eggs (up to 60 eggs) and

Table 1

Uptake of $[^3H]$-uridine by eggs before and after fertilisation.

Time of $[^3H]$ uridine addition (h)	$[^3H]$ uptake (cpm/egg/hour) Experiment					
	1	2	3	4	5	6
Unfertilised egg	6,420	3,022	7,864	12,947	8,470	15,374
Fertilised egg						
0	6,362	3,225	6,830	7,695	6,320	12,141
1	6,078	4,437	9,625	8,462	7,257	12,932
2	-----	4,490	9,169	9,109	7,962	12,250
3	8,726	3,572	12,629	20,450	13,637	24,632
4	9,414	4,407	-----	-----	-----	-----
16	-----	-----	8,992	12,167	-----	-----
17	-----	-----	-----	-----	8,089	11,650

----- no measurement taken

Eggs (5g) were incubated for 1h at each stage in 10ml sterile sea water at $8^{\circ}C$, containing either 50 µCi (experiment 1-5) or 100 µCi (experiment 6) $[5^3H]$ uridine; sp. act. 2Ci/mmol. After one hour the eggs were washed and 3 eggs or embryos per sample were counted as described in Methods. Experiments 1 and 2 were performed in 1972, 3, 4, and 5 in 1973 and 6 in 1974.

Table 2

Uptake of $[^3H]$-leucine by eggs before and after fertilisation.

Time of $[^3H]$ leucine addition (h)	$[^3H]$ uptake (cpm/egg/hour)					
	Experiment					
	1	2	3	4	5	6
Unfertilised egg	21,191	17,062	24,837	28,038	20,793	35,000
Fertilised egg						
0	14,491	8,380	8,855	15,670	10,470	18,110
1	13,590	9,560	11,650	16,728	11,656	21,755
2	15,330	9,890	13,627	17,140	14,067	21,240
3	15,840	10,500	16,498	24,933	18,717	24,639
4	20,264	12,625	------	------	------	------
16	------	------	12,500	15,358	------	------
17	------	------	------	------	10,864	12,962

------ no measurement taken

Eggs (5g) were incubated for 1h at each stage in 10ml sterile sea water at 8°C, containing either 50 µCi (experiments 1-5) or 100 µCi (experiment 6) L-[4, 5 ^3H] leucine; sp. act. 1Ci/mmol. After one hour the eggs were washed and 3 eggs or embryos per sample were counted as described in Methods. Experiments 1 and 2 were performed in 1972, 3, 4 and 5 in 1973, and 6 in 1974.

Table 3

RNA synthesis as measured by $\left[{}^{3}_{}\text{H}\right]$-uridine incorporation by eggs before and after fertilisation

Time of $\left[{}^{3}_{}\text{H}\right]$ uridine addition (h)	$\left[{}^{3}_{}\text{H}\right]$ incorporation (cpm/egg/hour)					
	Experiment					
	1	2	3	4	5	6
Unfertilised egg	0	0	0	0	0	0
Fertilised egg						
0	0	0	0	0	0	0
1	2	0	0	0	1	7
2	3	10	7	0	8	30
3	4	13	33	25	9	39
4	14	12	--	--	--	--
16	--	--	238	255	--	--
17	--	--	--	--	228	465

---- no measurement taken

Eggs (5g) were incubated for 1h at each stage in 10ml sterile sea water at 8°C containing either 50 µCi (experiments 1-5) or 100 µCi (experiment 6) $\left[5\text{-}{}^{3}\text{H}\right]$-uridine, sp. act. 2Ci/mmol. After one hour the eggs were washed and 10 eggs or embryos per sample were precipated with 5% T.C.A. The samples were then processed for counting as described in Methods. Experiments 1 and 2 were performed in 1972, 3, 4, and 5 in 1973, and 6 in 1974. The blanks (66 cpm/egg/hour) have been subtracted.

Table 4

Protein synthesis as measured by $[^3H]$-leucine incorporation by
eggs before and after fertilisation.

Time of $[^3H]$ leucine addition (h)	$[^3H]$ incorporation (cpm/egg/hour)					
	Experiment					
	1	2	3	4	5	6
Unfertilised egg	245	215	235	241	221	661
Fertilised egg						
0	195	158	212	217	189	567
1	210	201	231	247	213	639
2	218	167	263	277	217	651
3	245	235	288	297	266	---
4	293	257	---	---	---	---
16	---	---	238	227	---	---
17	---	---	---	---	225	675

----- no measurement taken

Eggs (5g) were incubated for 1h at each stage in 10ml sterile sea
water 8°C, containing either 50 µCi (experiments 1-5) or 100 µCi
(experiment 6) L-$[4, 5^3H]$ leucine; sp. act. 1 Ci/mmol. After
one hour the eggs were washed and 10 eggs or embryos per sample were
precipitated with 5% T.C.A. The samples were then processed for
counting as described in Methods. Experiments 1 and 2 were performed
in 1972, 3, 4 and 5 in 1973 and 6 in 1974. The blanks (56 /cpm/egg/hour)
have been subtracted.

also by examining fertilised eggs between the 4 - 16 hour period.
Unlike RNA, the results in Table 4 show that proteins are synthesised
in the unfertilised egg as well as in all other stages examined. If
one assumes proportionality between rates of protein synthesis and
leucine uptake, then total$\left[^3_{\cdot}H\right]$-leucine incorporation is roughly
doubled in the first hour after fertilisation. This level is maintained
in the later stages $\left[^3_{\cdot}H\right]$-leucine incorporation represents only about 2%
of the leucine uptake. Therefore, the proteins synthesised in the egg
and at least up to five hours after fertilisation are not dependent on
newly synthesised RNA but are coded for by m-RNAs synthesised at earlier
stages in oogenesis.

In order to confirm this observation, the levels of protein
synthesis were measured in the presence of RNA synthesis inhibitors. Various
concentrations of actinomycin D and ethidium bromide were added to embryos
to ascertain the minimum concentration required for inhibition of RNA
synthesis. Table 5 shows that a combination of actinomycin D and
ethidium bromide at concentrations as low as 2 µg/ml and 1µg/ml,
respectively, completely inhibit RNA synthesis in the 17 hour embryo.
These inhibitors had no effect on the levels of protein synthesis during
the first and second hours after fertilisation (Table 6). Therefore, as
previously concluded, protein synthesis in the early embryonic stages is
independent of concomittant RNA synthesis. That RNA inhibitors markedly
reduced the level of protein synthesis in the 17 hour embryo suggests
that newly synthesised RNA is required for some of the proteins·
synthesised at this time. However, the inhibitor-treated embryos appeared
to be morphologically identical to the control embryos (i.e. well formed
blastodiscs with normal shaped cells). This implies that the proteins
synthesised under the direction of newly synthesised RNA are not important
for development up to this stage. Summarising, the proteins synthesised
immediately after fertilisation are directed by stored m-RNA in the egg.

These results are identical to that found in amphibians (2) in
that proteins are synthesised in the eggs immediately before and after
fertilisation in the absence of RNA synthesis. Both amphibians and plaice
systems are similar in that the mature egg undergoes ovulation prior to
fertilisation. Plaice also resembles sea urchin in that fertilised eggs
synthesise proteins but not RNA (1, 3). The preovulated, mature egg of
plaice has not been examined in this study and therefore no direct
comparison can be made between the mature eggs of amphibians, sea urchin
and plaice.

Table 5

Effect of RNA synthesis inhibitors on $[^3H]$- uridine incorporation
by eggs before and after fertilisation.

Inhibitor concentration (µg/ml)		$[^3H]$ incorporation (cpm/egg/hour) Time of $[^3H]$-uridine addition to fertilised eggs (h)		
actinomycin D	ethidium bromide	0	2	17
0	0	--	9	266
2	1	--	0	0
5	2.5	--	0	0
20	10	--	0	0
100	50	--	0	0
0	0	0	9	208
5	2.5	0	0	0

-- no measurement taken

Experimental details are as described for Table 3. The inhibitors
at various concentrations, as seen in the first two columns at the
left, were added immediately after fertilisation and $[^3H]$ uridine
was added at the times shown. The blanks (69cpm/egg/hour) have
been subtracted. Results from two different experiments are given
in the Table.

Table 6

Effect of RNA synthesis inhibitors on $[^3\text{H}]$-leucine incorporation by eggs before and after fertilisation

Time of $[^3\text{H}]$ leucine addition (h)	Presence of inhibitors	$[^3\text{H}]$ incorporation (cpm/egg/hour)	
		Experiment	
		1	2
Fertilised egg			
0	–	706	604
	+	1095	731
1	–	553	789
	+	464	625
17	–	774	----
	+	116	----

--- no measurement taken.

Experimental details are as described for Table 4. Combinations of actinomycin D (5 µg/ml) and ethidium bromide (2.5 µg/ml) were added immediately after fertilisation, and $[^3\text{H}]$-leucine was added at the times shown.

4.3   Subcellular distribution and characterisation of newly
       synthesised proteins in the egg immediately before and
       after fertilisation.

As stated earlier, protein synthesis occurs in the unfertilised
and fertilised plaice egg (Table 4). To test the possibility that
specific proteins are synthesised as a consequence of fertilisation,
different protein fractions were isolated and examined from eggs
labelled with $\left[^{3}H\right]$-leucine at different stages after fertilisation.
Samples were homogenised in 100mM Tris-glycine buffer, pH8.3, at $4^{\circ}C$
and fractionated by successive centrifugations at 20,000g for 20 min.
and 105,000g for 90 min. The resulting supernatant was treated with
vinblastine sulphate to precipitate microtubulin-like proteins
according to the method of Olmsted et al. (172). This procedure is
represented diagramatically in Scheme 1. The different fractions were
precipitated with 10% (V/V) trichloro-acetic acid (TCA), digested with
NCS tissue solubiliser and counted for radioactivity (Table 7). In
experiment 3, the 20,000g pellet was sonicated and samples used directly
for counting.

The experiments confirmed earlier results (Table 4) in that
fertilisation stimulated total protein synthesis during the first hour.
All fractions showed increased protein synthesis at this time. This
was particularly true of the vinblastine-precipitable proteins which showed
marked increases in synthesis after the first hour, followed by a
decrease in the next hour. The microsomal pellets contained no
appreciable radioactivity at the stages examined. All fractions were
subsequently analysed by disc gel electrophoresis in an attempt to
characterise the proteins synthesised during the different stages.

Preliminary experiments were carried out using 7% polyacrylamide
gels prepared according to the method of Davis (176). After
electrophoresis, the gels were sliced into 1mm sections and prepared for
counting. Comparison of these electrophoretic patterns of labelled
proteins from the post-mitochondrial supernatants (Figure 1) clearly
shows that different proteins are synthesised by the egg before and
after fertilisation. Further, since RNA is not synthesised during
these stages, it would appear that specific stored m-RNAs are translated
at different times after fertilisation.

As stated earlier, the microsomal pellets contained no
measurable radioactivity. Therefore, the labelled, post-ribosomal
supernatant proteins would be expected to be similar in types and
quantities to those of the post mitochondrial supernatants. Unfortunately,
this was not always the case. Figure 2 represents such an example.
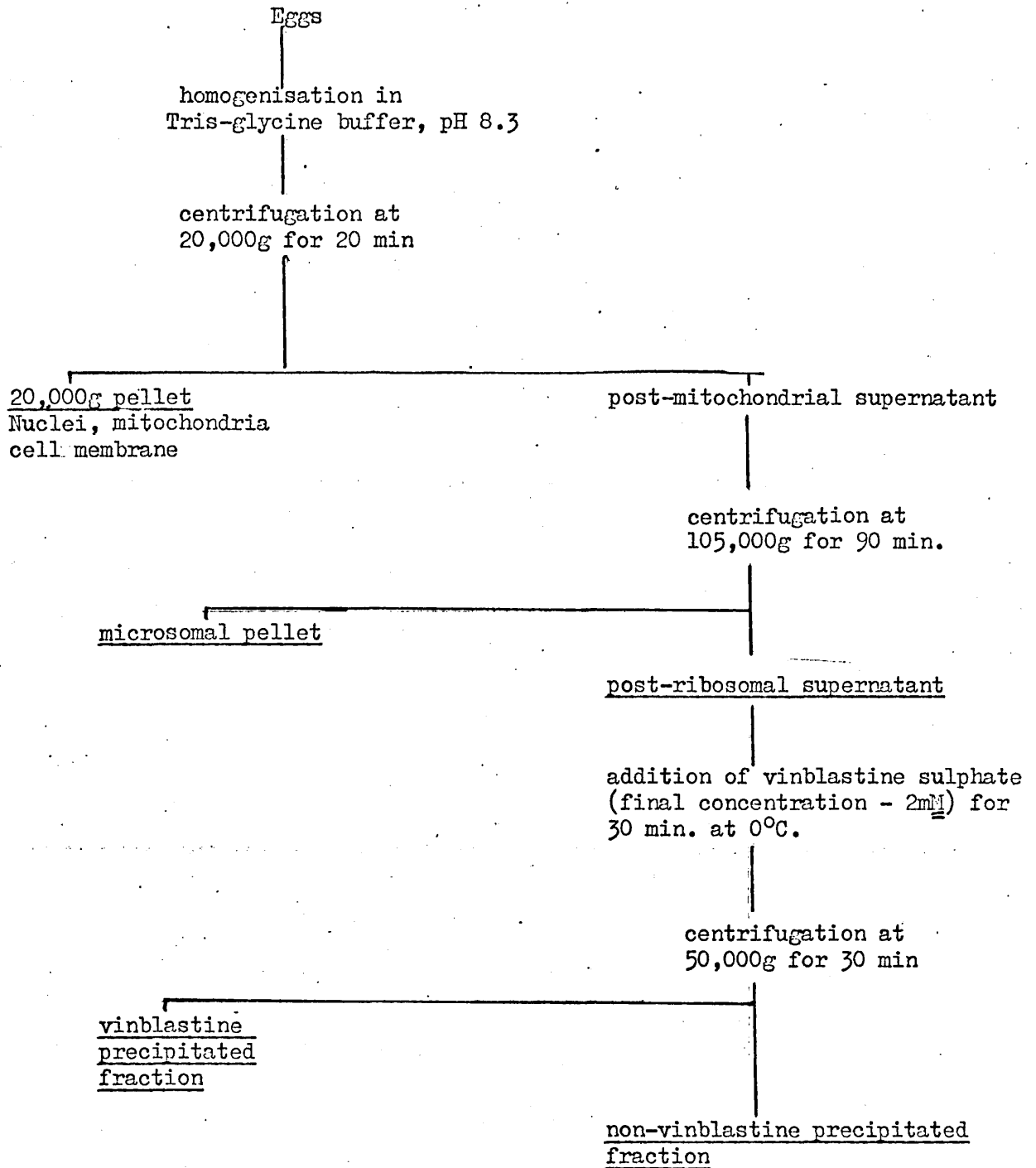
Scheme 1.

Eggs

|

homogenisation in
Tris-glycine buffer, pH 8.3

|

centrifugation at
20,000g for 20 min

20,000g pellet   post-mitochondrial supernatant
Nuclei, mitochondria
cell membrane
                                                          |

                                                  centrifugation at
                                                  105,000g for 90 min.

microsomal pellet

                                                  post-ribosomal supernatant

                                                          |

                                                  addition of vinblastine sulphate
                                                  (final concentration - 2mM) for
                                                  30 min. at 0°C.

                                                          |

                                                  centrifugation at
                                                  50,000g for 30 min

vinblastine
precipitated
fraction

                                                  non-vinblastine precipitated
                                                  fraction

Table 7

Distribution of $\left[^3H\right]$ labelled proteins in sub-cellular fractions isolated from eggs before and after fertilisation.

Time of $\left[^3H\right]$ leucine addition (h)	$\left[^3H\right]$ incorporation (cpm/egg/hour)				
			sub-cellular fractions		
	total	20,000g pellet	post-ribosomal supernatant	vinblastine precipitated fraction	non-vinblastine precipitated fraction
Unfertilised egg	624	338*	286	22	246
Fertilised egg					
0	614	218*	396	249	178
1	585	327*	258	41	206
2	367	128*	248	74	167
Unfertilised egg	661	437*	224	87	157
Fertilised egg					
0	567	215*	352	140	211
1	639	358*	281	89	192
2	651	462*	188	86	97
Unfertilised egg	653	301	279	63	237
Fertilised egg					
0	575	238	327	134	203
1	608	315	287	56	225
2	601	307	298	121	195

* 20,000g pellet (cpm) = total (cpm) - post-ribosomal supernatant (cpm)

(Table 7 continued)

Five grams of eggs were incubated in 10 ml of sterile sea water at 8°C containing 100 μCi of $L-[4, 5-^{3}H]$-leucine, sp. act. 1 Ci/mmol. for one hour. The eggs were then removed, washed and homogenised in 0.1 MTris-glycine buffer, pH 8.3 (1 g of eggs to 1 ml of buffer), fractionated and counted as described in Methods. Results from three different experiments are given in the Table.

As before, the electrophoretic profiles differ depending on the developmental stage but, in addition, they differ quantitatively and to a lesser extent, qualitatively from the corresponding stages in Figure 1. These changes presumably occurred during centrifugation, in spite of the low temperature. The post-mitochondrial supernatant proteins appear to be degraded in time, the degree varying from experiment to experiment. Degradation was not prevented by the addition of such chemicals as 2-mercaptoethanol, dithiothreitol or glycerol. Since it is important that the radioactive peaks represent in vivo proteins and not artefacts of preparation, only apparently stable post-mitochondrial preparations were used for further analysis. The 20,000g and vinblastine-precipitated fractions required solubilisation before electrophoresis. This was accomplished by heating the fractions in the presence of SDS. For this reason, the SDS-polyacrylamide gel electrophoresis system was routinely adopted as the method for protein analysis of all cell fractions.

Figure 3 confirms the earlier results (Figure 2) that different proteins in the post-ribosomal fraction are synthesised at different times during early embryogenesis. This is also true for the 20,000g pellet fraction (Figure 4). Therefore, in general terms, different proteins are synthesised in the unfertilised and fertilised eggs, and the types of protein synthesised also change during the first three hours after fertilisation.

If one accepts the view that all the newly synthesised proteins during this period are translated from pre-existing m-RNAs present in the mature egg, several interesting questions immediately present themselves. How is m-RNA stored in the egg? How does fertilisation activate the utilisation of stored m-RNA? What mechanisms are involved in the differential utilisation of stored m-RNAs during early embryogenesis? Answers to such questions obviously require detailed studies on the m-RNAs themselves. In the simplest situation, one would begin by studying the fates of two specific m-RNAs, one which is translated immediately at fertilisation and another which is translated at a later stage. Selection of the appropriate m-RNAs would first of all require the identification of a specific protein which follows this pattern of expression. Although the electrophoretic patterns clearly demonstrate that specific proteins are synthesised at specific times after fertilisation, the identities of such proteins remain unknown. In an attempt to identify specific proteins, two classes of proteins were examined, i.e. histones and microtubulins. These proteins have already been studied

in sea urchin eggs (see Introduction, section 1.6 and 1.7).

Histones were isolated from the 20,000g pellets by acid extraction and ethanol precipitation (148). The SDS-solubilised precipitates were analysed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. Protein bands with molecular weights expected for histones were present but lacked measurable radioactivity. Therefore, either histones were not synthesised during these stages or a more likely explanation is that leucine incorporation is not sensitive enough to demonstrate synthesis.

Figure 1.   Polyacrylamide gel electrophoresis of $[^{3}H]$-labelled
proteins isolated from the post-mitochondrial
supernatants of eggs before and after fertilisation.

Five grams of eggs were incubated in 10 ml of sterile sea water at
8°C containing 50 μCi $[5^{3}H]$-leucine (sp. act. 1 Ci/mmol) for one
hour. The eggs were then removed, washed and homogenised in 0.1
M Tris-glycine buffer, pH 8.3 (1 g of eggs to 1 ml of buffer), and
centrifuged at 20,000g for 20 min. Samples of the post-mitochondrial
supernatants (200 μl, equivalent to approximately 1 mg of protein)
were analysed directly on polyacrylamide gels as described in Methods.
The migration of proteins was from left to right.

Relative   = distance in mm moved by protein
mobility   distance in mm moved by Bromophenol blue

EGG
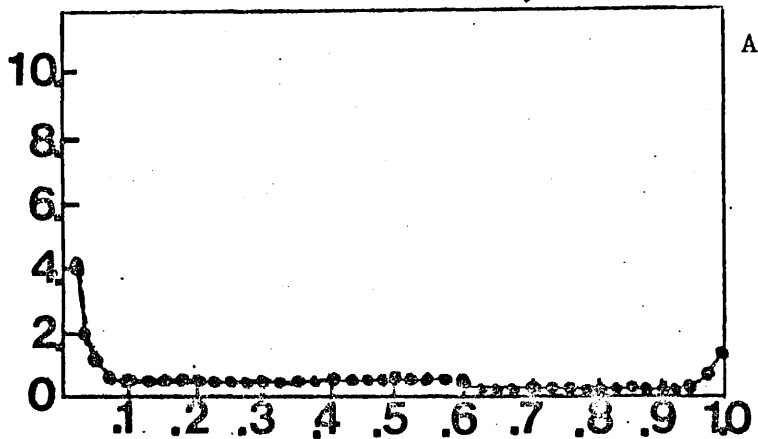
FERTILISED EGG
[H] leucine added at O hr.
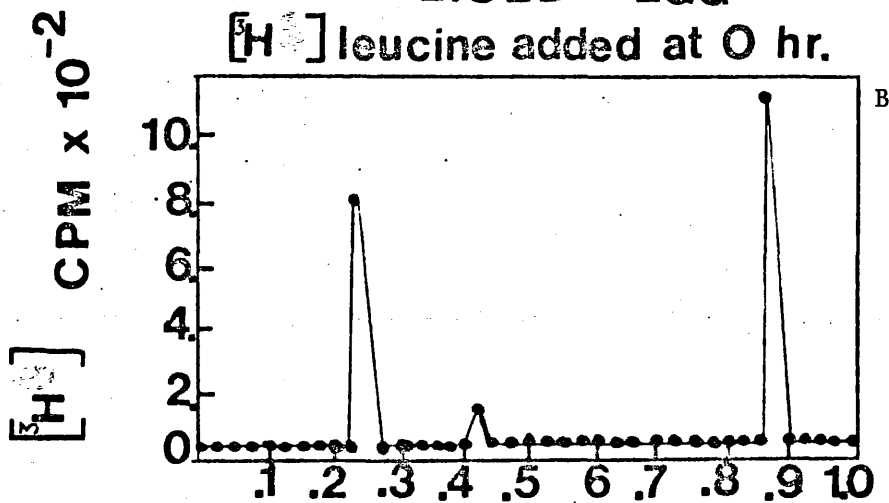
FERTILISED EGG
[H] leucine added after 1 hr.
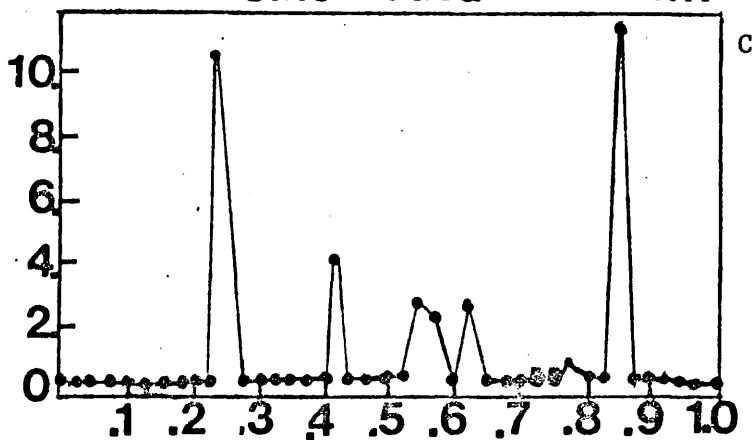
RELATIVE MOBILITY

Figure 2.   Polyacrylamide gel electrophosesis of $\left[^3\text{H}\right]$ - labelled proteins isolated from the post- ribosomal supernatants of eggs before and after fertilisation.

Experimental details are as described for Figure 1. The post- mitochondrial supernatants were centrifuged at 105,000g for 90 min at 2°C. 225 µl samples of the post-ribosomal supernatants (equivalent to approximately 1 mg of protein) were analysed directly on polyacrylamide gels as described in Methods. The anode is shown at the right.

EGG

FERTILISED EGG
[³H] leucine added at 0 hr

FERTILISED EGG
[³H] leucine added after 1hr

FERTILISED EGG
[³H] leucine added after 2 hrs
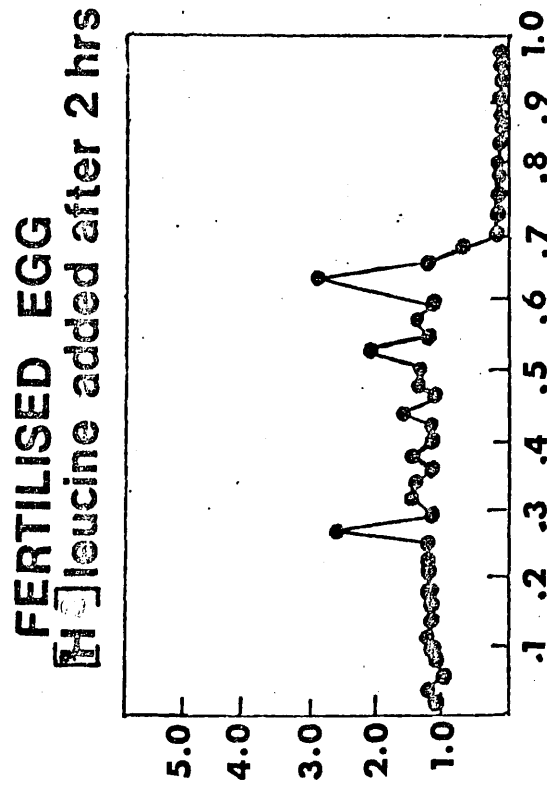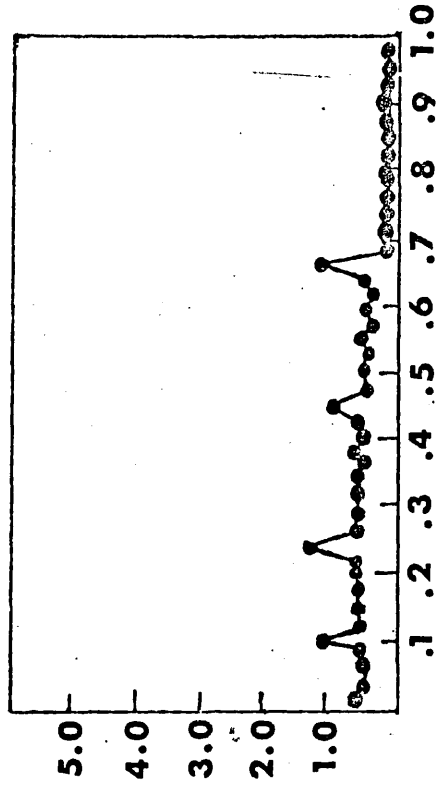
RELATIVE MOBILITY
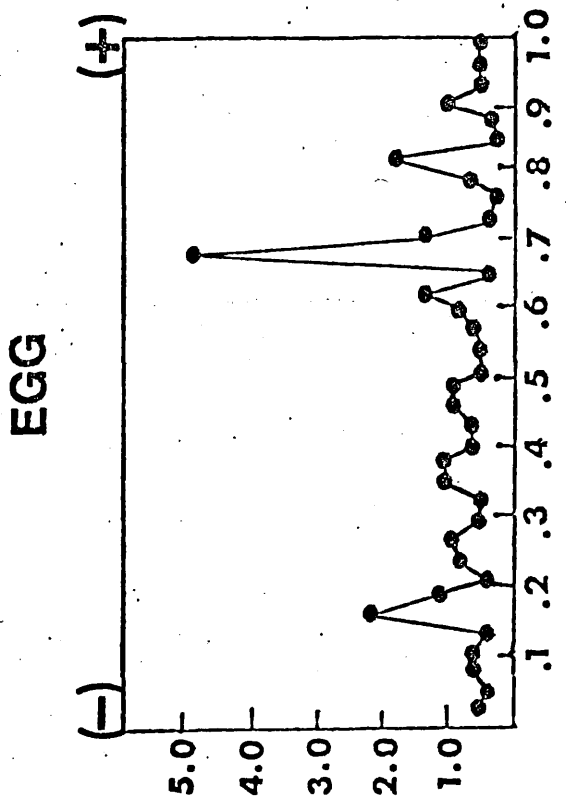
[³H] CPM x 10⁻²

Figure 3.   Sodium dodecyl sulphate (SDS)-polyacrylamide
                   electrophoresis of[^{3}H]-labelled proteins isolated
                   from the post ribosomal supernatants of eggs before
                   and after fertilisation.

Experimental details are as described in Figure 2. The post-
ribosomal supernatants were adjusted to 0.5% SDS and heated at
100°C for 5 min. After dialysis against SDS electrophoresis buffer,
100 μl samples (equivalent to approximately 0.1 mg of protein) were
analysed on SDS-polyacrylamide gels as described in Methods. All
proteins migrate towards the anode, shown at the right.

EGG

FERTILISED EGG
[³H] leucine added at O hour

FERTILISED EGG
[³H] leucine added after 1 hour

FERTILISED EGG
[³H] leucine added after 2 hours

[³H] CPM × 10⁻²
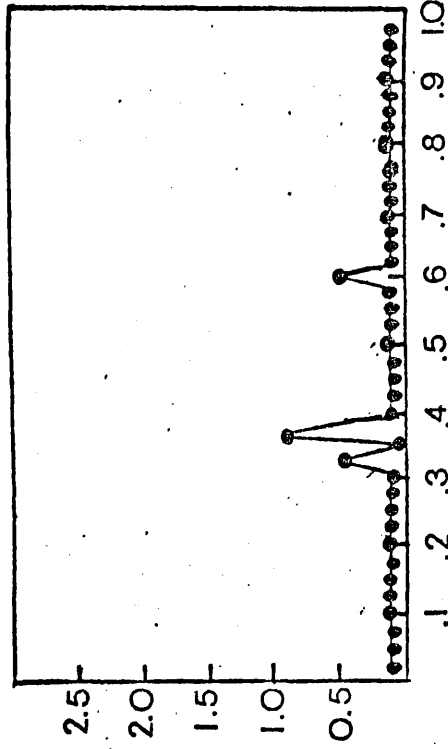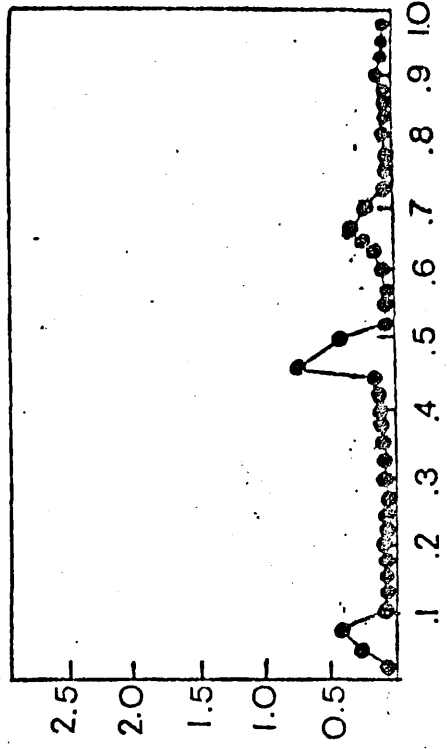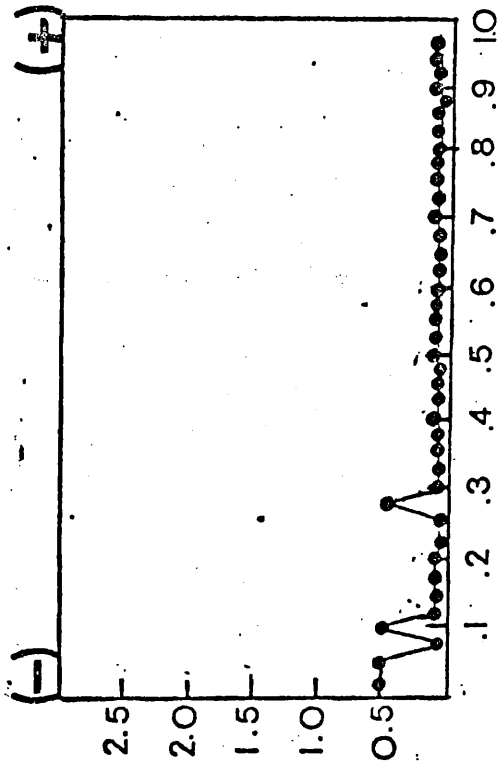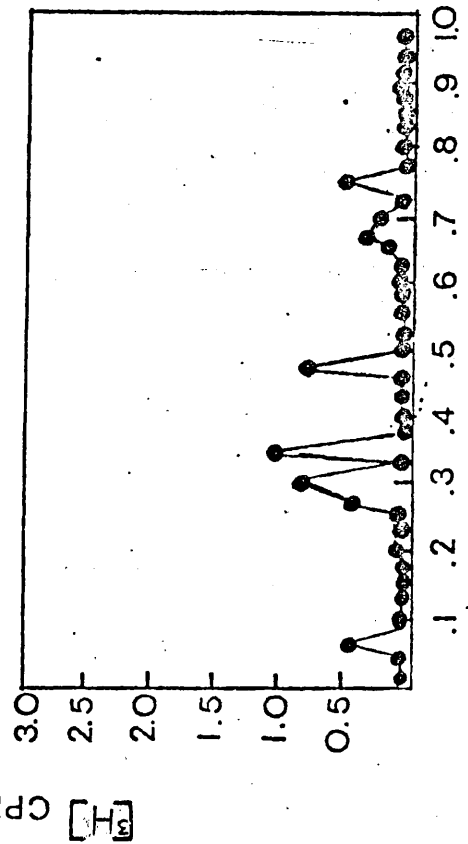
RELATIVE MOBILITY

Figure 4.   Sodium dodecyl sulphate (SDS)-polyacrylamide
electrophoresis of [^{3}H] -labelled proteins isolated
from the 20,000g pellets of eggs before and after
fertilisation.

Experimental details are as described for Figure 1. The 20,000g

pellets were resuspended in 5ml of SDS electrophoresis buffer,

homogenised and heated at 100°C for 5 min. After dialysis

against SDS electrophoresis buffer, 100 μl samples (equivalent

to approximately 0.1 mg of protein) were analysed on SDS-poly-

acrylamide gels as described in Methods.. All proteins migrate towards

the anode, shown at the right.

EGG

FERTILISED EGG
[³H] leucine added at 0 hr

FERTILISED EGG
[³H] leucine added after 1hr

FERTILISED EGG
[³H] leucine added after 2 hrs

FERTILISED EGG
[³H] leucine added after 2 hrs
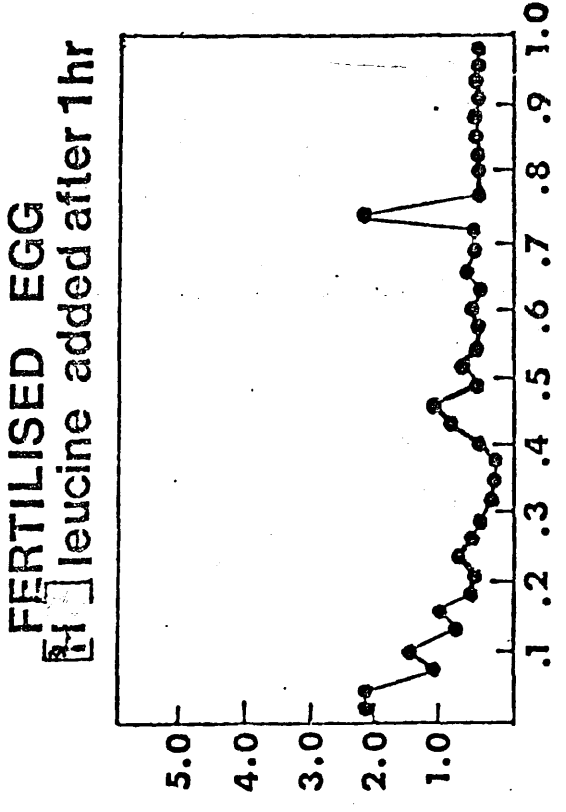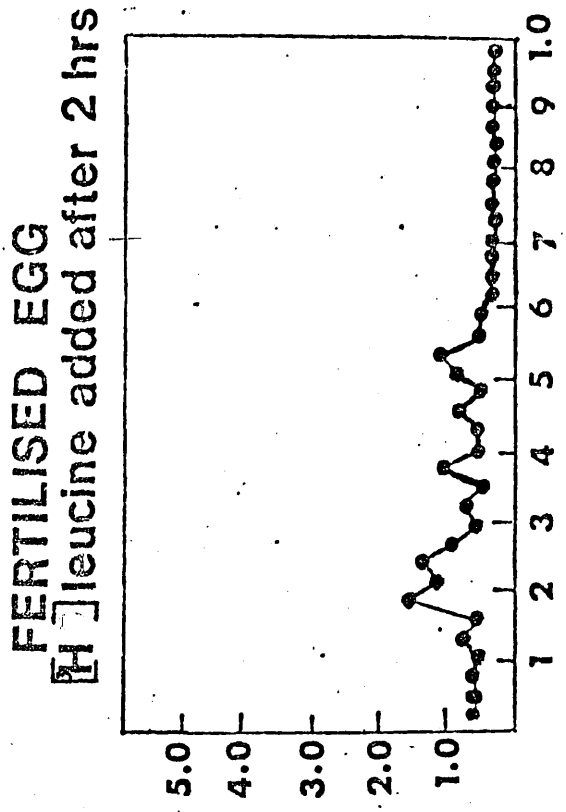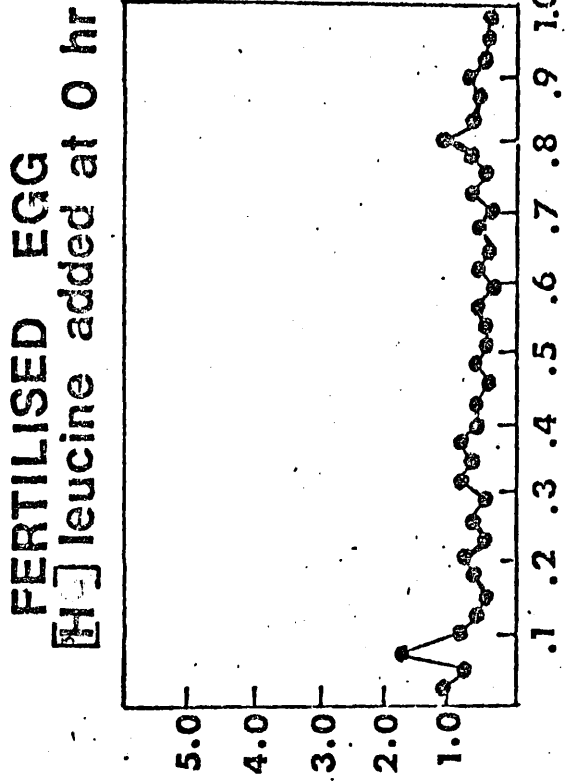
RELATIVE MOBILITY

[³H] CPM X 10⁻²

The selective precipitation of microtubulins by vinca
alkaloid was used as a method for isolating this group of proteins.
Post-ribosomal supernatants were treated with vinblastine sulphate
under appropriate conditions and the respective supernatant and
precipitated fractions separated by centrifugation. As stated earlier,
the greatest increase in $\left[^{3}H\right]$-leucine incorporation occurred in the
vinblastine-precipitated fraction (Table 7). The electrophoretic
profiles of the supernatant and vinblastine-precipitated fraction are
shown in Figures 5 and 6, respectively. Both fractions show differences
in the proteins synthesised depending on the developmental stage.
Vinblastine sulphate is obviously not specific for microtubulins (molecular
weight 52,000) since the vinblastine-precipitated fractions (Figure 6)
contain a range of radioactively labelled proteins (molecular weights,
10,000 - 100,000). This is in agreement with other reports (119).
In addition, it has been reported that experimental conditions such as
temperature, pH and ionic strength affect the qualitative and quantitative
nature of the proteins precipitated (119). In order to test the effect
of pH on vinblastine precipitation, post ribosomal supernatants were
treated with vinblastine at two different pHs. The electrophoretic
profiles of the labelled proteins precipitated by vinblastine at pH 8.3
(Figure 6) and pH 7.0 (Figure 7)are almost the same showing relatively minor
differences in the type and quantities of proteins. The reproducibility
of these experiments with different batches of eggs precipitates a range
of proteins rather than all proteins nonspecifically.

As shown in Figure 7, different vinblastine-precipitated proteins
are synthesised in the plaice egg before and after fertilisation. These pro-
teins were identified by their respective mobilities in SDS-polyacrylamide
gels. The radioactive peaks have been assigned alphabetical letters to
aid in the discussion. Each peak does not necessarily represent a single
protein species, nor do the same peaks from the different stages
necessarily represent the same protein. Nevertheless, each stage is
characterised by its own particular set of proteins (summarised in Table
8). Fertilisation results in the appearance of these new peaks (a, b and
d) in the first hour, followed by three (c, h and i) in the next hour, and
at least two (f and g) in the last hour examined. Therefore, the
original objective of identifying a number of proteins which are
differentially synthesised in the egg immediately after fertilisation
when RNA synthesis is absent has been fulfilled. The remainder of the
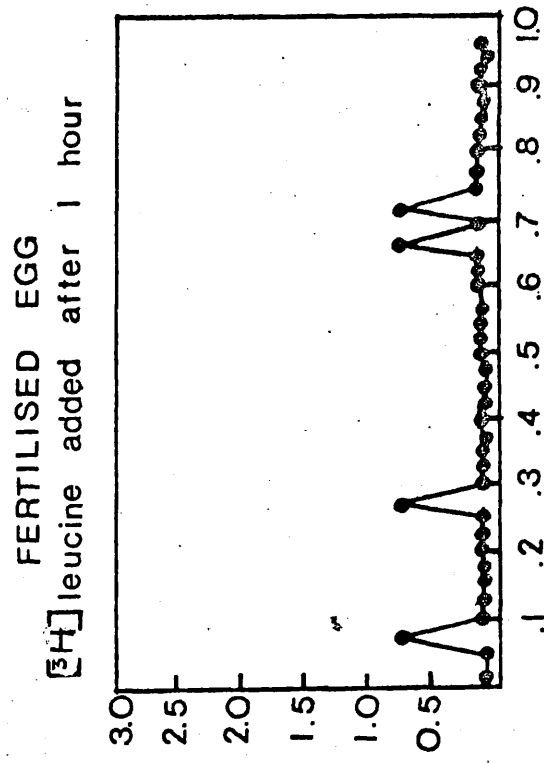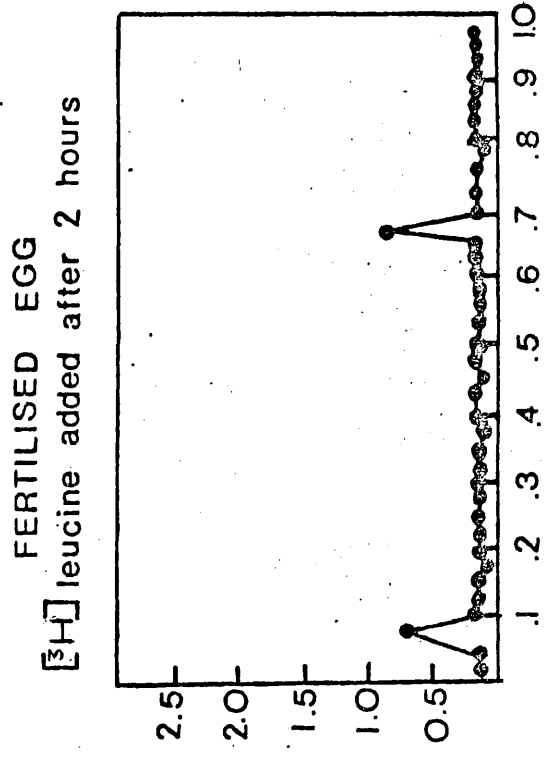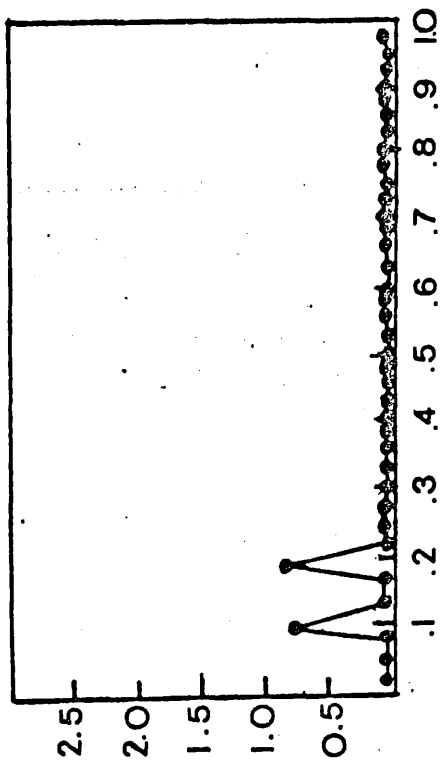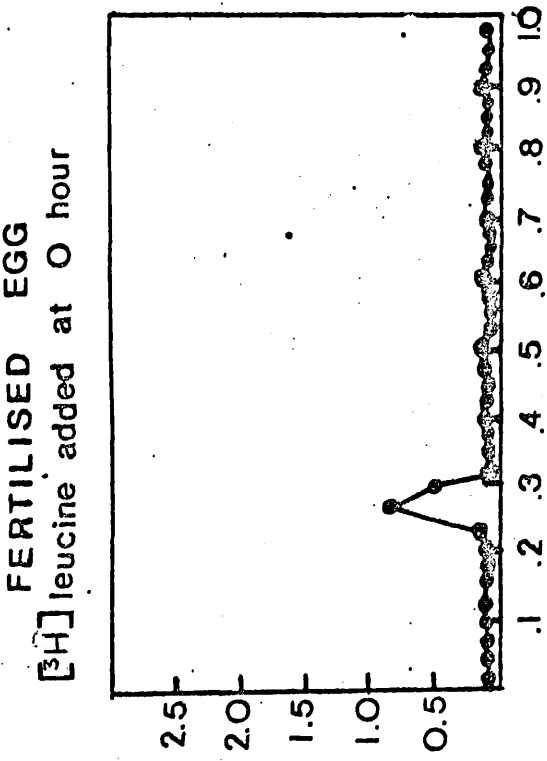study is an attempt to study the m-RNAs for such proteins.

Interestingly, microtubulins proved not to be suitable proteins
for study in this instance since microtubulin-like proteins (mt) were
synthesised in the egg both before and after fertilisation (Table 8).
The use of vinblastine instead of colchi⹂cine was rather fortuitous in
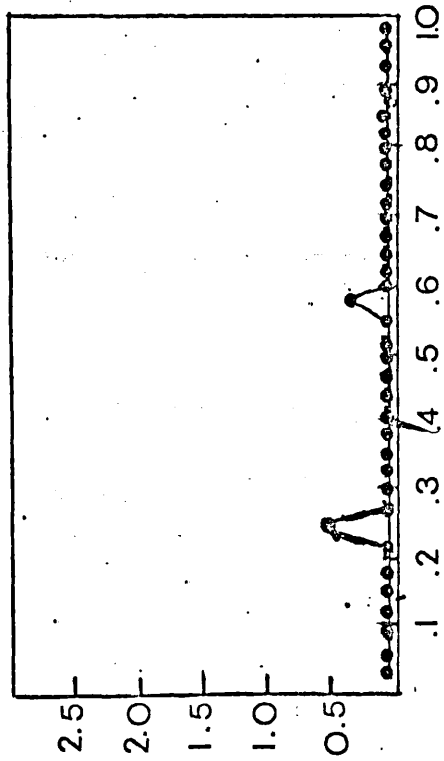view of the latter's extreme specificity.

Figure 5.   Sodium dodecyl sulphate (SDS)-polyacrylamide
electrophoresis of [^{3}H].-labelled proteins
isolated from the non-vinblastine precipitated
fractions of the post-ribosomal supernatant of
eggs before and after fertilisation

Experimental details are as described for Figure 2. The post-
ribosomal supernatants (pH 8.3) were adjusted to 2 x 10^{-3}M
vinblastine sulphate and left for 30 min at 0°C. The vinblastine-
precipitated proteins were collected by centrifugation at 50,000g
for 30 min.

The supernatants (containing the non-vinblastine precipitated
proteins) were adjusted to 0.5% SDS, and 100 µl samples were analysed
on SDS-polyacrylamide gels as described in Methods. All proteins migrate
towards the amode, shown at the right.

FERTILISED EGG
[³H] leucine added at O hour

EGG

FERTILISED EGG
[³H] leucine added after 2 hours

FERTILISED EGG
[³H] leucine added after 1 hour

RELATIVE MOBILITY

[³H] CPM × 10⁻²

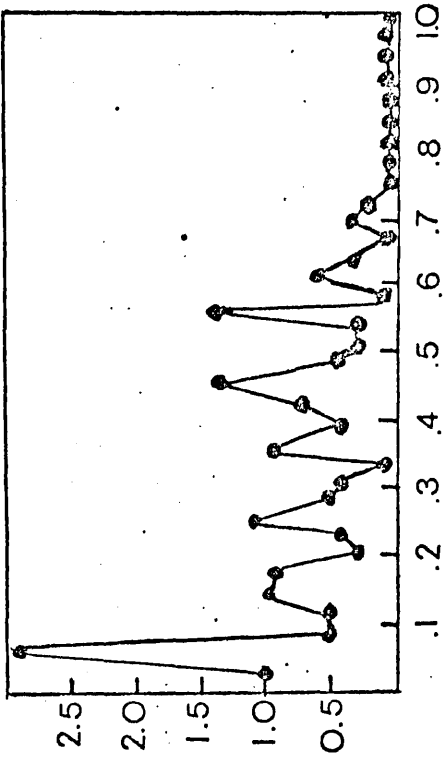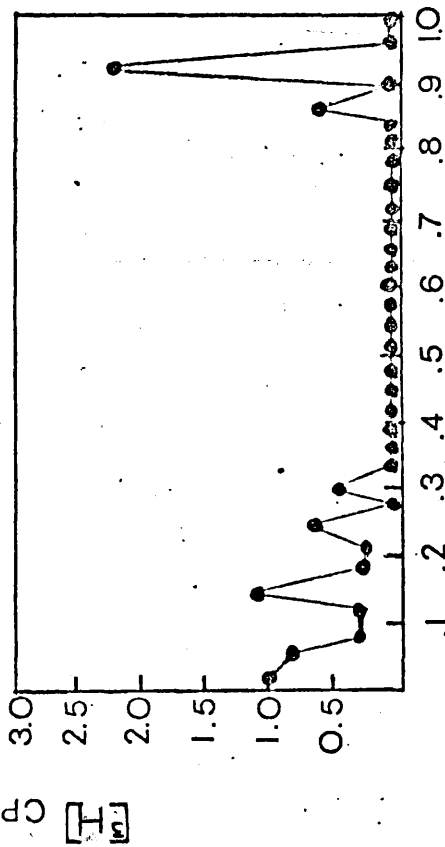Figure 6.   Sodium dodecyl sulphate (SDS)-polyacrylamide
              electrophoresis of [^{3}H] -labelled proteins isolated
              from the vinblastine precipitated fractions (at
              pH 8.3) of the post-ribosomal supernatants of
              eggs before and after fertilisation.

Experimental details are as described in Figure 5. The vinblastine
precipitated pellets were resuspended in 100μl SDS electrophoresis
buffer and heated at 100°C for 5 min. After dialysis against SDS
electrophoresis buffer, 100 μl samples were analysed on SDS-
polyacrylamide gels as described in Methods. All proteins migrate
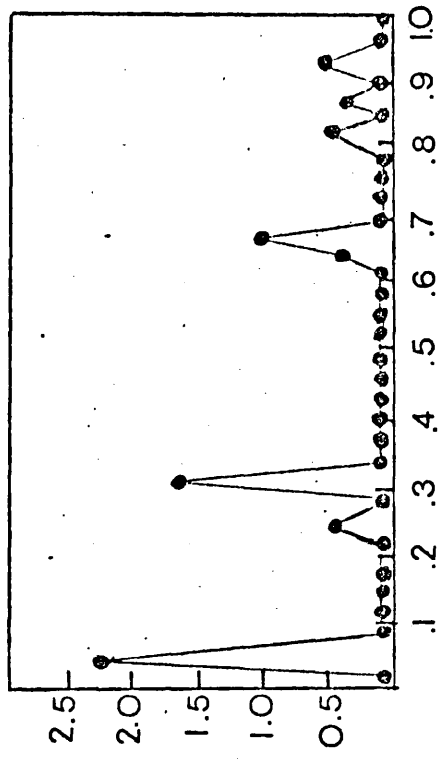towards the anode, shown on the right. (Microtubulin proteins =
m.t.)

EGG

FERTILISED EGG
[³H] leucine added at O hour

FERTILISED EGG
[³H] leucine added after 1 hour

FERTILISED EGG
[³H] leucine added after 2 hours
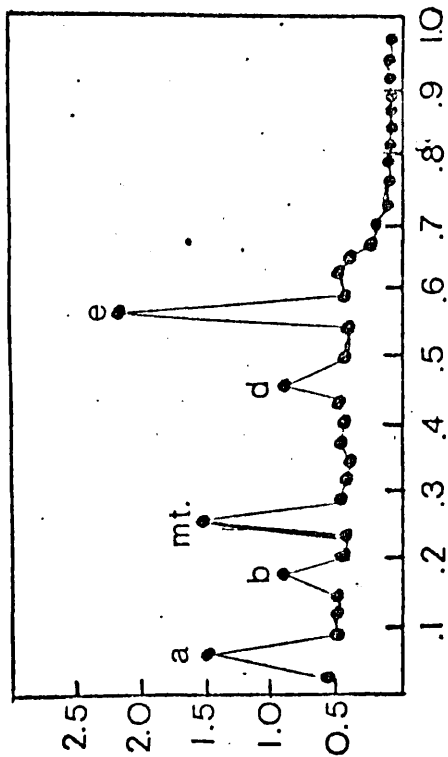
[³H] CPM x 10⁻²

RELATIVE MOBILITY

Figure 7.   Sodium dodecyl sulphate (SDS)-polyacrylamide
electrophoresis of [^{3}H] -labelled proteins isolated
from the vinblastine precipitated fractions (at
pH 7.0) of the post-ribosomal supernatants of eggs
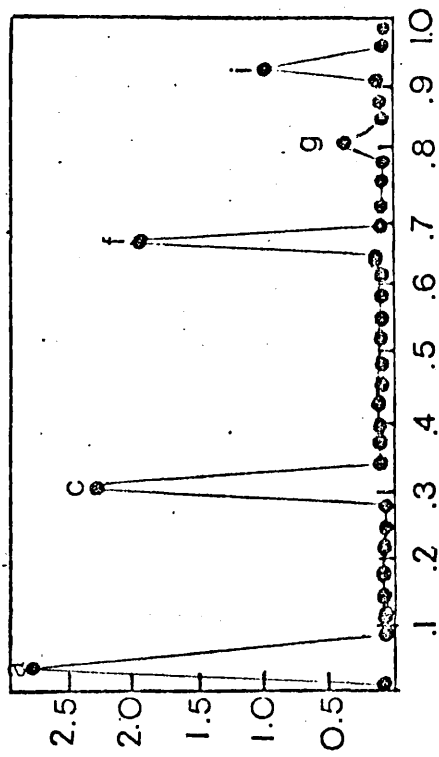before and after fertilisation.

Experimental details are as described for Figure 6, except that the
post-ribosomal supernatants were first adjusted to pH 7.0 with 1 MHCl
before addition of vinblastine sulphate.

The peaks are labelled alphabetically, a to i, according to the
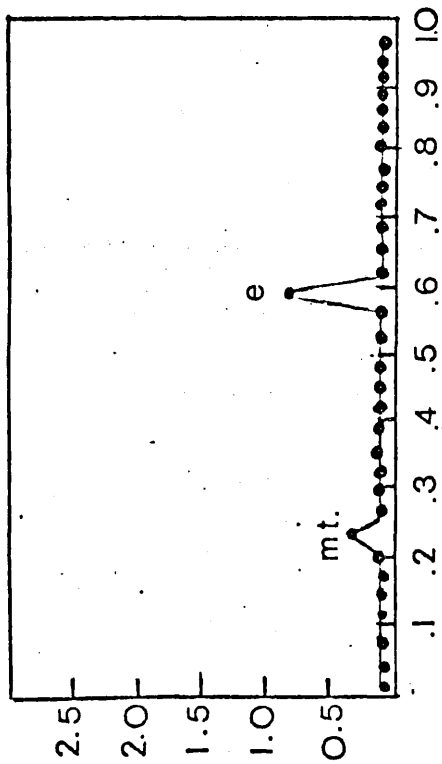increasing mobilities. Microtubulin = m.t.
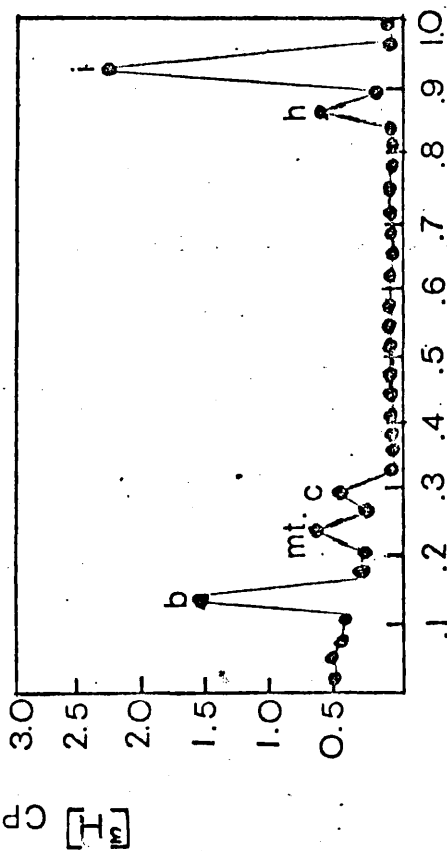
EGG

FERTILISED EGG
[3H] leucine added at O hour

FERTILISED EGG
[3H] leucine added after 1 hour

FERTILISED EGG
[3H] leucine added after 2 hours

RELATIVE MOBILITY

[3H] CPM x 10-2

Table 8

A summary of the vinblastine precipitated, $[^3H]$-labelled proteins from the post-ribosomal supernatants of eggs before and after fertilisation (based on Figure 7).

			Stage of development			
Peak	Relative mobility	M.W.	Egg	Fertilised egg		
				0-1h	1-2h	2-3h
a	0.05	85,000	−	+	+	+
b	0.17	68,000	−	+	+	−
mt	0.23	53,000	+	+	+	−
c	0.30	48,000	−	−	+	−
d	0.46	36,500	−	+	−	−
e	0.59	27,000	+	+	−	−
f	0.68	19,000	+	−	−	+
g	0.80	15,000	−	−	−	+
h	0.86	13,000	−	−	+	−
i	0.94	11,000	−	−	+	+

+ present; − absent.

5. Studies on stored m-RNAs for vinblastine-precipitable proteins.

From the data so far, it would appear that specific m-RNAs, as identified by their ability to synthesis vinblastine-precipitable proteins in vivo, are stored in the egg. As a possible approach to understanding the mechanisms involved in their storage and differential utilisation after fertilisation, their cellular distributions during early embryogenesis were studied.

5.1 Cell-free systems

m-RNA activity were assayed in either the rabbit reticulocyte or wheat germ cell-free systems. The results shown in Figure 8 confirm earlier reports (162) that hemin stimulates protein synthesis by the reticulocyte system and maintains a linear rate during the first hour of incubation. Hemin was routinely added to assay mixtures using this system.

Various properties of the wheat germ cell-free system were studied using ovalbumin and globin m-RNAs as the exogenous m-RNAs. For example, these RNAs increased the incorporation of $\left[{}^{14}C \right]$ - leucine into TCA-precipitable proteins as compared to the controls (Figure 9). Evidence that exogenous m-RNAwas specifically responsible for this stimulation is presented in Figures 10 and 11. Purified E.coli ribosomal RNA (in amounts up to 12.5 μg per assay) only marginally stimulated leucine incorporation over control (Figure 10). In contrast, ovalbumin m-RNA markedly increased leucine incorporation, the increase being linear up to 5 μg m-RNA per assay (Figure 11).

Earlier reports that wheat germ system is prone to "premature termination" (see Introduction) are obviously a matter of concern. It is not clear whether "premature termination" results in the production of nascent or released protein fragments. Identification of the m-RNAs in question is solely dependent upon the recognition of radioactively labelled proteins which are precipitated by vinblastine and have particular mobilities on SDS-gels. The presence of major quantities of partially completed proteins would make identification very difficult, if not impossible. The observation that leucine incorporation was only linear for the first 30-40 min (Figure 9) suggests that some component(s) of the system becomes limiting. This conclusion is supported by the results shown in Figure 12. The same quantity of ovalbumin m-RNA was much less effective in stimulating leucine incorporation when added to a pre-incubated wheat germ system. A possible explanation is that an important component(s) is relatively unstable at the incubation temperature, 25°C

Figure 8. Effect of hemin on the kinetics of protein synthesis in the rabbit-reticulocyte cell-free system.
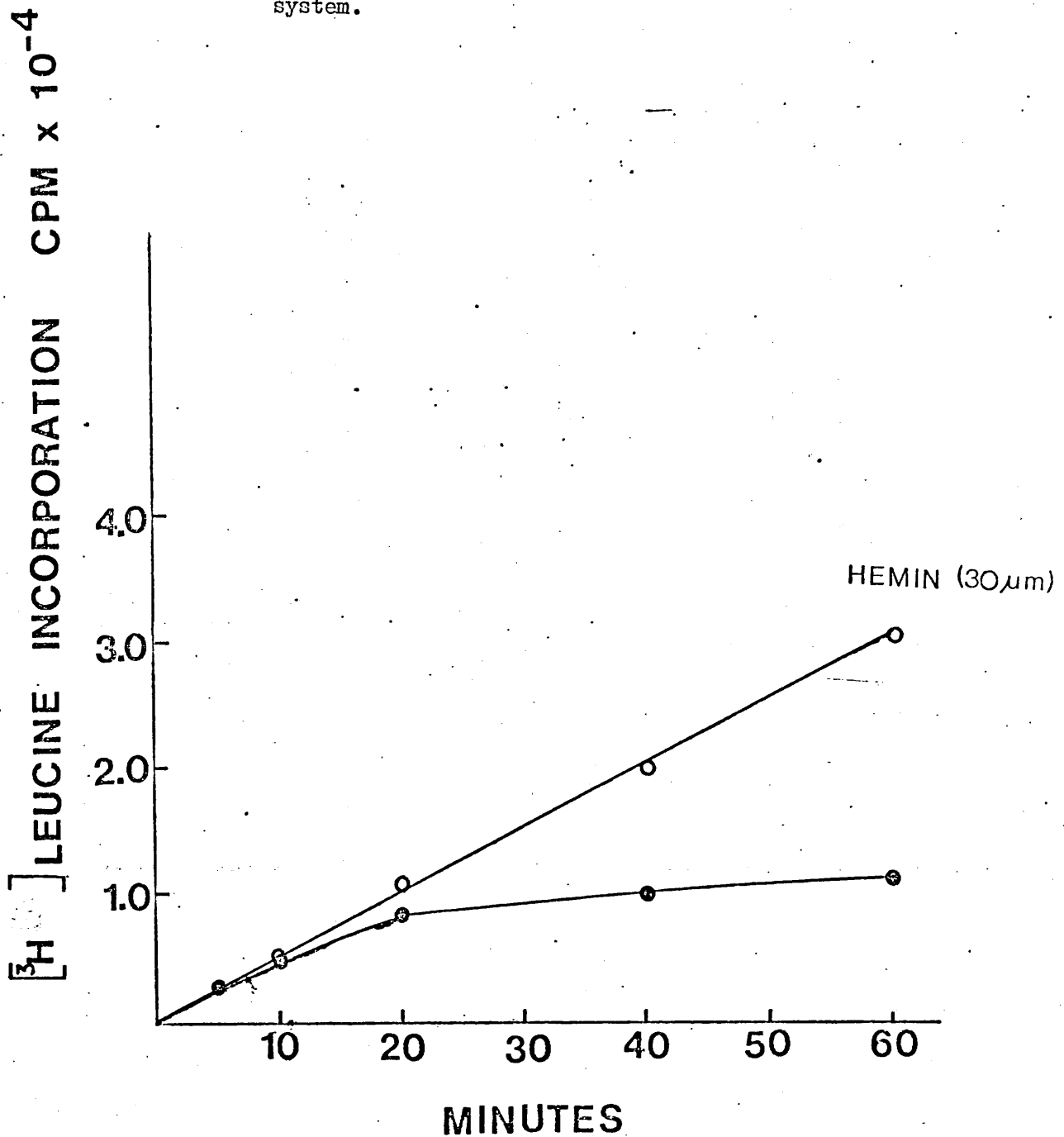
Reaction mixtures (50 μl) containing 4 μCi[^{3}H]-leucine (sp.act. 1 Ci/mM with (0) and without (●) added hemin were incubated at 25°C. At the times indicated 20 μl aliquots were withdrawn and counted as described in Methods. The values have been corrected for 50 μl samples.
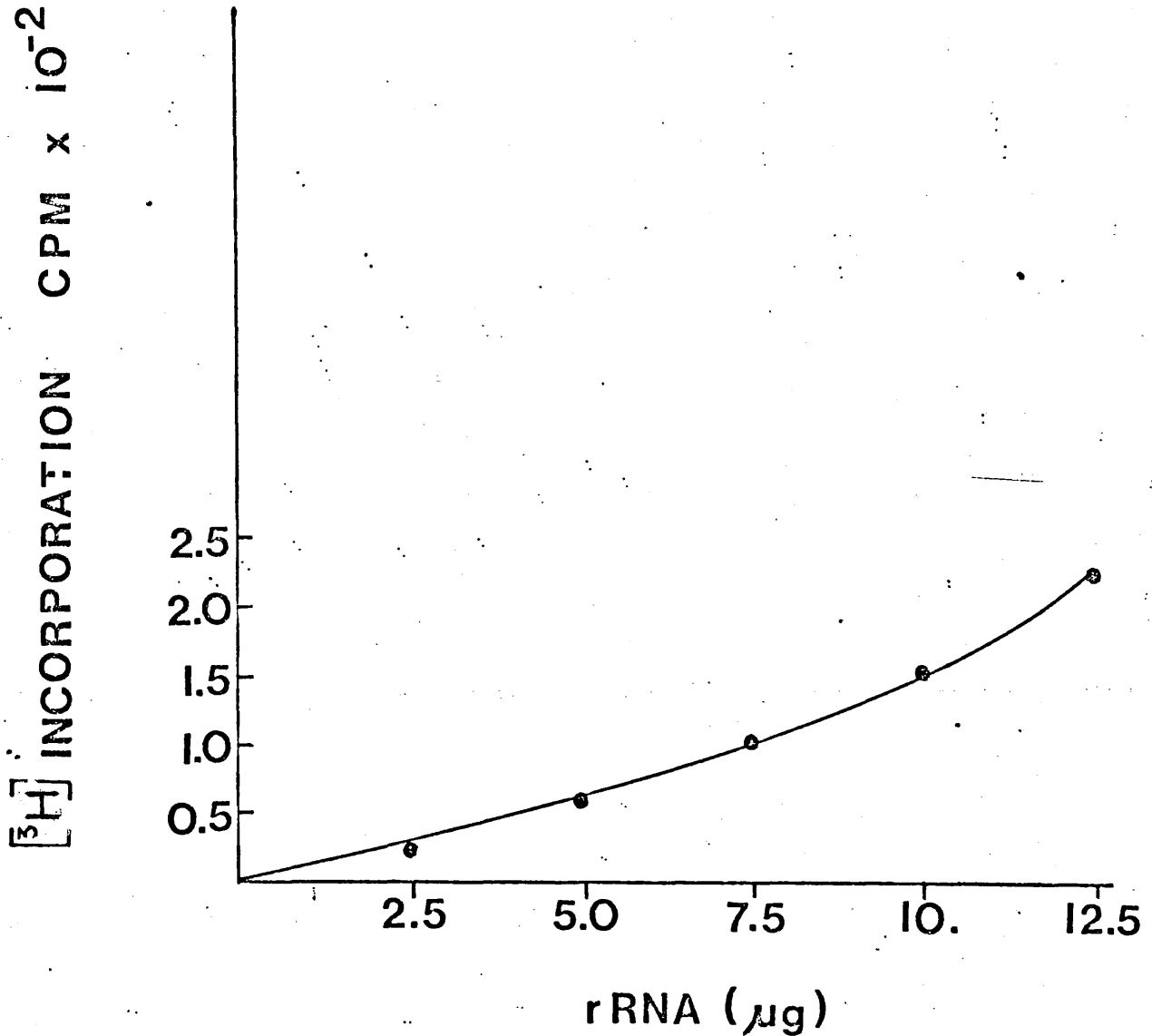
Figure 9.   Kinetics of protein synthesis directed by rabbit
             globin and ovalbumin m-RNA in wheat germ extracts.
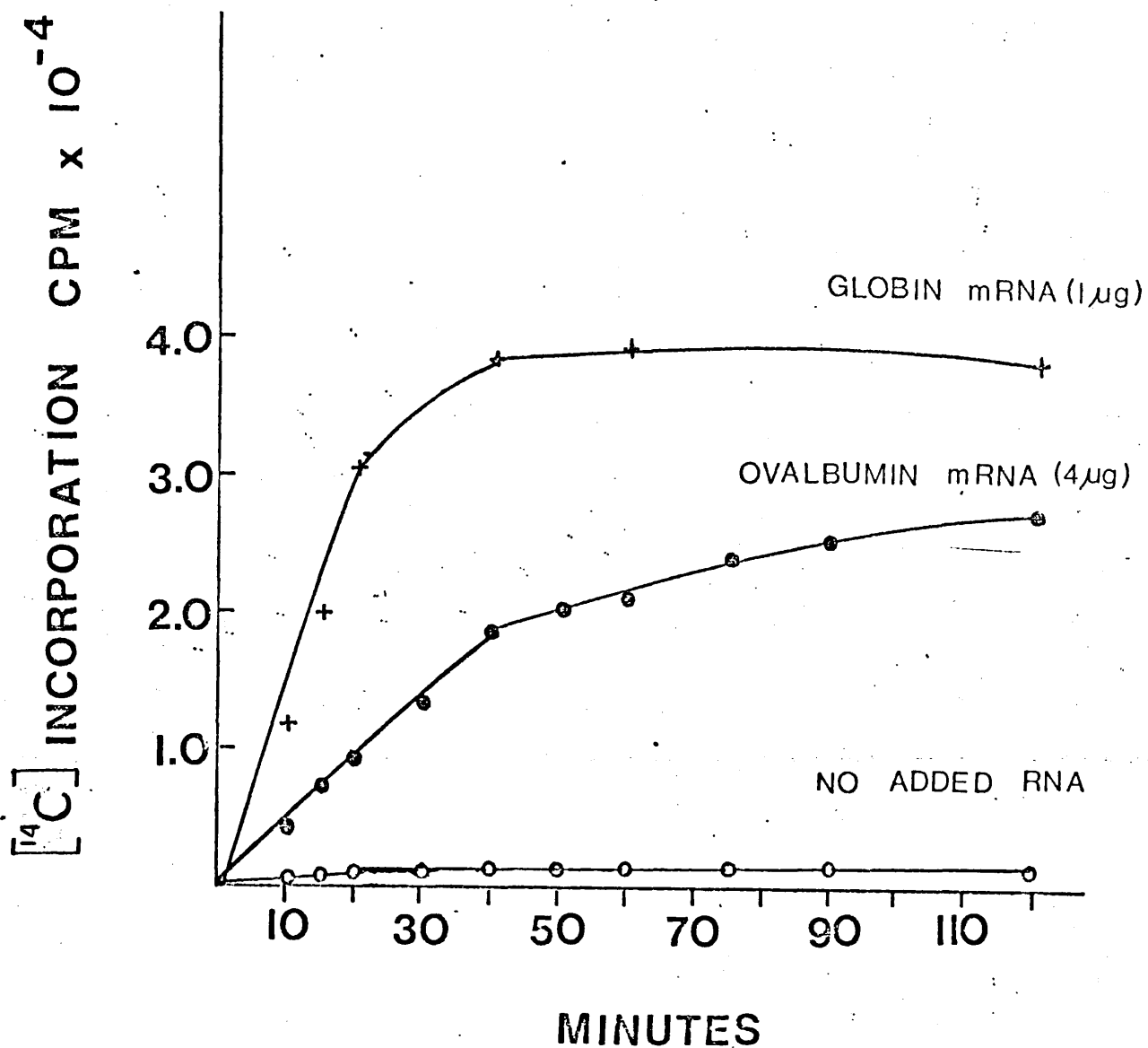
Reaction mixtures (50 μl) containing 1 μCi $\begin{bmatrix} {}^{14}C \end{bmatrix}$ - leucine (sp. act. 348
mCi/mmol). with and without added m-RNAs were incubated at 25°C. At
the times indicated 20 μl aliquots were withdrawn and counted as
described in Methods. The values have been corrected for 50 μl samples
and the backgrounds have been subtracted.

Figure 10.   Effect of E. coli ribosomal RNA on protein synthesis
in wheat germ extracts.

Reaction mixtures (50 μl) containing 4 μCi $[^3H]$-leucine (sp. act.
1Ci/mmol). and different amounts of E.coli r-RNA were incubated at
25°C for 30 min. 20 μl aliquots were withdrawn and counted, as
described in Methods. The values have been corrected for 50 μl
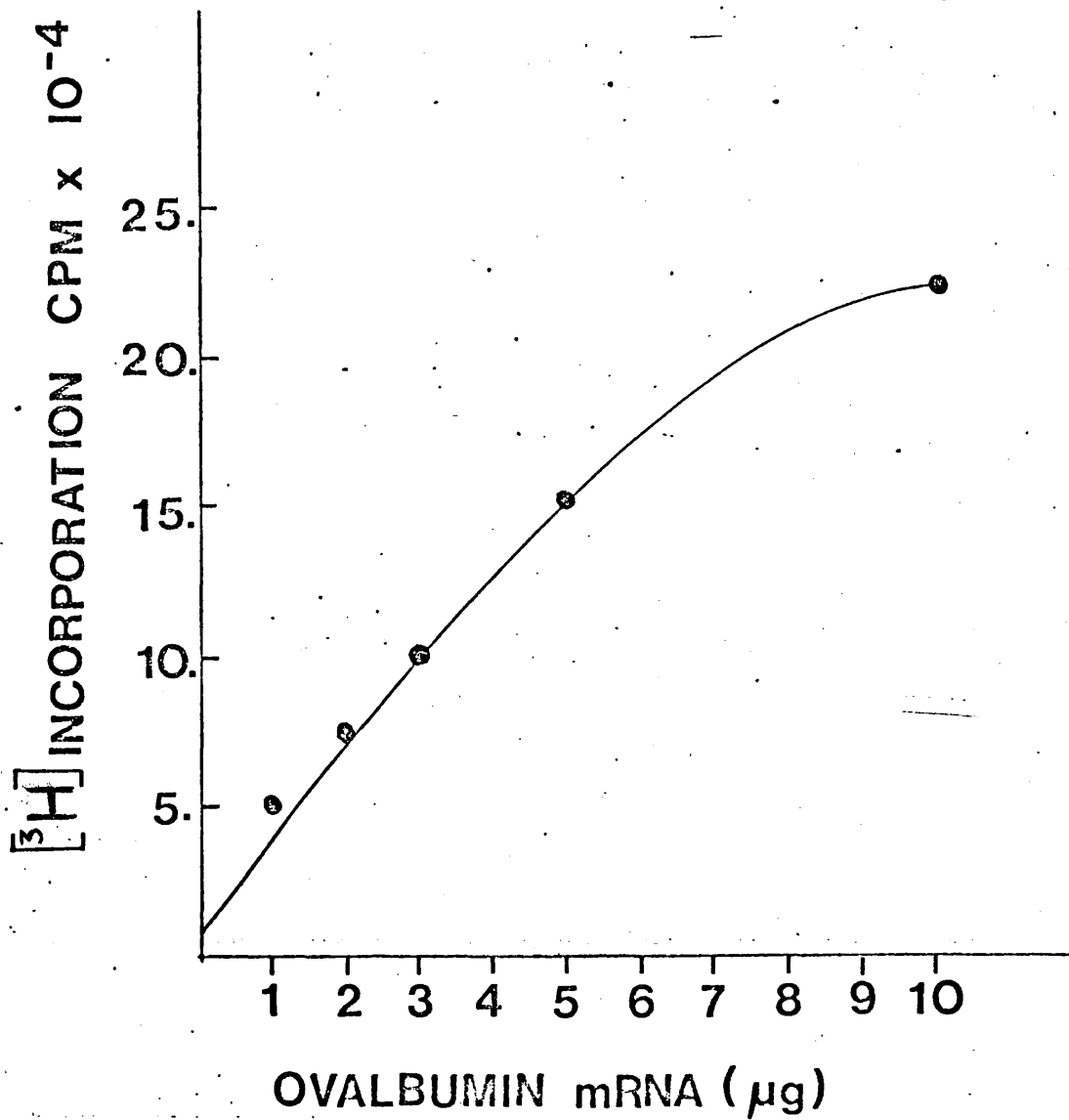samples and the backgrounds have been subtracted.

Figure 11. Effect of ovalbumin m-RNA on protein synthesis in wheat germ extracts.

Experimental details are as described for Figure 10 except that different amounts of ovalbumin m-RNA were used.

Figure 12.   Effect of pre-incubation of the wheat germ extract
              on the kinetics of protein synthesis directed by
              ovalbumin m-RNA.
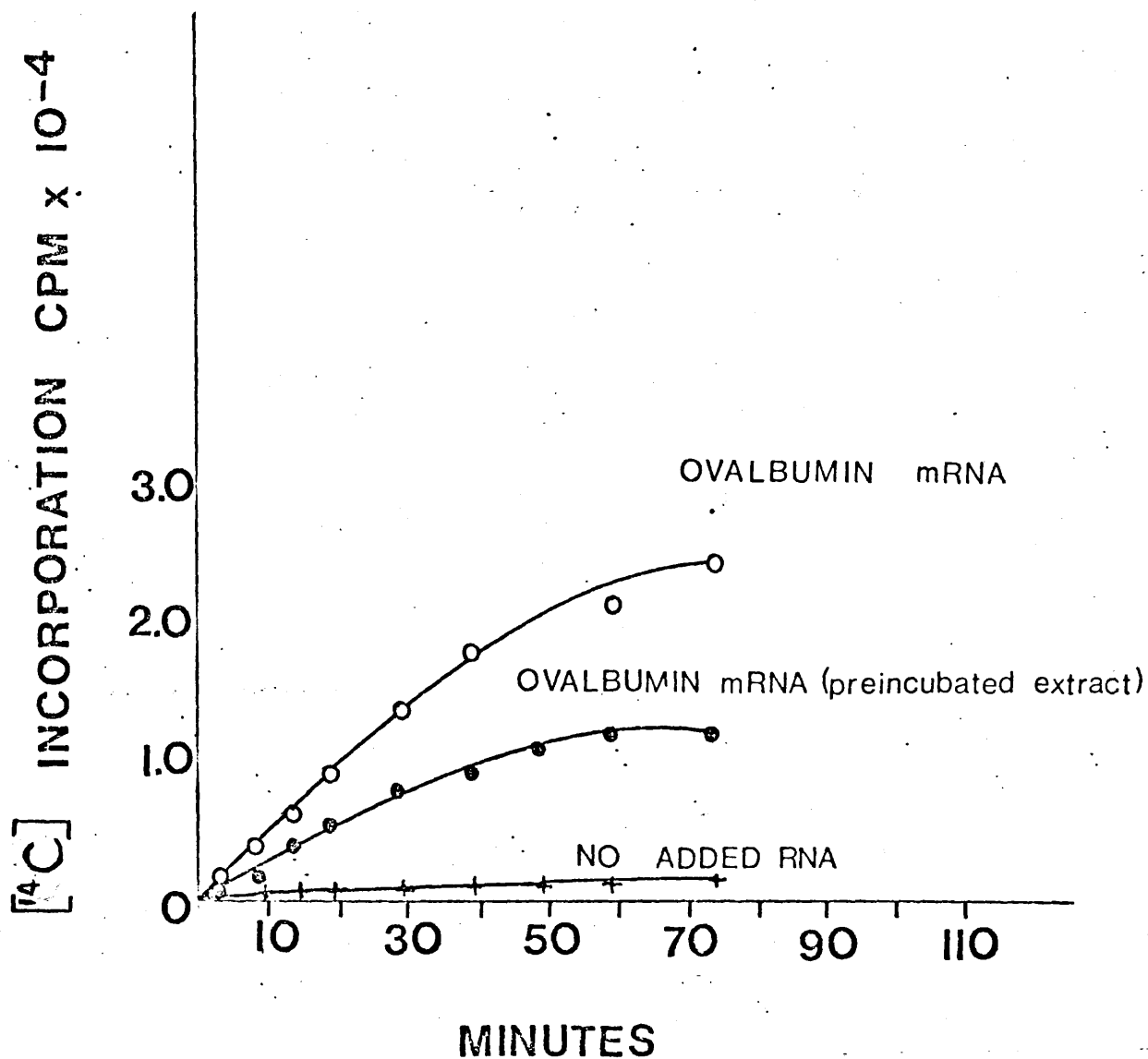
Two sets of reaction mixtures (50 µl) containing 1 µCi of $[^{14}C]$-
leucine (sp. act. 348 mCi/mmol) and 5 µg ovalbumin m-RNA were
prepared so that in one set the wheat germ extract had been pre-incubated
at 25°C for 60 min. A control set had no added m-RNA. The reaction
mixtures were then incubated at 25°C and at the times indicated, 20 µl
aliquots were withdrawn and counted as described in Methods. The values
have been corrected for 50 µl samples.

and eventually limits some aspect of protein synthesis. In order to test that the effect of incubation time on the types of products synthesised in vitro under the direction of ovalbumin m-RNA, reaction mixtures incubated for different time periods were analysed by SDS-gel electrophoresis (Figure 13). The labelled products included both released (supernatant) and nascent (polysome-associated) poly peptides. The m-RNA preparation predominantly codes for ovalbumin (m.w. 44,000) but also contains conalbumin and lysozyme m-RNAs as minor components. About 20-25% of the labelled proteins migrated at the position expected for ovalbumin. Most of the ovalbumin was synthesised in the first 30 min of incubation. Conalbumin was not synthesised in the wheat germ system, as observed by other workers (179). Except for lysozyme, the remaining radioactivity may either represent nascent polypeptide frag-ments,prematurely released polypeptide fragments or a combination of both. Measurements of TCA-precipitable labelled protein in the supernatant fractions showed that only 30-35% of that incorporated into total protein was released after 30 minutes. Prematurely released polypeptides were not, therefore, a major problem in interpreting the electrophoretic profiles. In retrospect, electrophoretic analysis of the supernatants alone would have been a better method of resolving this problem.

Several lines of evidence suggest that some component involved in protein elongation rather than initiation is responsible for the decline in leucine incorporation after 30 minutes. Firstly, only small amounts of completed ovalbumin were synthesised after 30 minutes (Figure 12). Secondly, electrophoretic analysis of the products synthesised in the preincubated system showed thatproportionately less completed ovalbumin (about 10% of total) was synthesised than in the corresponding assay without preincubation (Figure 14). The proportion would have been the same, if the decreased incorporation was due to impaired initiation.

Polysomal RNA isolated from plaice embryos was tested for m-RNA activity in both cell-free systems. Plaice RNA increased the level of leucine incorporation into TCA and vinblastine-precipitable proteins as compared to the controls in the wheat germ system. The rate of incorporation was linear for 40 minutes and proportional to the amount of RNA added (up to 12.5 μg per 50 μl assay). Therefore, the wheat germ system is useful in assaying both total and vinblastine-precipitable protein m-RNA activities. In contrast, the reticulocyte system could not be used for assaying total m-RNA activity since addition of plaice

RNA decreased the rate of leucine incorporation into TCA-precipitable proteins. However, the observation that plaice RNA stimulated the synthesis of vinblastine-precipitable proteins did suggest the usefulness of the reticulocyte system for assaying these particular m-RNAs.

<u>Figure 13</u>   Sodium dodecyl sulphate (SDS)-electrophoresis of the proteins synthesised in the wheat germ cell-free system in the presence of ovalbumin m-RNA.

The reaction   mixture (100 µl) containing 2 µCi$\left[^{14}C\right]$ - leucine (sp.act. 348 mCi/mmol) and 11.5 µg of ovalbumin m-RNA was incubated at 25°C.   25 µl samples were removed after 30, 90 and 120 min and diluted with 25 µl SDS electrophoresis buffer. After heating at 100°C for 5 min, the samples were analysed on SDS-polyacrylamide gels as described in <u>Methods</u>.   All proteins migrate towards the anode, shown at the right.   The arrows indicate the relative mobilities for conalbumin, ovalbumin and lysozyme.

30 minute incubation

90 minute incubation

120 minute incubation
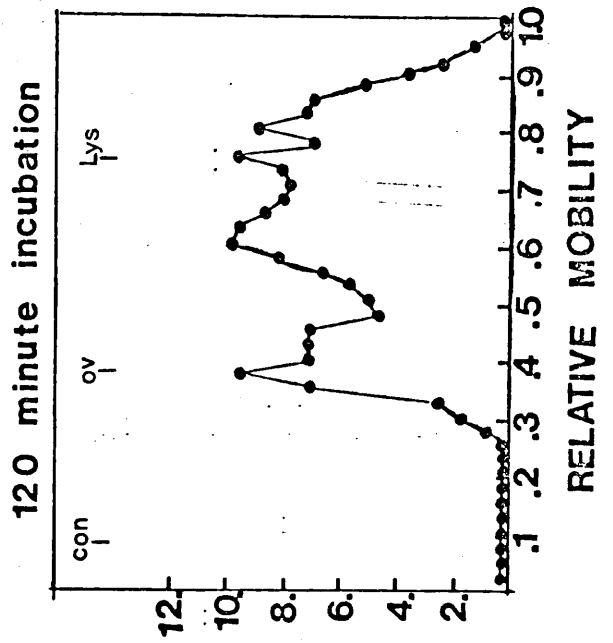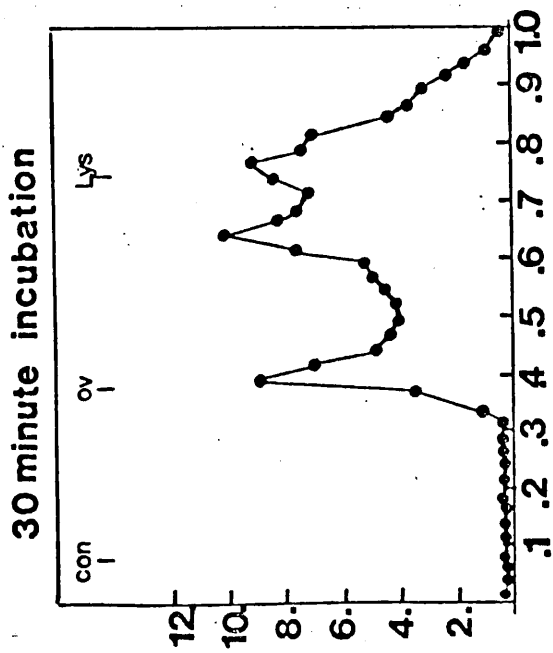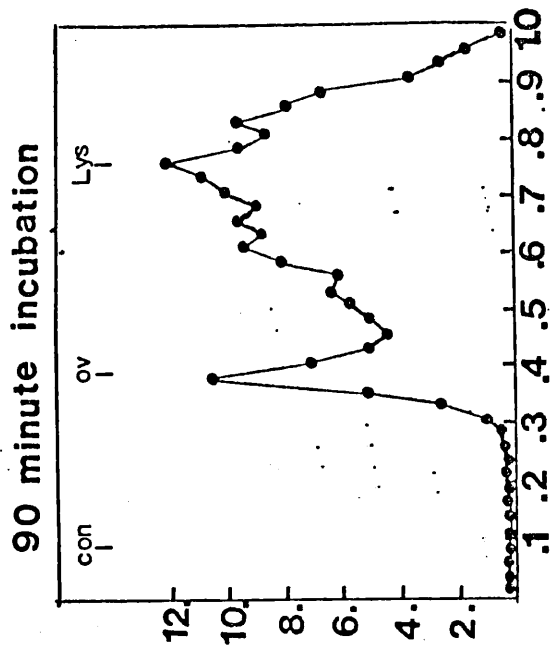
[^{14}C] INCORPORATION CPM × 10^{-2}

RELATIVE MOBILITY

Figure 14. Sodium dodecyl sulphate (SDS)-electrophoresis of the
proteins synthesized in the pre-incubated wheat germ
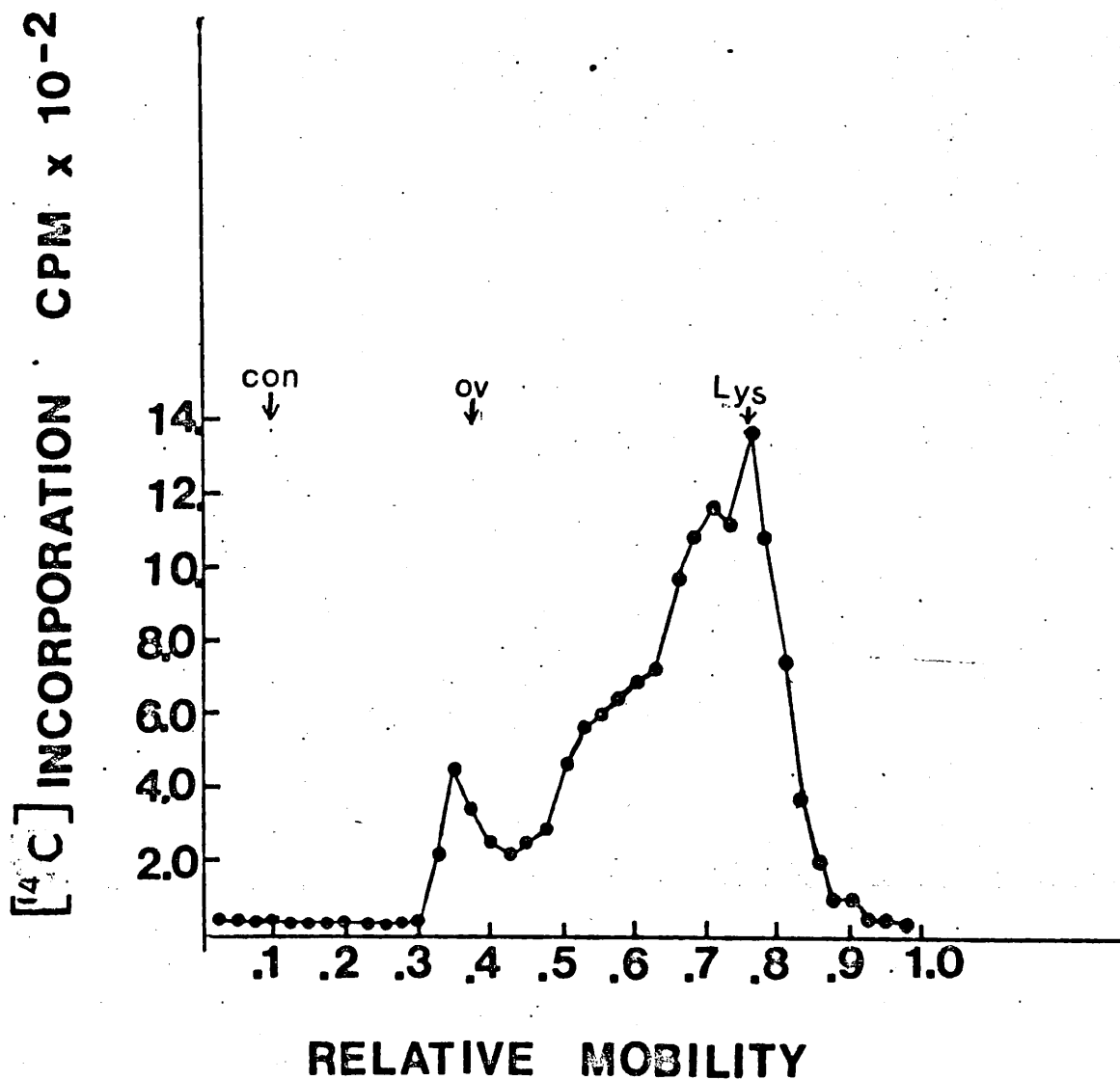cell-free systems in the presence of ovalbumin mRNA.

The reaction mixture (50 μl) containing 1 μCi of [^{14}C]leucine (sp.
act. 348 mCi/mmol), 5 μg of ovalbumin mRNA and wheat germ extract
(pre-incubated at 25°C for 60 min) was incubated at 25°C for 40
min. A 25 μl sample was removed, diluted with 25 μl of SDS
electrophoresis buffer, and heated at 100°C for 5 min. The sample
was then analysed on an SDS-polyacrylamide gel as described in
Methods. All proteins migrate towards the anode, shown at the right.
The arrows indicate the relative mobilities for conalbumin, oval-
bumin and lysozyme.

* The samples were hand-homogenised in a Teflon-glass
homogeniser (clearance 0.45 mm) in two volumes (w/v)
of the buffer..

5.2   Isolation of plaice RNA

RNA was isolated from microsomal and supernatant fractions
obtained from unfertilised and fertilised eggs (see Scheme 1 for
fractionation procedure, except a $\not\times$ TMK buffer was used, see Methods).
Special precautions were taken to minimise RNase activity. All glass-
ware and solutions were routinely sterilised and the latter treated
with diethylpyrocarbonate. It was also necessary to add heparin (900μg/ml)
to the homogenisation buffer. RNA isolated in the absence of heparin was
extensively degraded, as shown by electrophoretic analysis in SDS - 2.6%
polyacrylamide gels. Heparin, which also inhibits protein synthesis in
vitro was removed from the ethanol-precipitated RNA by washing in 3 M
sodium acetate. Sucrose gradient analysis confirmed earlier reports
(159.) that the K^+/Mg^{++} ratio was important in maintaining polysomal
integrity. Concentrations of 300mM K^+ and 10mM Mg^{++} were found to be optimum.
Nevertheless, the microsomal fraction was predominantly monosomal at the
stages examined. The phenol/chloroform method (155) produced the best
quantitative and qualitative yields of RNA of the phenol methods tested.
Poly (A)-containing RNA was isolated from polysomal or supernatant RNAs
by affinity chromatography with oligo (dT)-cellulose. Formamide-RNA
samples were also fractionated on 5-20% sucrose gradients.

5.3   Intracellular distribution of m-RNA in unfertilised and fertilised eggs.

To examine the effect of fertilisation on the intracellular
distribution of m-RNA, polysomal and supernatant RNAs isolated from
unfertilised and fertilised eggs were assayed for m-RNA activity in
the wheat germ system (Table 9). m-RNA activity was almost exclusively
associated with polysomal RNA, except for the unfertilised egg where a
significant amount was found in the supernatant RNA fraction. Almost all
m-RNA activity was found in the non-poly (A)-containing RNA fractions.
These observations were true for both total and vinblastine-precipitable
protein m-RNAs. The relative proportion of poly (A) to non-poly (A)-
containing RNA in polysomal RNA increased after fertilisation. In addition,
the relative proportion of vinblastine-precipitable protein m-RNAs to
other m-RNAs was also increased after fertilisation.

The simplest explanation for these results is that two classes
of m-RNA exist in the mature egg, one class which is being translated in
the polysomes and a second class which is stored in the cytoplasm.
Fertilisation results in the activation of the stored m-RNA which is then
transported to and translated in the polysomes. At least a portion of

Table 9

M-RNA activity in RNA fractions isolated from eggs before and
after fertilisation.

Polysomal and supernatant RNAs were isolated from 100 g quantities
of eggs. The RNAs were further fractionated on oligo (dT) –
cellulose columns into poly A and non-poly A containing fractions.
All RNA fractions were tested for m-RNA activity in the wheat germ
cell-free system. Reaction mixtures (50 μl) containing 4 μCi of
$[^3H]$-leucine (sp. act. 1 Ci/mmol) and 8 μg RNA were incubated at
25°C for 40 min. $[^3H]$ incorporation into TCA-precipitated proteins
and vinblastine-precipitated proteins was estimated with separate
reaction mixtures. Experimental details are as described in Methods.
1 mg of RNA was assumed to be equivalent to 20 absorbance units at
260 nm.

Table 9

Messenger RNA activity in RNA fractions isolated from eggs before and after fertilisation.

	RNA (mg)	mRNA activity-$[^3H]$ incorporation (cpm x 10^{-3})			
		TCA-precipitated		Vinblastine-precipitated	
		Total	Sp. act.*	Total	Sp. act.*
Egg					
Polysomal RNA					
total	19.50	7664	393	-----	-----
poly A	0.65	129	199	-----	-----
non-poly A	17.74	6209	350	2803	158
Supernatant RNA					
total	4.65	1507	324	-----	-----
poly A	0.07	----	---	-----	-----
non-poly A	2.18	985	452	490	225
Fertilised egg (after 1 hour)					
Polysomal RNA					
total	16.90	6321	374	----	----
poly A	1.09	534	490	143	131
non-poly A	14.90	5900	396	2816	189
Supernatant RNA					
total	0.50	123	246	-----	-----
poly A	0.02	----	----	-----	-----
non-poly A	0.45	86	190	27	60
Fertilised Egg (after 3 hours)					
Polysomal RNA					
total	26.35	9802	372	----	----
poly A	1.83	573	313	249	136
non-poly A	21.40	8881	415	5157	241
Supernatant RNA					
total	0.41	41	99	----	----
poly A	0.01	----	---	-----	-----
non-poly A	0.28	21	76	4	14

---- no measurement taken

* Sp. act. = Specific activity (cpm x 10^{-3}/mg of RNA)

these require polyadenylation. The appearance of relatively more
vinblastine-precipitated protein m-RNAs in the polysomes after
fertilisation may result from new RNA synthesis although the results
with inhibitors (Table 5) suggest this is not the case. However,
interpretation of these results is limited to some extent by the
semi-quantitive nature of the RNA isolation procedure as well as the
translational variability of the wheat germ system with different
m-RNA populations.

5.4 Identification of specific m-RNAs for vinblastine-precipitable proteins.

Earlier studies have shown that specific vinblastine-precipitable
proteins were synthesised in the plaice egg at specific times before and
after fertilisation (Table 8). The object of the remaining experiments
was an attempt to identify the presence of their respective m-RNAs in the
different RNA fractions prepared as in Table 9. RNA samples were assayed
with either the reticulocyte or wheat germ cell-free systems and the
vinblastine-precipitable products were analysed by SDS-polyacrylamide gel
electrophoresis. Both sets of results, represented in Figure 15
(reticulocyte system) and Figure 16 (wheat germ system), were extremely
disappointing because of the lack of clearly defined, labelled peaks. It
was hoped to compare the electrophoretic mobilities of the in vitro
labelled proteins with those obtained for the in vivo labelled proteins.
Nevertheless, the electrophoretic profiles did provide some useful informa-
tion. For example, the observation that all RNA fractions examined
directed the synthesis of vinblastine-precipitable proteins in vitro
suggests the presence of vinblastine-precipitable protein m-RNAs in
each RNA fraction. Further, the reticulocyte and wheat germ cell-free
systems have different translational efficiencies with the same RNA.
preparations since different electrophoretic profiles were obtained from
assays with identical RNA samples. That the "poly A" polysomal RNA
fraction of the fertilised egg (1 hour) contains microtubulin m-RNAs
is supported by the presence of a well defined peak with the electro-
phoretic mobility expected of microtubulin protein (mt, Rf = 0.23).
This peak was identified in the electrophoretic profiles obtained from both
cell-free systems. No microtubulin-like peak was distinguishable in
other profiles. One is tempted to suggest that microtubulin m-RNA is
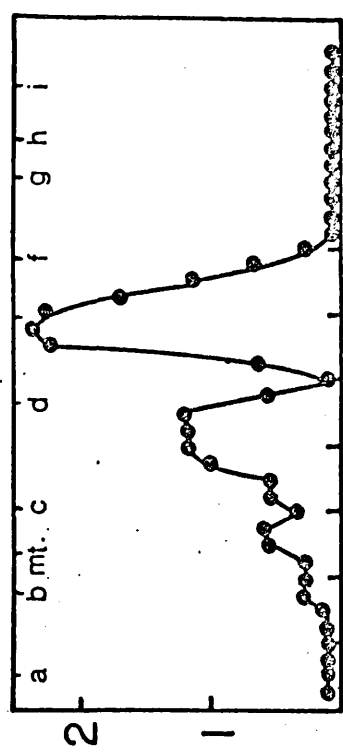polyadenylated prior to translation.

Several factors may be responsible for the inadequate resolution
of discrete vinblastine-precipitable proteins synthesised in vitro.

Figure 15.   Sodium dodecyl sulphate (SDS)-polyacrylamide
              electrophoresis of the vinblastine-precipitated
              proteins synthesised in the rabbit-reticulocyte
              cell-free system in the presence of RNA fractions
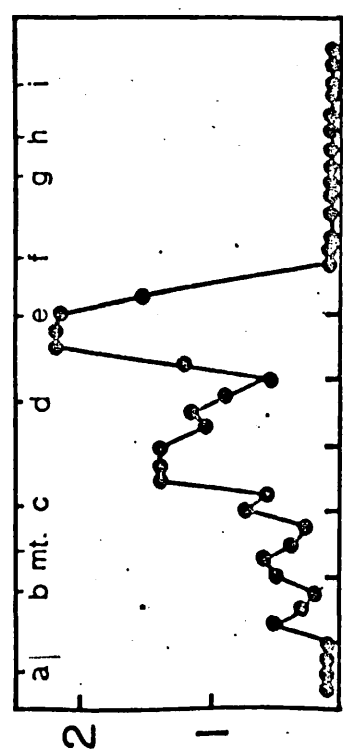   isolated from different developmental stages.

Assays (100 μl) containing 8 μCi of $\left[^{3}H\right]$-leucine (sp. act. 1
Ci/mmol) and 35 μg RNA were incubated for two h. at 25°C. The
RNA was isolated and fractionated as described in Methods. The
vinblastine-precipitated proteins were subjected to SDS-
polyacrylamide electrophoresis. Protein migration is towards
the anode, shown at the right. The control values (in the absence
of added RNA) have been subtracted. The relative mobilities of
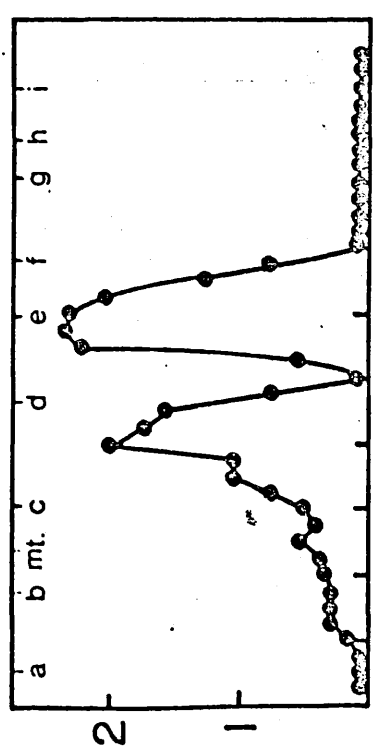the in vivo labelled proteins are designated as in Table 8.
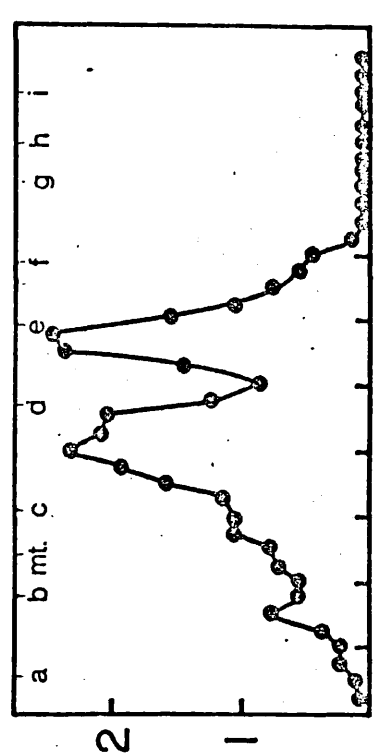
STAGE: EGG

TOTAL POLYSOMAL RNA

"POLY A" POLYSOMAL RNA

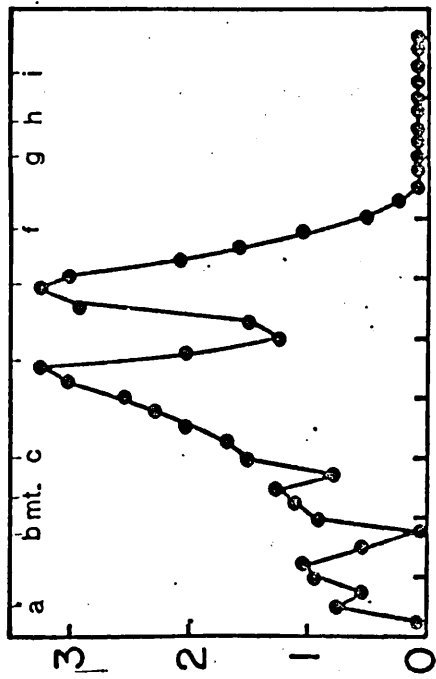"NON-POLY A" POLYSOMAL RNA

SUPERNATANT RNA

[³H] CPM x 10⁻²

RELATIVE MOBILITY

STAGE: EGG – ONE HOUR AFTER FERTILISATION

TOTAL POLYSOMAL RNA

"POLY A" POLYSOMAL RNA
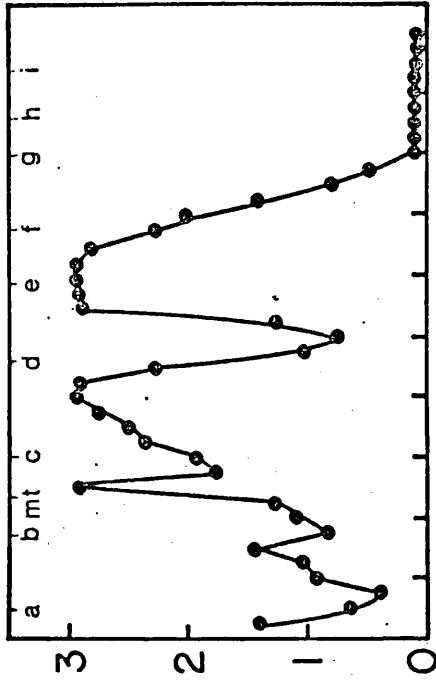
SUPERNATANT RNA

"NON – POLY A" POLYSOMAL RNA

[³H] CPM x 10⁻²

RELATIVE MOBILITY

STAGE: EGG - THREE HOURS AFTER FERTILISATION

TOTAL POLYSOMAL RNA

"POLY A" POLYSOMAL RNA

"NON-POLY A" POLYSOMAL RNA

SUPERNATANT RNA

$[^3H]$ CPM x 10^{-2}
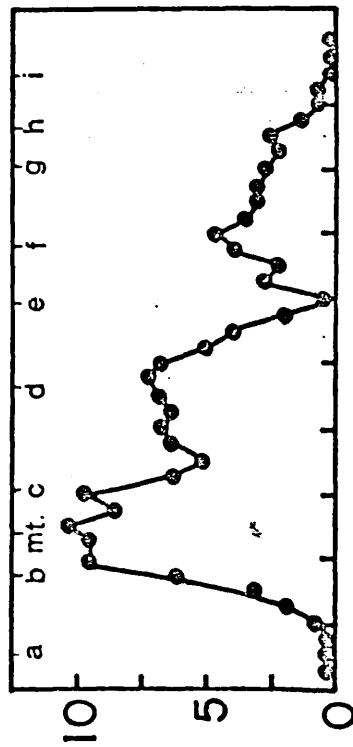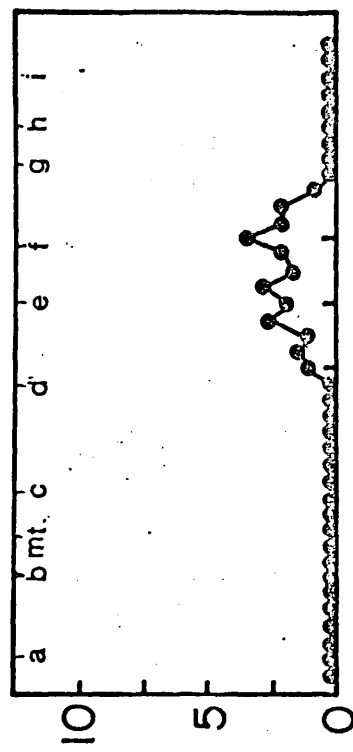
RELATIVE MOBILITY

Figure 16.   Sodium dodecyl sulphate (SDS)-polyacrylamide
electrophoresis of the vinblastine-precipitated
proteins synthesised in the wheat germ cell-free
system in the presence of RNA fractions isolated
from different developmental stages.

Assays (100 μl), containing 8 μCi of $\left[^3H\right]$-leucine (sp. act.
1 Ci/mmol.) and 16 μg of RNA were incubated for 40 min at 25°C.
The RNA was isolated and fractionated as described in Methods.
The vinblastine-precipitated proteins were subjected to SDS-
polyacrylamide electrophoresis. Protein migration is towards
the anode, shown at the right. The control values (in the absence
of added RNA) have been subtracted.

STAGE: EGG
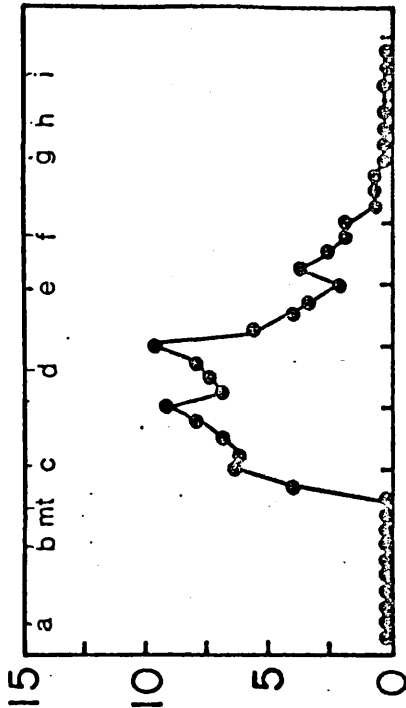
"POLY A" POLYSOMAL RNA

SUPERNATANT RNA

TOTAL POLYSOMAL RNA

"NON-POLY A" POLYSOMAL RNA

$[^3H]$ CPM x 10^{-2}
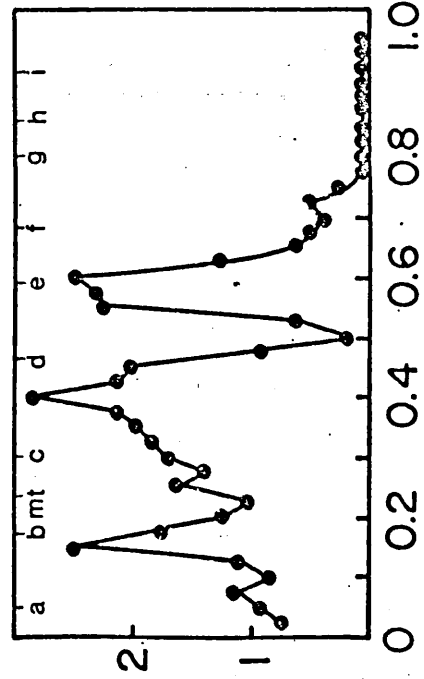
RELATIVE MOBILITY

STAGE: EGG – ONE HOUR AFTER FERTILISATION

STAGE: EGG - THREE HOURS AFTER FERTILISATION

TOTAL POLYSOMAL RNA

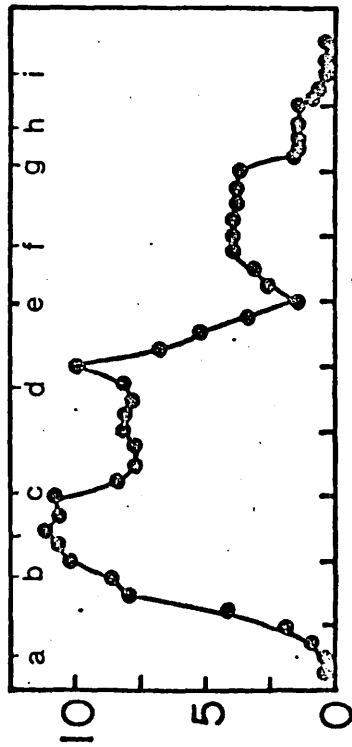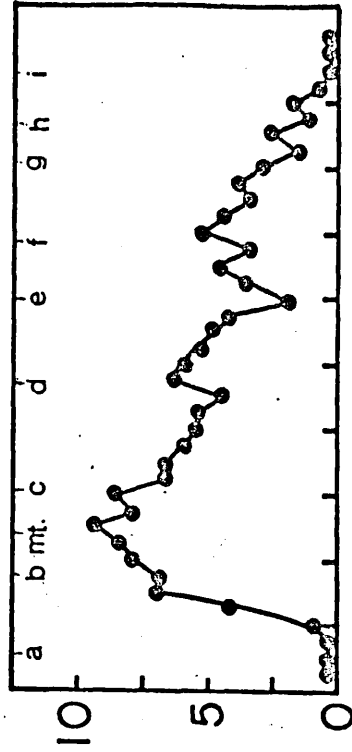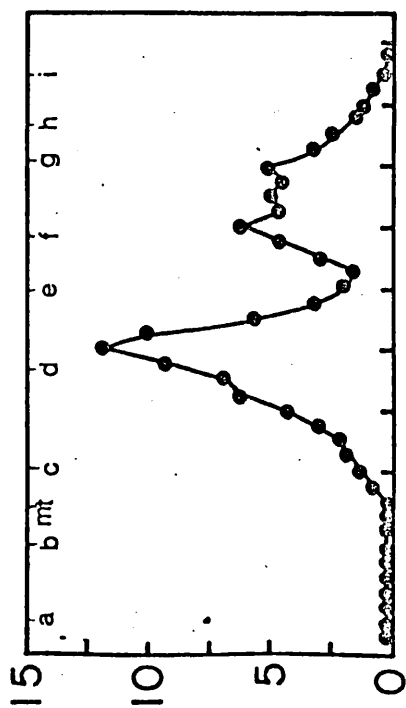"POLY A" POLYSOMAL RNA

"NON-POLY A" POLYSOMAL RNA

SUPERNATANT RNA

[³H] CPM x 10⁻²
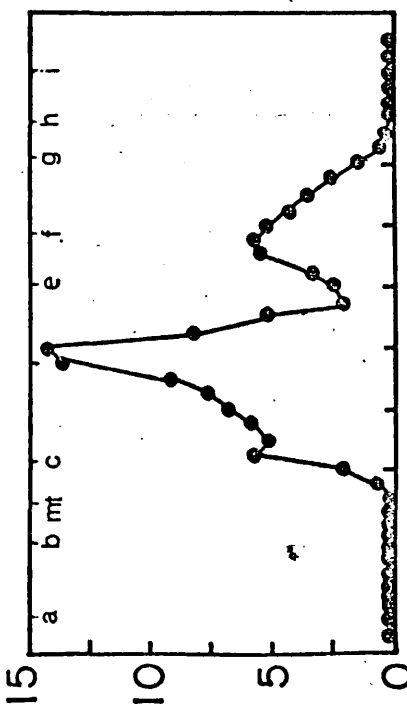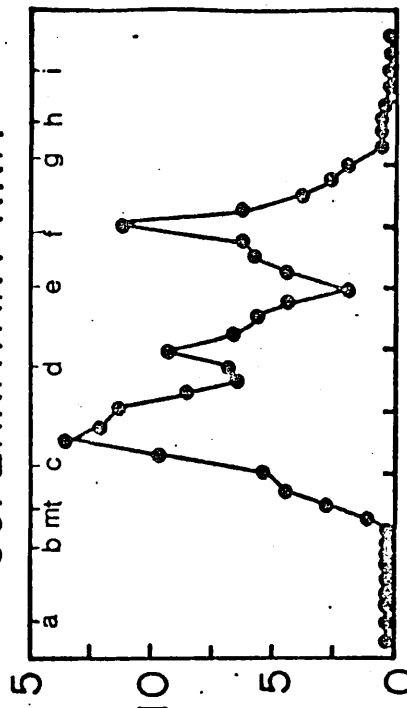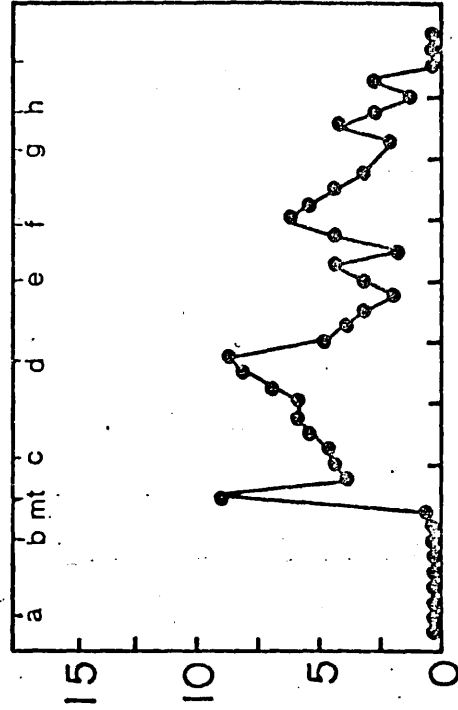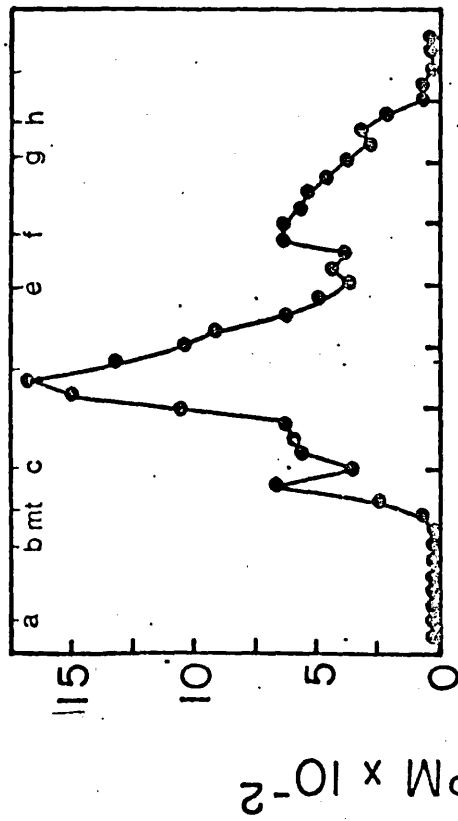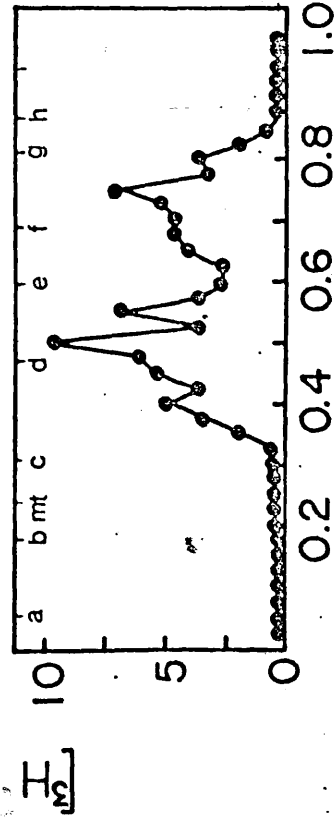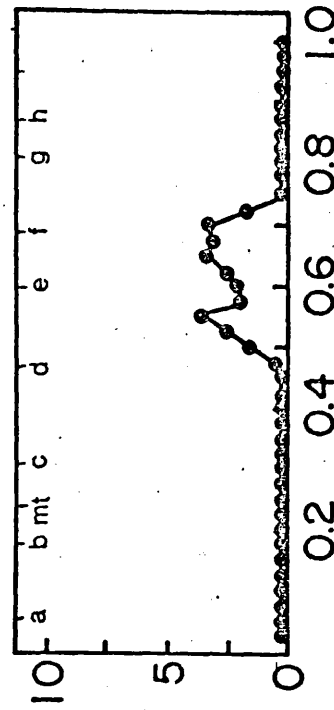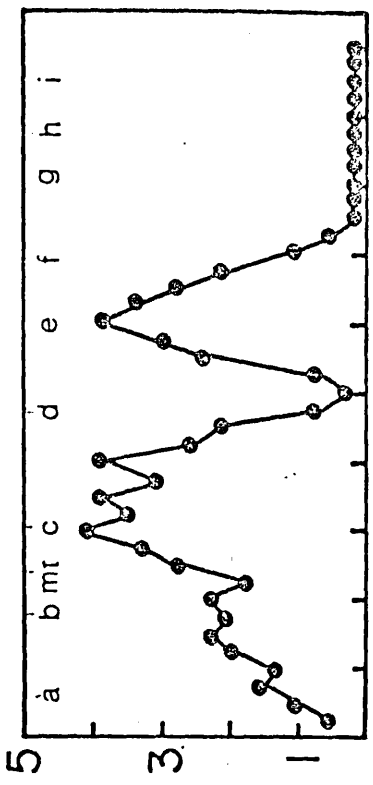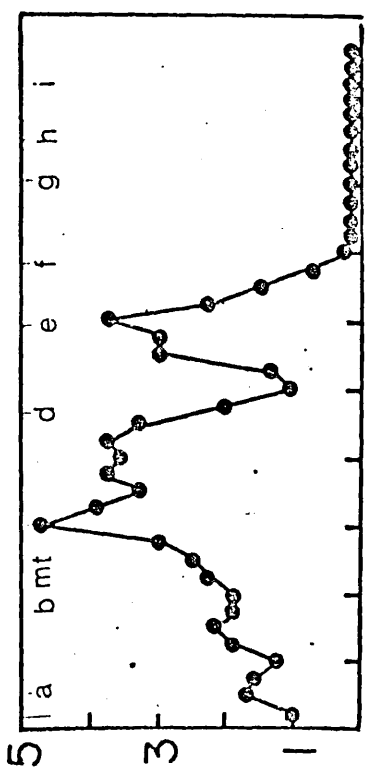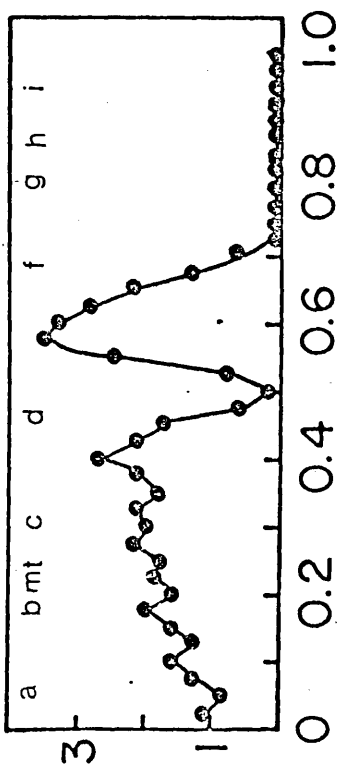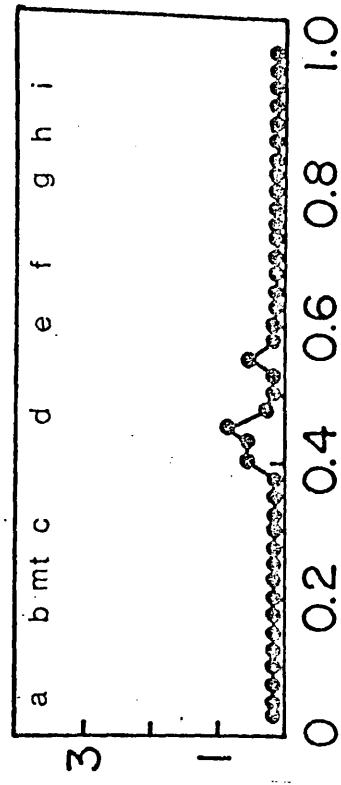
RELATIVE MOBILITY

Firstly, many more species of vinblastine-precipitable protein m-RNAs
may be present in isolated RNA than those translated in the embryonic
stages examined. Secondly, if different vinblastine-precipitable
protein m-RNAs are present in widely different amounts, then the products
of some may obscure the products of others. Differential translational
efficiencies in the in vitro systems would produce a similar effect.
These studies are complicated by the broad specificity of vinblastine.
Although the vinblastine-precipitable protein m-RNAs represent a restricted
range compared to the total m-RNA population, they still represent a
considerable number of different m-RNA species.

In an attempt to restrict the range of m-RNAs under study at a
particular time, the RNA samples were fractionated in 5-20% sucrose
gradients. Purified radioactively-labelled E. coli ribosomal RNAs were
used as molecular weight markers. The distribution of m-RNA activities
throughout the gradients was determined and appropriate fractions having
greatest m-RNA activity were combined. The RNA fractions were then
assayed with the wheat germ cell-free system and the products analysed by
SDS-polyacrylamide gel electrophoresis, as before. This was only possible
with polysomal and egg supernatant RNAs, the quantities of the other RNA
samples being too small for gradient analysis.

The vinblastine-precipitable in vitro products directed by
fraction "A" (see Figure 17) of the egg supernatant RNA are shown in
Figure 18. Four discernible peaks had similar mobilities to the in
vivo peaks mt, c, d and f. This suggests that m-RNAs coding for these
proteins are present in the egg supernatant. Three RNA fractions from
the egg polysomal RNA (see Figure 19) were analysed. The in vitro
synthesis of products similar to b and f were directed by fraction "A",
similar to d by fraction "B" and similar to g and i by fraction "C"
(Figure 20). Therefore, m-RNAs for proteins b, d, f, g and i appear
to be associated with unfertilised egg polysomes. Similar experiments
were carried out with RNA fractions isolated from fertilised egg
polysomal RNAs (Figure 21 - one hour; Figure 23 - three hours).
Fractions "A" and "B" from polysomal RNA obtained from eggs fertilised for
one hour directed the in vitro synthesis of protein g and proteins mt,
e and f, respectively (Figure 22). Synthesis of proteins a, c and f
was directed by polysomal RNA fraction "A" from eggs fertilised for three
hours (Figure 24). A summary of these results is presented in Table 10.

The mature egg clearly contains m-RNAs which are not expressed until after fertilisation. Further, these m-RNAs are stored in polysomes as well as the cytoplasm. M-RNAs continue to be stored in the polysomes after fertilisation. "Polysomes" in this context refers to the 105,000g microsomal fraction and therefore does not conclusively prove that stored m-RNAs are associated with polysomes.

The interpretation of the results is very dependent on the accuracy of comparing the mobilities of in vivo and in vitro products on SDS-gels. The possibility that some or even all of the in vitro peaks are artefactual was described earlier in this section. Only well-defined peaks were used in this discussion. Peaks with mobilities other than those recognised in vivo were also identified (e.g. Rf values of 0.40, 0.49, 0.65 and 0.74). This study is limited by the relatively low specificity of the techniques for product recognition, especially in view of the low specific activities of the m-RNA fractions.

Figure 17.   Sucrose gradient analysis of m-RNA activities
isolated from egg supernatant RNA.

Formamide-treated egg supernatant RNA (500 μg), together with
10 μg$\begin{bmatrix} ^3H \end{bmatrix}$-labelled E..coli ribosomal RNAs as markers, were
analysed on a 5-20% sucrose gradient as described in Methods.
Fractions (125 μl) were collected and alternate fractions were
analysed for radioactivity (25 μl) and m-RNA activity (100 μl)
in the wheat germ cell-free system, as described in Methods.
The arrows indicate the position of the E. coli ribosomal RNAs.
Sedimentation was from right to left. Fractions 15 to 27 were
pooled (A) and used for product analysis (see Figure 19).

STAGE: EGG
SUPERNATANT RNA
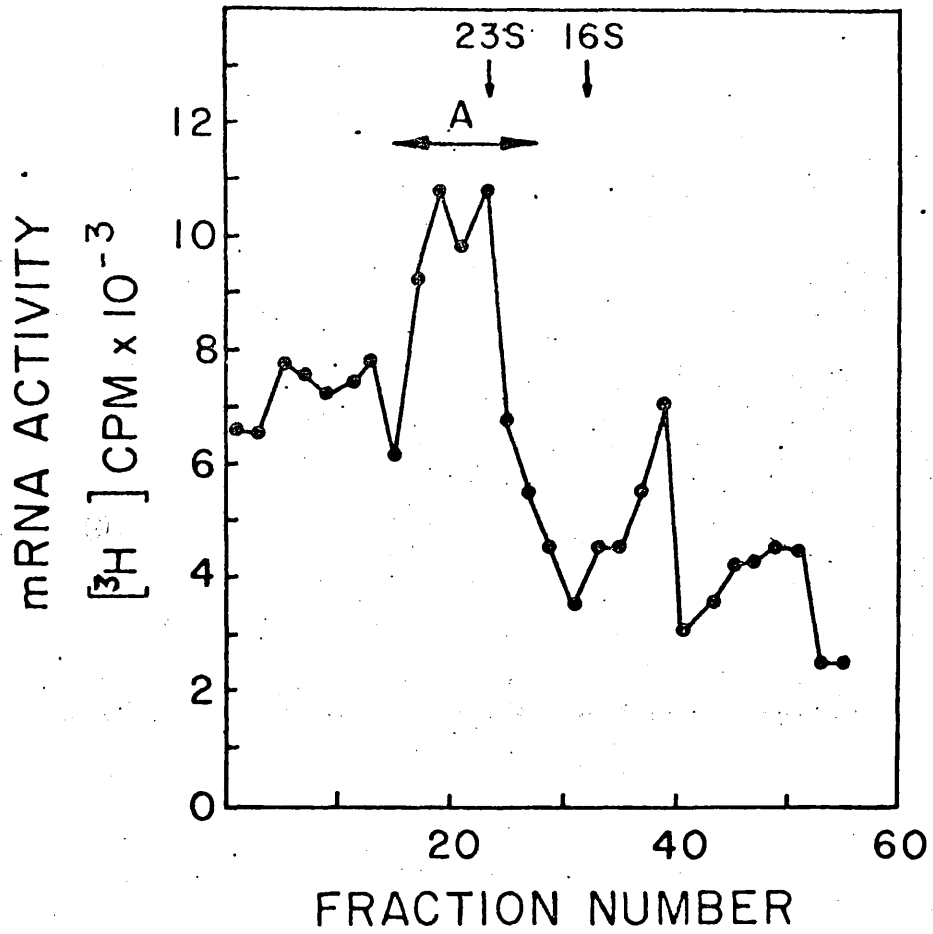
Figure 18.   Sodium dodecyl sulphate (SDS)-polyacrylamide
electrophoresis of the vinblastine-precipitated
proteins synthesised in wheat germ cell-free
system in the presence of egg supernatant RNA
purified by sucrose gradient analysis.

Experimental details as in Figure 16. (A) represents the
combined RNA fraction as in Figure 17.

STAGE: EGG

SUPERNATANT RNA
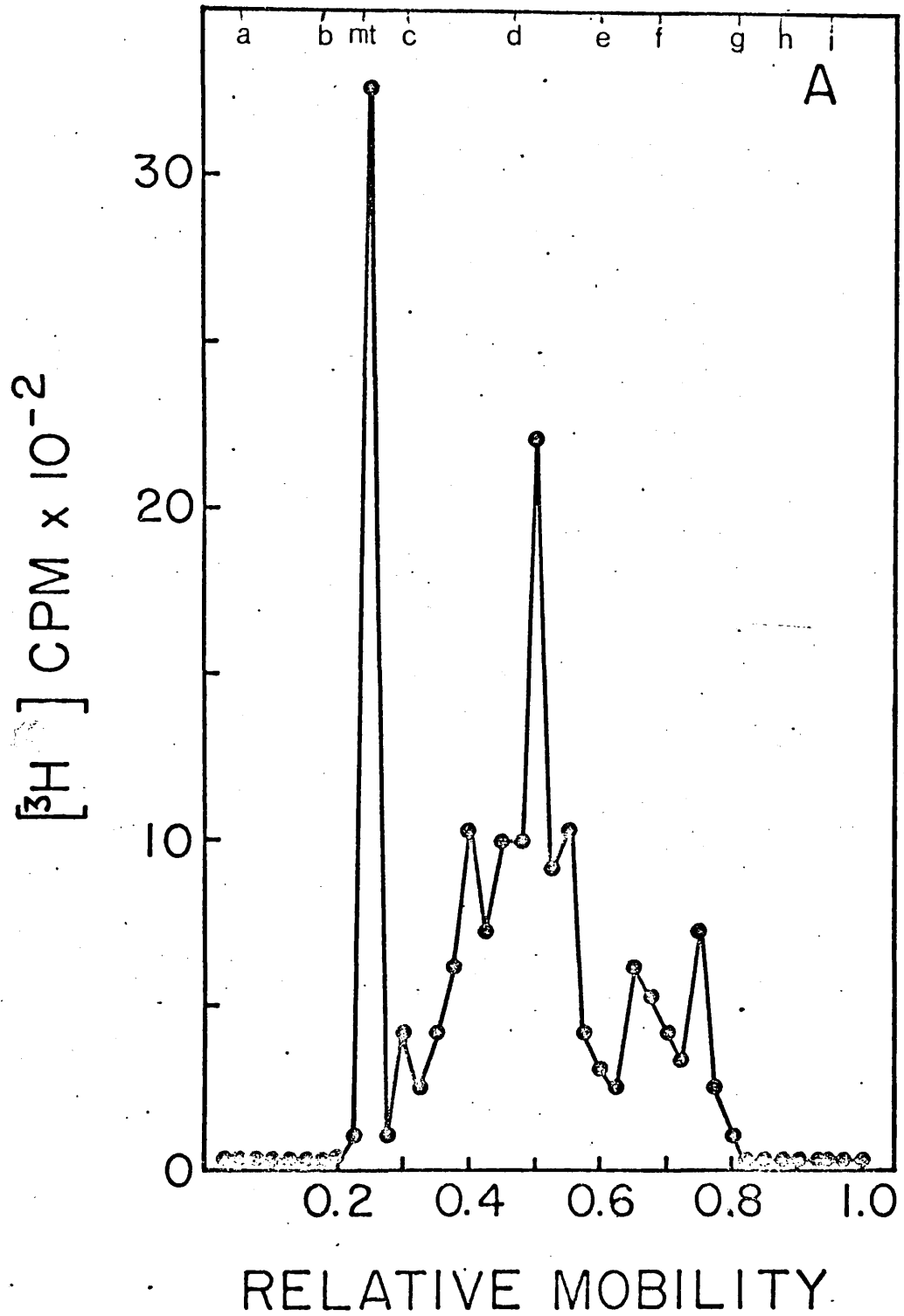
Figure 19.   Sucrose gradient analysis of m-RNA activities
              isolated from egg polysomal RNA.

Experimental details are as in Figure 17. Fractions 5-11 (A),
13-21 (B) and 35-45 (C) were pooled and used for product
analysis (see Figure 20).

Figure 19.   Sucrose gradient analysis of m-RNA activities
              isolated from egg polysomal RNA.

STAGE: EGG
TOTAL POLYSOMAL RNA
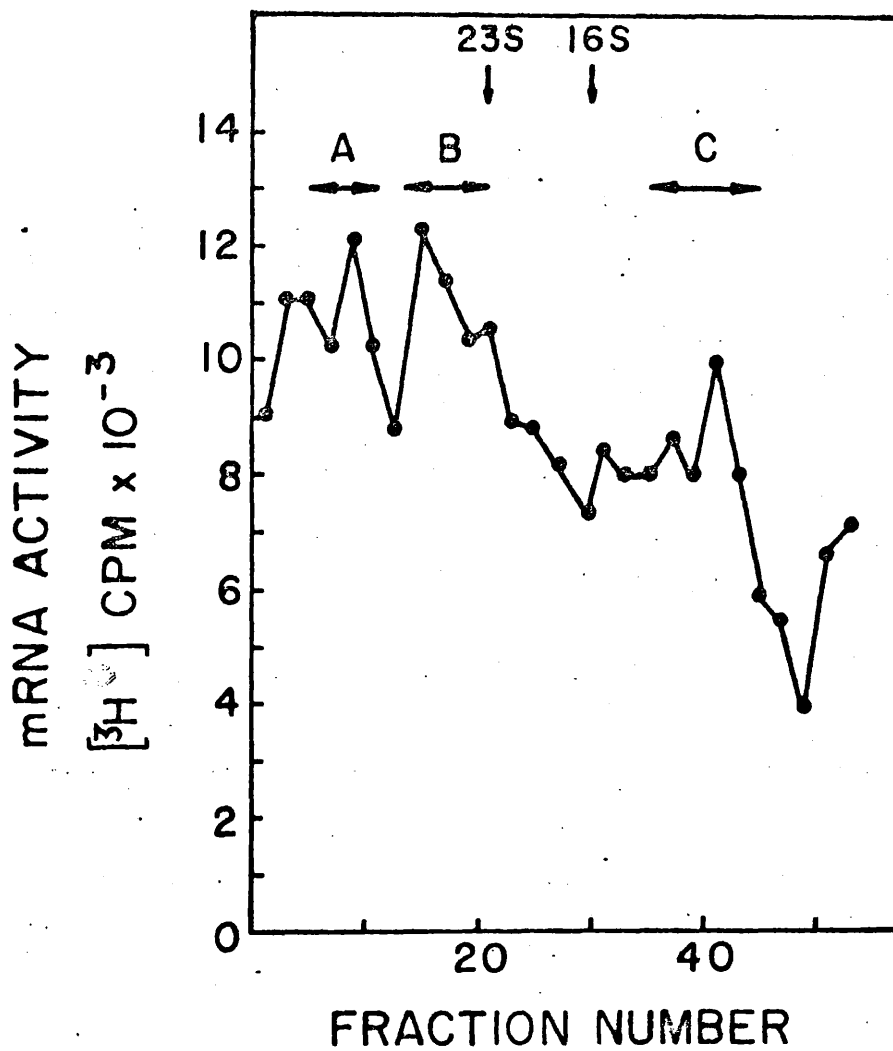
Figure 20.   Sodium dodecyl sulphate (SDS)-polyacrylamide
              electrophoresis of the vinblastine-precipitated
              proteins synthesised in the wheat germ cell-free
              system in the presence of egg polysomal RNA
              purified by sucrose gradient analysis.

Experimental details are as in Figure 16. (A), (B), and (C)
represent the combined RNA fractions as in Figure 19.

STAGE: EGG

TOTAL POLYSOMAL RNA
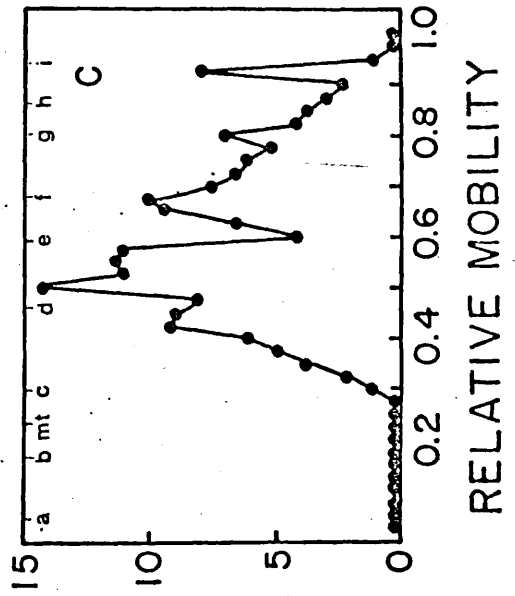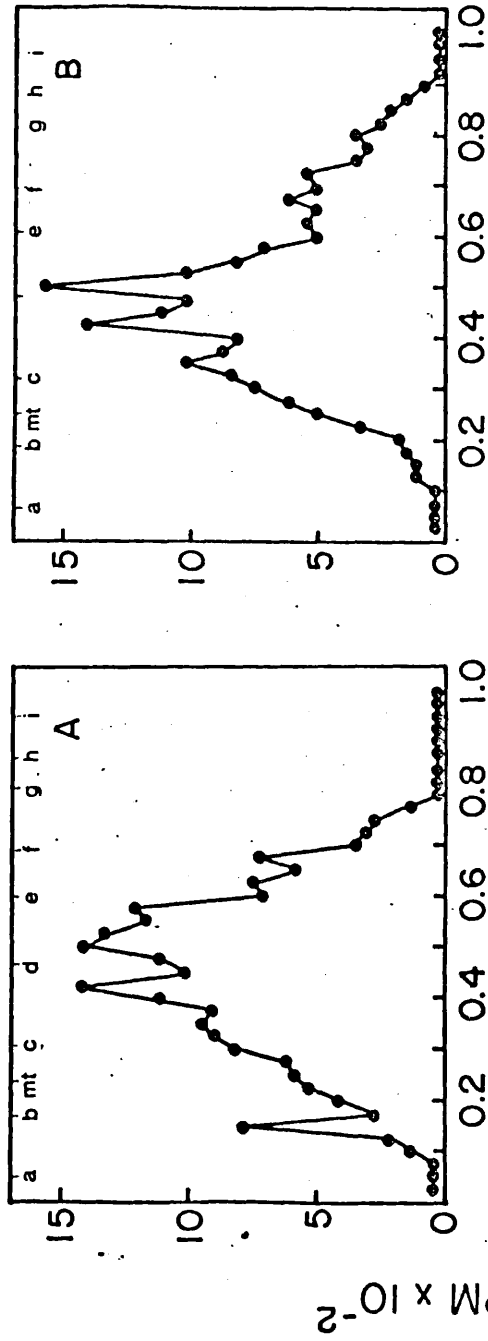
Figure 21.   Sucrose gradient analysis of m-RNA activities in
              polysomal RNA isolated from eggs one hour after
              fertilisation.

Experimental details are as in Figure 17. Fractions 9-13 (A),
15-17 (B) and 29-35 (C) were pooled and used for product
analysis (see Figure 22).

STAGE: EGG - ONE HOUR
AFTER FERTILISATION
TOTAL POLYSOMAL RNA
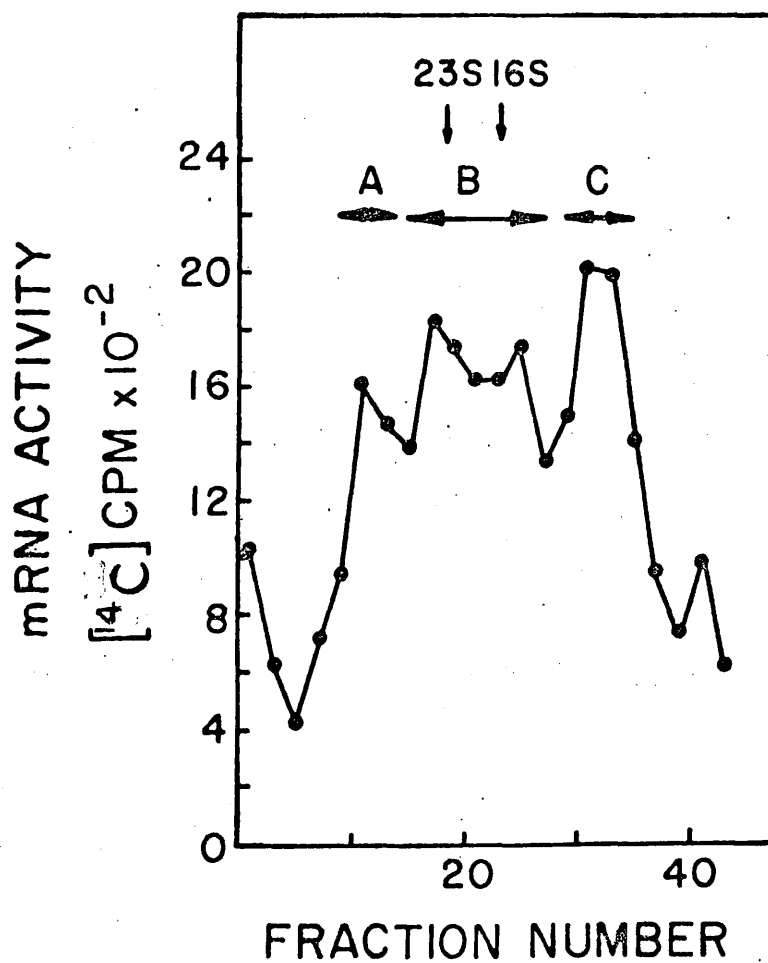
Figure 22.   Sodium dodecyl sulphate (SDS)-polyacrylamide
electrophoresis of the vinblastine-precipitated
proteins synthesised in the wheat germ cell-free
system in the presence of polysomal RNA isolated
from eggs one hour after fertilisation and
purified by sucrose gradient analysis.

Experimental details are as in Figure 16. (A), (B) and (C)
represent the combined RNA fractions as in Figure 21.

STAGE: EGG-ONE HOUR AFTER FERTILISATION

TOTAL POLYSOMAL RNA

$[^3H]$ CPM x 10^{-2}
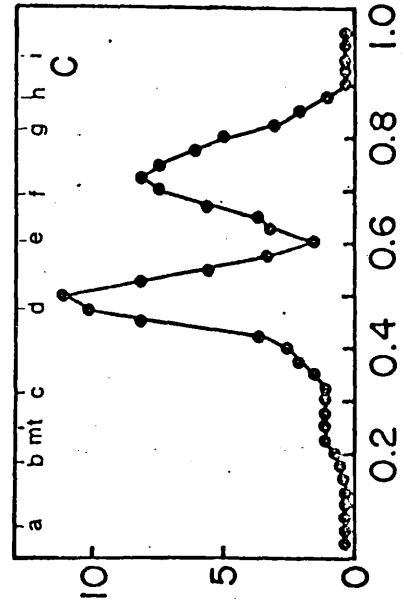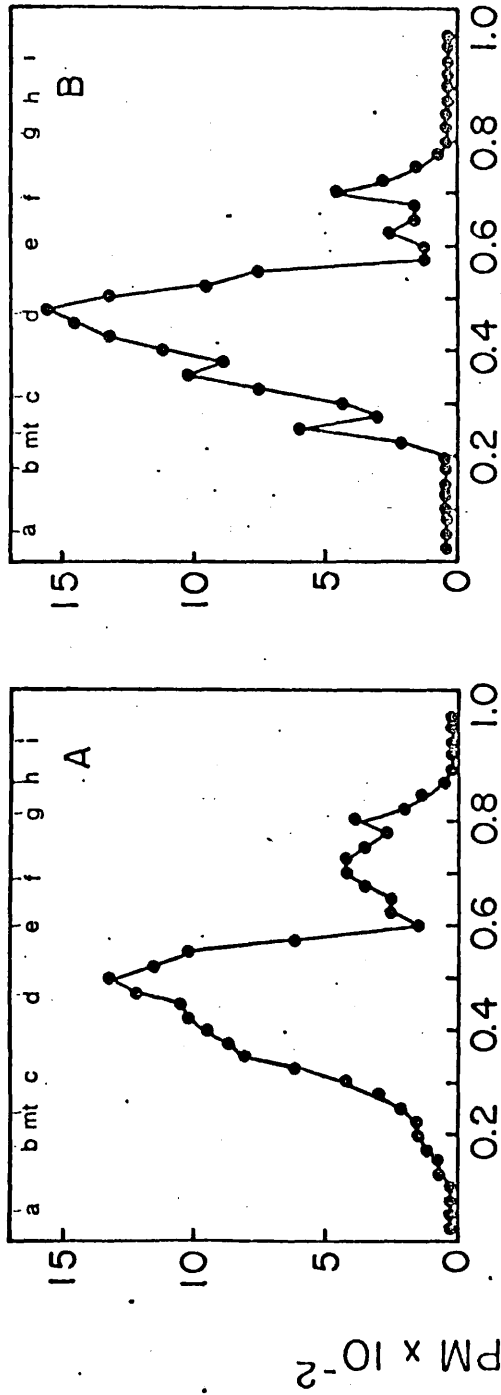
RELATIVE MOBILITY

Figure 23.   Sucrose gradient analysis of m-RNA activities in
              polysomal RNA isolated from eggs three hours after
              fertilisation.

Experimental details are as in Figure 17. Fractions 9-11 (A),
13-21 (B), 23-31 (C) and 33-39 (D) were pooled and used for
product analysis (see Figure 24).

STAGE: EGG-THREE HOURS AFTER FERTILISATION TOTAL POLYSOMAL RNA
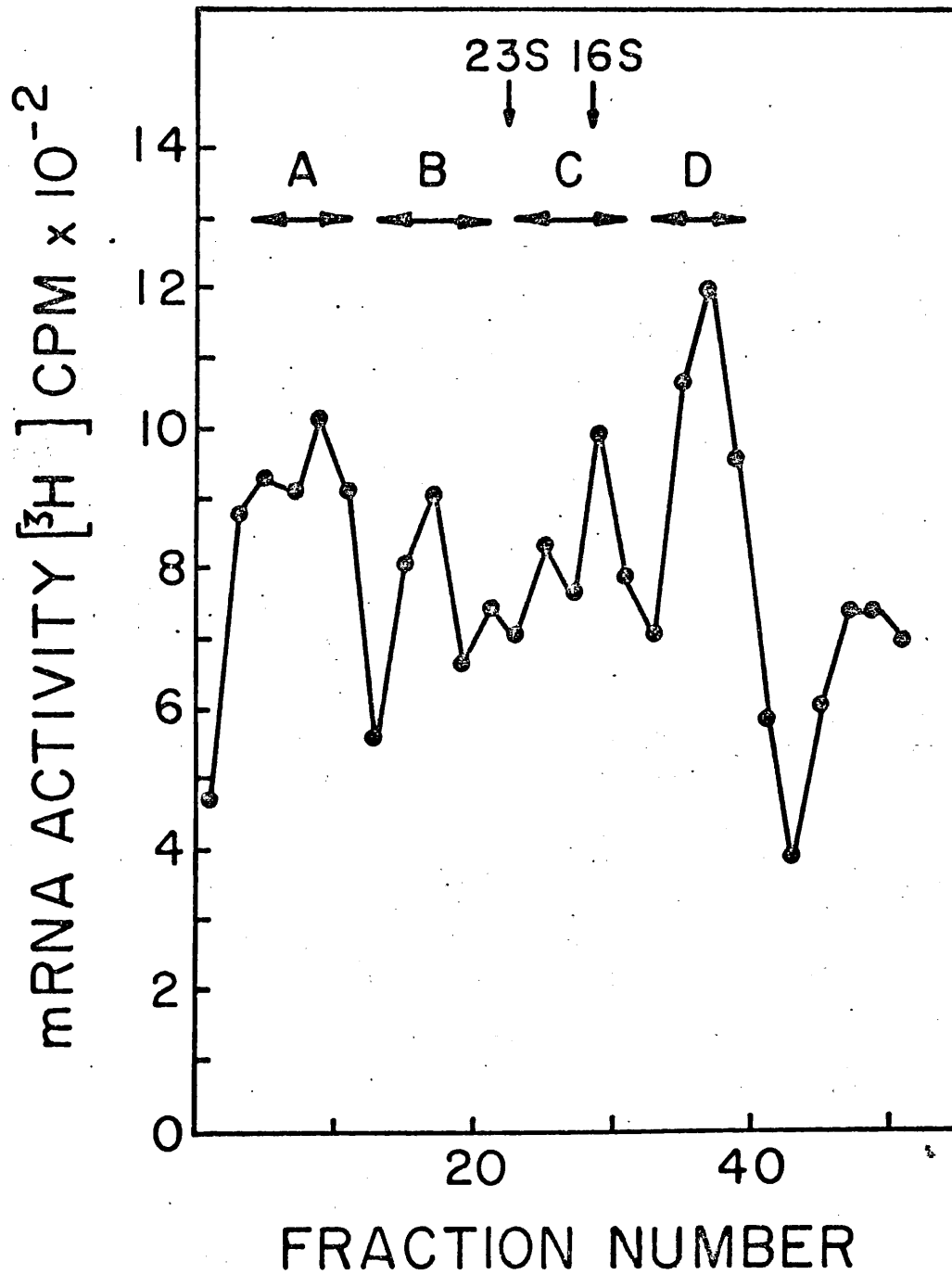
Figure 24.   Sodium dodecyl sulphate (SDS)-polyacrylamide
              electrophoresis of the vinblastine-precipitated
              proteins synthesised in the wheat germ cell-free
              system in the presence of polysomal RNA isolated
              from eggs three hours after fertilisation and
              purified by sucrose gradient analysis.

Experimental details as in Figure 16. (A), (B), (C) and (D) represent
the combined RNA fractions as in Figure 23.

STAGE: EGG — THREE HOURS AFTER FERTILISATION

TOTAL POLYSOMAL RNA

$[^3H]$ CPM $\times 10^{-2}$

RELATIVE MOBILITY
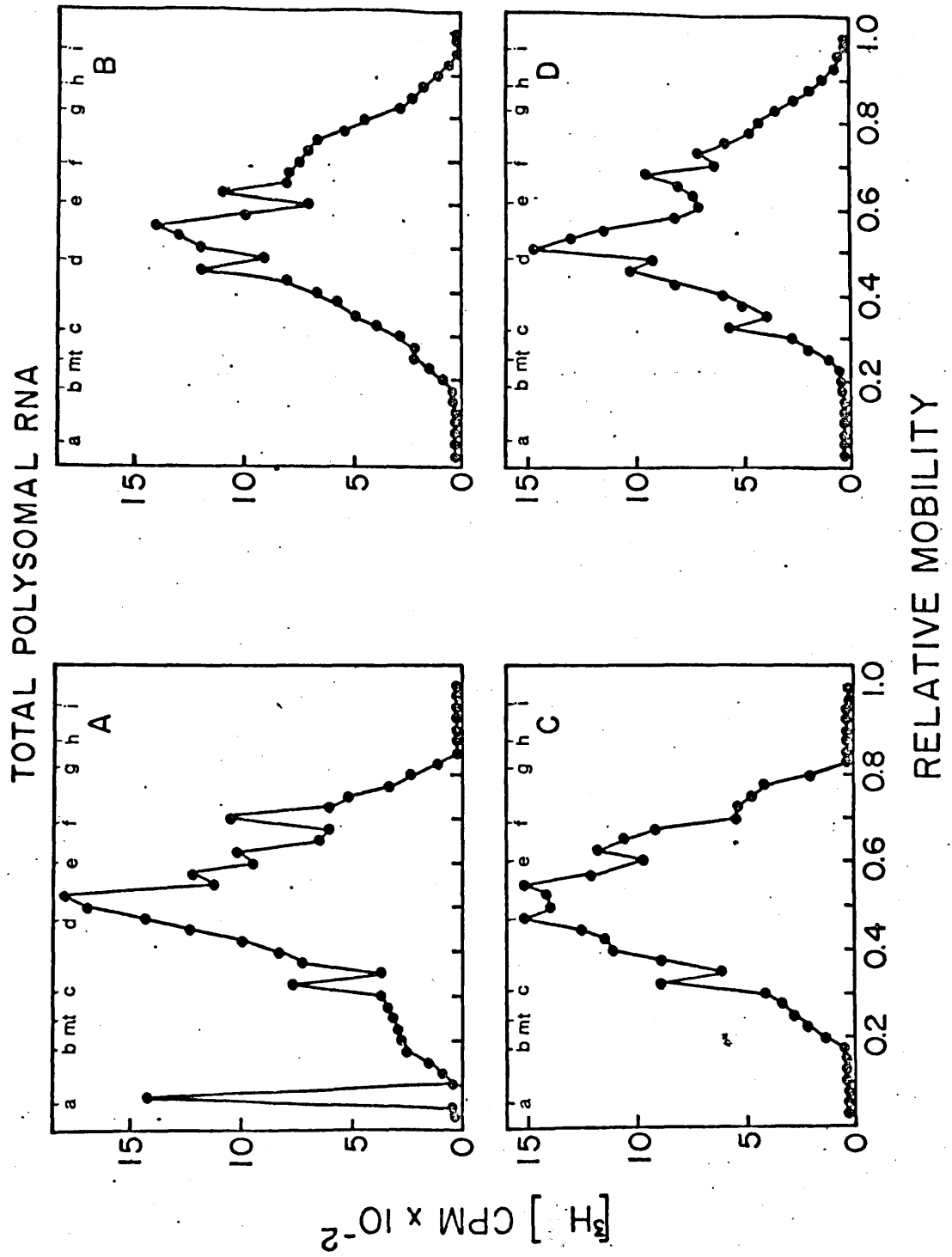
Table 10

Comparison of vinblastine-precipitable protein m-RNAs in unfertilised and fertilised eggs as determined by in vivo and in vitro analyses.

| m-RNA	Egg			Fertilised egg (1 hr)		Fertilised egg (3hr)	
	in vivo	in vitro cytoplasm	polysomes	in vivo	in vitro polysomes	in vivo	in vitro polysomes
a	-	-	-	+	-	+	+
b	-	-	+	+	-	-	-
mt	+	+	-	+	+	-	-
c	-	+	-	-	-	+	+
d	-	+	+	+	-	-	-
e	+	-	-	+	+	-	-
f	-	+	+	-	+	+	+
g	-	-	+	-	+	+	-
h	-	-	-	-	-	-	-
i	-	-	+	-	-	+	-

+ detected; - undetected.

6.   Summary

Plaice eggs were fertilised under controlled conditions in the laboratory and various molecular parameters were studied during early embryogenesis. Both unfertilised and fertilised eggs were permeable to radioactively labelled uridine and leucine supplied in the medium. This made it possible to use the incorporation of radioactive precursors into RNA and protein as a measure of macromolecular synthesis, as well as a means of detecting the newly synthesised molecules themselves. Ideally, the synthesis of specific proteins could be followed over discrete time periods as a function of development. Hitherto earlier studies with sea urchin and amphibians have been limited by the impermeability of the mature eggs to radioactive precursors (1-5).

Little or no measurable RNA synthesis occurred in the mature egg or up to five hours after fertilisation. In contrast, low levels of protein were synthesised during these developmental stages. Fertilisation roughly doubled the level of protein synthesis. The administration of RNA synthesis inhibitors, actinomycin D and ethidium bromide, to the egg had no effect on protein synthesis or on subsequent development up to late blastula, stage. Protein synthesis and normal development were severely affected after this stage. These results support the conclusion that protein synthesis and development during early embryogenesis is dependent on m-RNAs synthesised and stored in the egg. Similar conclusions have been drawn from previous studies with sea urchin and amphibian systems (1, 3).

Fertilisation increased the synthesis of proteins found in both the organelle and supernatant fractions, especially those supernatant proteins precipitated by vinblastine. The microsomal fraction contained very little radioactivity, which was not surprising since the unfertilised and fertilised eggs possessed very low quantities of polysomes. The possibility that some of the newly synthesised proteins in the 20,000g fraction were histones was investigated and proved negative. However, different results may have been obtained with radioactive arginine , this amino acid being a more prominent histone component. Electrophoretic analysis of supernatant fractions in 7% polyacrylamide gels clearly showed that different proteins were synthesised in the egg before and after fertilisation. Similar results were obtained with vinblastine-precipitated proteins analysed by SDS-5%

polyacrylamide gel electrophoresis. Vinblastine was originally selected because of its selective precipitation reaction with microtubulin proteins, but other proteins were also precipitated. Nevertheless, the specificity of vinblastine-precipitation did provide a method for recognising a number of proteins which were differentially expressed as a consequence of fertilisation. These proteins were characterised by their precipitability with vinblastine sulphateand relative mobilities on SDS-gels. Microtubulin-like proteins were synthesised before and immediately after fertilisation. The other proteins were more interesting in that synthesis did not occur until after fertilisation, and further, at different times. These results suggest that not only are specific m-RNAs stored in the egg, but also mechanisms exist which control their selective utilisation after fertilisation.

Plaice RNA was assayed for m-RNA activity using the rabbit reticulocyte and wheat germ cell-free systems. The wheat germ system proved to be most useful since the low endogenous levels of protein synthesis enabled the direct assay of exogenous m-RNA activity. Although this system suffered from "premature termination" due to defective elongation, the presence of prematurely-released polypeptides was considered to be minimal. The reticulocyte system was only useful for assaying m-RNAs for vinblastine-precipitable proteins.

Polysomal and cytoplasmic RNA preparations were isolated from unfertilised and fertilised eggs and then separated into poly (A)- and non-poly (A)-containing fractions by oligo (dT)-cellulose chromatography. Each RNA fraction was assayed for total and vinblastine-precipitable protein m-RNA activities. The first rather surprising result was that most m-RNA activity was found in the non-poly (A)-containing RNA fractions. This is contrary to the generally held view that eukaryotic m-RNAs, except for histone m-RNA, are characterised by the presence of 3'-terminal poly (A) sequences. Assuming that the experimental procedures followed are not at fault, it appears that m-RNAs other than those coding for histones also lack poly (A) sequences. It may be that non-poly (A)-containing m-RNAs are characteristic of gametic and embryonic systems. Several other reports support this contention (54-60). The presence of poly (A)-containing RNA suggests that the eggs possess the enzymatic machinery for polyadenylation. The observation that the proportion of poly (A)- to non-poly (A) RNA increased in the polysomal fraction after fertilisation may indicate that at least certain of the m-RNAs are polyadenylated prior to translation. Whether or not polyadenylation is directly involved in regulating

translation is still open to question. At least one species of
m-RNA, that which codes for microtubulins, was positively identified
in the polysomal poly (A)-RNA fraction isolated from eggs one hour
after fertilisation.

The second, but less surprising result, was that m-RNA activity
in the egg was distributed between the supernatant and polysomal RNA
fractions. After fertilisation, the supernatant RNA fraction was con-
siderably reduced and most of the m-RNA activity was found associated
with the polysomal fraction. Specific m-RNAs for vinblastine-
precipitable proteins were identified in the supernatant and poly-
somal RNA fractions. That certain of these m-RNAs were not translated
in vivo suggests that m-RNA is stored both in the cytoplasm and in the
polysomes themselves. This raises the possibility that more than one
type of regulatory mechanism may control m-RNA utilisation in the plaice
egg. Apart from histone m-RNA in sea urchin egg (20), the vinblastine-
precipitable protein m-RNAs represent the best documented examples of
"maternal m-RNA".

This study represents only a small step towards an understand-
ing of the mechanisms which regulate gene expression in early embryo-
genesis. The important questions still remain unanswered. For example;
what determines which m-RNAs are stored in the egg? How are the m-RNAs
stored in the cytoplasm and polysomes of the egg? What molecular events
connect the act of fertilisation with the release of stored m-RNAs? What
mechanisms control the differential utilisation of stored m-RNAs?

MATERIALS AND METHODS

7. Material and Methods.

7.1 The Organism.

Eggs and sperm from plaice (Pleuronectus platessa) were obtained from Dr.C. Purdom at the Fisheries Laboratory, Lowestoft, Suffolk, England.

Plaice were taken from the North Sea in December and kept in fish tanks at the Lowestoft Fisheries Laboratory during early January through March, when the plaice mature sexually. Samples of these mature eggs were packed in ice and transported to our laboratory. Sea water was also obtained from Lowestoft, and for all experiments was filtered through a Millipore filter, autoclaved and the antibiotics streptomycin sulphate (0.05mg/ml) and sodium penicillin G (50 I.U.) were added to prevent bacterial contamination. The eggs and embryos at various stages of development were frozen on solid CO_2 and stored. The viability of the eggs and embryonic development were examined by microscope.

7.2 Biochemicals and reagents.

Rabbit heamoglobin messenger RNA was a gift from Dr. Robert Williamson, Beatson Institute, Glasgow, Scotland. Ovalbumin m-RNA was a gift from Dr. Richard Palmiter, Searle's Research Laboratories, High Wycombe, Bucks., England. Radioactive isotopes, $L-[4,5^{3}H]$-leucine (sp. act. 1Ci/mmol), $5[^{3}H]$-uridine (sp. act. 2Ci/mmol) and $L[^{14}C]$-leucine (sp. act. 348mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., England. These chemicals were stored at $-20^{\circ}C$ under conditions designed to minimise contamination or rapid decomposition. For liquid-scintillation counting, the scintillants used were 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazol-2-yl) benzene (POPOP) from BDH Chemicals Ltd., and the solvents were Soluene from Beckman, and Triton X-100 from the Sigma Chemical Company.

Hemin, acrylamide and N,N^{1}-methylenebisacrylamide were from Eastman Kodak Co.; Amberlite MB-1, formamide and sodium dodecyl sulphate were from BDH Chemicals Ltd.; vinblastine sulphate and diethylpyrocarbonate were from Sigma Chemical Co.; NCS tissue solubiliser was a product of Amersham- Searle.

All other chemicals used in this investigation were AnalaR grade, or better. The solutions were made with glass-distilled deionised water and all glassware was rinsed in this water and heat-sterilised.

7.3   Fertilisation techniques

Immediately on arrival at our laboratory, the eggs were
washed several times with sea water and artificially fertilised at
8°C according to the methods used at Lowestoft - the semi-wet method
(175). For fertilisation, approximately 100g of eggs were placed in
a shallow dish containing 200 ml of sea water, milt was then added and
the solution agitated gently for 2 min. After standing for 10 min, the
fertilised eggs were washed free of sperm. The end of the washing
procedure was taken as zero time after fertilisation. The fertilised
eggs were then incubated as described in Section 7.4. Development was
studied in all cases for at least 24h after fertilisation to determine
the quality of development. In all experiments, fertilisation was 95%
or better.

7.4   In vivo labelling of cells.

Eggs and embryos (5g), treated as described in Section 7.3, were
transferred by means of a nylon screen into 10ml of Antibiotic
filtered sea water containing 50µCi of $\left[^3H\right]$-labelled leucine (sp. act.
1Ci/mmol) and incubated for 1h at 8°C. The eggs or embryos were then
removed from the medium and washed with sea water and transferred to
fresh sea water at 0°C containing the respective unlabelled precursor
(0.1%) for 10 min.

In some experiments, the effect of inhibitors upon RNA
synthesis in embryos was examined. Immediately after fertilisation,
various concentrations of actinomycin-D and ethidium bromide were added
to samples (1g) in sea water (2ml) and the samples were incubated at the
appropriate times with the radioactive precursors (10µCi per sample)
for 1h at 8°C. All incubations were then stopped and processed for
RNA or protein synthesis as described.

7.5   Uptake of labelled precursors by eggs and embryos.

Eggs and embryos (5g) were incubated as described in Section
7.4. At this point three-egg or three-embryo samples (in duplicate)
were taken, washed with sea water, and stored frozen at -20°C. The
remaining samples of eggs and embryos were processed as described in
Section 7.6. The permeability of the eggs and embryos to radioactivity
was determined by thawing the frozen samples, adding 1ml of NCS tissue
solubiliser and heating to 47°C overnight, cooling and adding 10ml of
toluene scintillant (5.5g of PPO and 100mg of POPOP per litre of toluene)
and counting in a Packard Tri-Carb liquid scintillation counter. The

counting efficiency was 42-47% as determined by the external standard ratio method.

7.6   In vivo RNA and protein synthesis.

. 5g samples of radioactively labelled eggs and embryo were prepared as in section 7.5. After their final wash, 10-egg samples were precipitated in 5% trichloroacetic acid (TCA). The samples were centrifuged at maximum speed in a MSE Minor centrifuge for 10 min, to remove unincorporated labelled precursors in the supernatant. The samples for RNA synthesis were hand homogenised in a teflon-glass homogeniser, spotted on Whatman 3MM filter discs and sequentially washed in 10% TCA at $0^{\circ}C$, 70% ethanol at $40^{\circ}C$, ethanol: diethyl ether (1:1, v/v) at $40^{\circ}C$ and finally with diethyl ether at room temperature. The dried filters were digested overnight in 0.5 ml of NCS at $47^{\circ}C$, cooled and 10ml of toluene scintillant was added and the samples were counted. Blanks were determined by the same method as described above, using unlabelled eggs or embryos as starting material. TCA- insoluble material was treated as above, except that it was treated for 30 mins at $80^{\circ}C$ in 5% TCA, immediately after homogenisation. This was done to determine non-specific adherence of labelled precursors. It was also demonstrated that the labelled material was completely solubilised by treatment with 0.5N, NaOH at $37^{\circ}C$ for 30 mins, indicating that it was RNA.

The same procedure was followed for estimation of protein synthesis except that after the samples were homogenised in 5% TCA they were heated in the same solution for 30 mins at $80^{\circ}C$. The blanks were determined by the same method, using unlabelled eggs or embryos as starting material.

N.B.   Using paper chromatography labelled leucine was identified in the cytoplasm of radioactively labelled cells, implying that large scale modification of the precursor had not occurred.

* The samples were hand-homogenised ten times in a Teflon-
glass homogeniser (clearance 0.45 mm) in two volumes (w/v)
of the buffer.

* The 7% polyacrylamide gels were run in Tris-glycine
buffer, pH 8.3.

* The 5% (SDS) polyacrylamide gels were run in sodium
phosphate buffer, pH 7.0.

7.7 <u>Subcellular fractionation of eggs and embryos and</u>
<u>quantitation of in vivo labelled protein in the</u>
<u>various fractions.</u>

Samples (1-10g) were prepared as in section 7.4 and
homogenised in 100 mM Tris-glycine buffer, pH 8.3 (1:1 w/v). The
homogenate was centrifuged at 20,000g for 20 min in an MSE 18
centrifuge at 0°C. The resulting pellet (20,000g pellet) was resuspended
in Tris-glycine buffer, pH 8.3 (1gm of sample/ml of buffer) and the
supernatant (20,000g supernatant) was then further centrifuged at
105,000g for 90 min at 0°C. Vinblastine sulphate was added to 1ml of
the 105,000g supernatant to give a final concentration of 2mM. The
solution became turbid immediately but was allowed to stand at 0°C for
30 min; the precipitate was then sedimented at 50,000g for 30 min at
0°C. The supernatant (vinblastine-soluble fraction) was removed and
stored at -20°C, and the pellet (vinblastine-insoluble protein fraction)
was re-suspended in the SDS buffer defined in section 7.8.2. Then 100 µl
aliquots of the different fractions were precipitated with 10% TCA,
digested with 1ml NCS tissue solubiliser and counted in 10ml toluene
scintillant. The microsomal pellet was solubilised in 1ml NCS and
counted in 10ml toluene scintillation.

7.8 <u>Electrophoretic analysis of in vivo labelled proteins from</u>
<u>various subcellular fractions.</u>

7.8.1 <u>Discontinuous polyacrylamide gels (173)</u>

The gel electrophoretic system of Davis was used. Samples of
protein (200 µl, approx. 1mg of protein) were electrophoresed at 2mA
per tube for 10 mins, followed by 4mA per tube for 60 min at 0-4°C.
Bromophenol blue was used as a marker dye and migrated about 6cm under
these conditions. Gels were removed from the tubes using a syringe.

Optical scanning of the gels was found to be too insensitive
to detect the presence of individual proteins from plaice eggs. The
gels were either stained or sliced and counted after solubilisation
as described in section 7.8.4 and 7.8.3 respectively.

7.8.2 <u>Sodium dodecyl sulphate-polyacrylamide gels (174)</u>

The molecular weights of various proteins were determined
using the method of Weber and Osborn ., except that 10% gels were
replaced by 5% gels. The known protein standards used were cytochrome
C (mol. wt. 11,700). heamoglobin (mol. wt. 15,500) trypsin (mol. wt.
23,000), pepsin (mol. wt. 35,000), aldolase (mol. wt. 40,000), glutamic

dehydrogenase (mol. wt. 53,000), pyruvate kinase (mol. wt. 57,000) and human serum albumin (mol. wt. 68,000). One or more of these was always used.

7.8.3 Determination of radioactivity in polyacrylamide gels.

Gels were removed from the electrophoresis tubes, placed in aluminium foil tubes and frozen at $-20^{\circ}C$ for 30 min. Each frozen gel was placed on a damp Whatman no. 3 filter paper on a Mickle gel slicer, and sliced into 1mm sections (176). These slices were transferred with forceps to glass scintillation vials so that they rested against the inside bottom edge of the vial. They were allowed to dry, the gel slices completely covered by addition to each vial of 250μ.1 of a freshly prepared mixture of conc. NH_4OH and 30% H_2O_2 (1:99, v/v) at $0^{\circ}C$. The slices were digested by overnight incubation at 45°. After cooling to room temperature, 10ml of either Soluene-toluene scintillant (Soluene, 200ml; PPO, 5g; POPOP, 100mg; toluene, 800ml) or Triton X-100-toluene scintillant (Triton X-100, 330ml; PPO, 5g; POPOP, 100mg; toluene, 660ml) were added to each vial. The contents of the vial were mixed thoroughly and counted in a refrigerated scintillation counter. Both cocktails gave counting efficienty of 38-45% as determined by external standard ratio.

7.8.4 Staining procedures for polyacrylamide gels.

Method 1

Gels were removed from the electrophoresis tubes and the proteins fixed by immersing the gels in 12.5% TCA (20 ml) for at least 1 h at room temperature. Then 1ml of a solution of 1% Coomassie brilliant blue was added to the fixing solution and the gels gently shaken in this solution for 1-4 h. The gels were de-stained in 10% TCA for 6-24 h.

Method 2

The SDS gels were placed in test tubes and stained at room temperature overnight in a solution of Coomassie brilliant blue (1.25g) in a mixture of 50% methanol (454ml) and glacial acetic acid (46ml), from which any insoluble material had been removed by filtration through Whatman no. 1 filter paper. The gels were de-stained in a solution of 5% methanol and 7.5% glacial acetic acid in water.

7.9   General methods.

7.9.1   Paper chromatography of amino acids (177)

To determine if the H^3-labelled leucine was converted
into any other form when it was transported into the egg or embryo,
paper chromatograms were run of the amino acids from the post-
mitochondrial supernatant. Samples (250μl) were run with standard
acids on Whatman no. 3 paper in two different solvent systems:
n-butanol:acetic acid:water (120:30:50, v/v); n-butanol:
pyridine:water (60:60:60, v/v). After drying, the chromatograms
were cut into 0.5cm strips and each strip was counted for
radioactivity in toluene scintillant.

7.9.2   Protein estimation.

The protein content of all samples was measured spectrophoto-
metrically by the method of Lowry et al.(178) except that the Folin-
Ciocalteau reagent was diluted in the ratio 1:1 with water. Human serum
albumin was used as a standard. Absorbance of the coloured solutions
was measured at 750nm using a Unicam S.P. 500 spectrophotometer.. All
readings were performed in triplicate.

7.10   Cell-free systems for determining m-RNA activity

7.10.1 Rabbit reticulocyte system (161)

Rabbit reticulocyte lysate was prepared according to the
method of Lingrel, except that blood was obtained by bleeding from the
ear. Hemin is known to protect the cell-free system from inactivation
and was included in the lysate to a final concentration of 2.5×10^{-5}M.
The concentration of hemin had previously been determined by adding 60mg
of hemin to 5ml of 0.1M NaOH and slowly adding 0.2M Tris. pH 7.5, until
a pH of 7.7 was reached, centrifuging at 2000g for 20 min to remove any
insoluble material, and comparing the absorbance of samples (10μl) of this
solution against known amounts of hemin in 10% pyridine at 550nm. After
stirring for 1 min to ensure complete lysis, the cells were centrifuged
at 12,000g for 10 min at 2°C, and the supernatant was stored in liquid
nitrogen. Incubation mixtures were set up as described by Lingrel and
reactions were run at 25°C for 2h, cooled to 0°C, 20μl samples were
spotted on to Whatman 3MM filter paper discs. The discs were washed in
5% TCA, heated at 90°C in 5% TCA, dried under an i.r. lamp

and digested overnight in 500µl of NCS at 47°C. Each digest was mixed with 5ml of toluene scintillation fluid and counted.

This system was tested for its ability to translate exogenous m-RNA. A 250µl incubation mixture was set up containing 25 µCi of $[^3H]$ leucine. Ovalbumin m-RNA (25µg) was added and the mixture incubated for 60 min at 25°C. Samples (25µl) were subjected to electrophoresis on SDS gels. The gels were sliced and counted as in section 7.8.3.

7.10.2 Wheat germ system (163)

Wheat germ cell-free system was prepared and incubation mixtures set up according to the method of Roberts and Patterson except that unlabelled leucine was replaced by radioactive leucine, and the final concentrations of $MgAc_2$ and KCl were 2mM and 100mM respectively. Reactions were incubated for various times at 25°C. Samples of 20 µl were pipetted immediately on to 2.3cm discs of Whatman 3 MM paper, the discs were dried, placed in 10% TCA and then transferred to 10% TCA at 90°C for 10 min, rinsed several times in cold 5% TCA and dried by sequential rinsing in ethanol, ethanol-ether (3:1, v/v), and ether. The discs were counted in 5ml of toluene scintillant.

7.10.3 Product analysis by SDS-polyacrylamide-gel electrophoresis. (174)

Proteins from the cell-free systems were precipitated by the addition of 2mM vinblastine sulphate, centrifuged through a 1M sucrose pad (500µl) at 50,000g for 30 min at 0°C, and the pellet was dissolved in SDS buffer containing NaH_2PO_4, H_2O (1.95g), $Na_2 HPO_4, 7H_2O, (9.9g)$, SDS (0.5g) per litre and 0.5M Tris (unadjusted) to deacylate transfer RNA. Samples were heated in SDS electrophoresis buffer, pH 7.0 (see Method 7.8.2) at 100°C for 5 min, diluted in the ratio 1:1 with this buffer, electrophoresed, sliced and counted in toluene scintillant as described in section 7.8.2.

The total amount of labelled vinblastine sulphate-precipitated protein was determined by solubilising aliquots of the dissolved pellet in 10 volumes of NCS and counting in 10ml toluene scintillant.

The samples were hand-homogenised ten times in a Teflon-glass homogeniser (clearance 0.45 mm) in two volumes (w/v) of the buffer.

7.11 Subcellular fractionation of whole cells.

7.11.1 Plaice

Unfertilised and fertilised eggs were homogenised in two volumes (w/v) of freshly prepared TMK buffer ($300mM$ KCl, $10mM$ MgAc$_2$, $50mM$ Tris, pH 7.6) by use of a motor-driven Teflon pestle in a glass tube (clearance .45mm). It was found that the addition of heparin ($900\mu g/ml$) was necessary to inhibit RNase activity. The homogenate was fractionated by successive centrifugations at $0-4^{\circ}C$: 1.5 min at 200g; 10 min at 1500g; 10 min at 5000g and 15 min at 12,000g. The postmitochondrial supernatant was then centrifuged at 200,000g for 60 min and the RNA was extracted from the translucent pellet and the supernatant as described in sections 7.12.1.1 and 7.12.1.2 respectively.

For fractionation of plaice polysomes, eggs and embryos were homogenised in 2 volumes (w/v) of buffer ($50mM$ Tris, pH 7.6; $10mM$ MgAc$_2$; 300 mM KCl; 0.2% Triton X-100) by use of a motor-driven Teflon-glass homogeniser (clearance 0.75mm). The homogenate was centrifuged at 20,000g for 20 min at $0^{\circ}C$. The resulting supernatant was diluted to give an absorbance at 260nm of 5 units, then layered over an 8ml 10-35% linear sucrose gradient and centrifuged at 80,000g for 2h in an MSE (3 X 10ml) swing-out rotor. The gradient was passed through a Pye-Unicam SP 1800 spectrophotometer and absorbance at 260nm was measured in a 1cm flow-through cell.

The best ionic strength (monovalent ion concentration) for maintaining the integrity of the polysomes was determined as follows: the concentration of the divalent ion (Mg^{2+}) was held constant and the concentration of the monovalent ion (K^{+}) was varied between $0-450mM$ to determine the best ratio for polysomes. The results showed that for plaice egg and embryo polysomes, a $10mM$ McAc$_2$ and 300 mM concentration gave the optimal polysome profile.

7.11.2 Rat liver

Rat liver was the initial material used to work out the techniques for RNA isolation, and this RNA was used for standards and markers in various techniques used for RNA characterisation.

Rats were starved overnight and the livers were removed after cervical fracture. Livers were blotted with filter paper and trimmed of connective tissue, immediately placed on ice and homogenised in a Teflon-glass homogeniser in 3 vol. (w/v) of buffer containing $300mM$ sucrose, $20mM$ Tris (pH 7.5), 100 mM KCl, $3.5mM$ MgCl$_2$, $3.5mM$

$CaCl_2$, $0.7mM$ EDTA, 0.5% Triton X-100. Dextran sulphate ($50\mu g/ml$) was added to the homogenate, passed through a double layer of muslin and centrifuged at 5,000g for 5 min. The nuclear pellet was re-homogenised in the buffer and recentrifuged. The resulting supernatants were combined and treated with 1% of Triton X-100 and 0.5% of deoxycholate, incubated for 10 min at $0^\circ C$ and centrifuged at 20,000g for 20 min. The supernatant was then re-centrifuged at 100,000g for 2h and the resulting microsomal pellet used for RNA extraction as described in section 7.12.2.

7.12   Isolation of RNA

All glassware used to isolate RNA was heat-sterilised overnight at $110^\circ C$. All solutions were sterilised by boiling for 15 min with 0.1% diethyl pyrocarbonate. All operations were carried out at $2^\circ C$, unless otherwise stated.

7.12.1 Isolation of plaice RNA

7.12.1.1 Plaice polysomal RNA

Plaice microsomes were prepared as in section 7.11.1 and dissolved in NETS buffer ($100mM$ NaCl, $1mM$ EDTA, 0.5% SDS, $10mM$ Tris pH 7.4), heated at $37^\circ C$ for 5 min and made up to a concentration of 25 absorbance units at 260nm with NETS buffer. The solution was then extracted at room temperature with equal volumes of an ANE buffer ($10mM$ NaCl, $1mM$ EDTA, $10mM$ NaAc, pH 6.0)- saturated phenol/chloroform mixture until there was no interphase, according to the method of Perry (155). The aqueous phase was precipitated overnight at $-20^\circ C$ by addition of NaCl to a final concentration of $0.1M$ and 2.5 vol. of ethanol Precipitates were collected by centrifugation for 10 min at 30,000g, and washed twice by resuspension in $3M$ NaAc, $5mM$ EDTA, pH 6.0 followed by centrifugation at 30,000g for 5 min. The RNA was dissolved in $0.1M$ NaAc, pH 6.9, and precipitated with ethanol at $-20^\circ C$ and redissolved in sterilised distilled water to a final concentration of 50 absorbance units at 260nm/ml. RNA had an absorbance ratio ($A260/A280$) of 2.1.

7.12.1.2   Plaice post-ribosomal supernatant RNA.

Plaice post-ribosomal supernatant was prepared as in Section 7. 11.1. An equal volume of ethanol was added to the supernatant and the mixture stored at $-20^\circ C$ for 2-4h. The resultant precipitate was collected by centrifugation at 30,000 for 15 mins. The resultant pellet was re-

suspended in NETS buffer and further processed as in section 7.12.1.1.

7.12.2 Isolation of rat liver RNA.

Microsomes were prepared as in section 7.11.2, and RNA extracted by the phenol-chloroform method and stored as described in section 7.12.1.1. RNA was characterised on 2.6% polyacrylamide gels to determine the quality of the RNA.

7.12.3 Isolation of E. coli RNA.

E. coli B was incubated in the presence of 200μCi of $[^3H]$-labelled uridine in 1.5 litres of broth at 37°C for 18h. Cells were spun down at 5,000g for 10 min. The pellet was dissolved in 3 vol. of SDS buffer (50mM Tris-HCl buffer, pH 7.4, 5mM MgAc$_2$,0.2% SDS) and sonicated. The solution was shaken and spun down using a bench centrifuge. The resulting supernatant was removed and equal volumes of 90% phenol were added, shaken for several min. and centrifuged at 10,000g for 5 min at 0°C. The aqueous phase was removed without disturbing the interphase and re-extracted in phenol. This procedure was continued until a clear interphase was obtained.

The nucleic acids were precipitated in 2 vol. of ethanol at -20°C overnight. This solution was then centrifuged at 30,000g for 25 min. The precipitate was washed twice with ethanol and dried under nitrogen. The pellet was redissolved in 0.15M NaAc buffer, pH 6.0. Solid NaCl to a final concentration of 4M was added to this solution and left overnight at 0°C. The pellet was collected by centrifuging for 25 min at 30,000g, was redissolved in 0.15M NaAc buffer (pH 6.0) and adjusted to 4M NaCl. This was again left overnight at 0°C and then centrifuged at 30,000g for 25 min. The pellet was resuspended in 3M NaAc (pH 6.0) and centrifuged at 30,000g for 5 min, was washed twice in ethanol-water-NaAc (75:25:2, v/v/w) and the RNA pellet was dissolved in water. (E. coli ribosomal RNA $[^3H]$ = 23,400 cpm/ absorbance unit at 260nm).

E. coli ribosomal RNA was also prepared as described above only in the absence of $[^3H]$-labelled uridine.

7.13. Quantitation of RNA.
7.13.1 The orcinol method.

Orcinol (100mg), which had been recrystallised from benzene, and FeCl$_3$, 6H$_2$O (100mg) were dissolved in conc. HCl (100ml) and 1 ml of this solution was mixed with 1ml of standard yeast RNA solution (1mg/ml) or an unknown amount of RNA. It was placed in a boiling

water bath for 45 min, cooled, and the absorbance at 670nm was read against a water blank.

7.13.2 Spectrophotometric method

The absorbance of RNA samples was measured in the u.v. region of the spectrum (230-310nm). The RNA concentration was estimated by using the relationship $E_{1cm}^{1\%} = 230$ ie. a 1%(w/v) solution of RNA gives an absorbance of 230 at 260nm with a 1cm path length. If the 260/230 and 260/280 ratio was greater than 1.8, this indicated that the solution contained a fairly pure sample of nucleic acids.

7.14. Characterisation of RNA

7.14.1 Characterisation of RNA by polyacrylamide gel electrophoresis.

The gel electrophoretic system of Loening (158) was used. RNA samples (0.5 E_{260nm} units) were electrophoresed on 2.6% polyacrylamide gels for 15 mins at 30V, followed by 60-75 mins at 60V. The gels were scanned at 265 nm in a Joyce Lobel Chromoscan fitted with a medium-pressure mercury lamp (ST 75), a 265nm interference filter and a liquid filter containing p-dimethylamino-benzaldehyde (15mg/100ml of AnalaR methanol).

Gels were placed in a 50% methanol-20% acetic acid-30% water mixture overnight. They were then rehydrated in water for 1h and then transferred into a 2% Toluidine blue-50% methanol-20% acetic acid-28% water mixture for 1h and subsequently destained in several changes of 7% acetic acid. (176).

7.14.2 Characterisation of RNA by polyacrylamide gel electrophoresis in formamide.

Amberlite monobed resin MB-1 (2g) was added to 60ml of formamide and stirred for about 2h to ensure complete mixing of resin throughout the formamide. When the specific conductivity had decreased from 400 to approx. 5μmho, the resin was filtered off and discarded and the formamide was used that day. The formamide was adjusted to pH 9.0 by addition of diethyl barbituric acid (0.0921 g per 25 ml formamide).

4% polyacrylamide gels were prepared by dissolving acrylamide (0.85g), bisacrylamide (0.15g), diethylbarbituric acid (0.0921 g) and TEMED (0.06 ml) in 20 ml formamide. After addition of 18% aqueous ammonium persulphate (0.2 ml), the volume was adjusted to 25 ml with formamide.

Lyophilised samples were dissolved in formamide, pH 9, containing 5% sucrose and a trace of Bromophenol blue as a tracker dye.

The applied sample (25µg) was overlayed with 1 ml formamide, pH 9, and then overlayed with 0.02M NaCl. Electrophoresis was carried out for about 1 h at 5mA/gel, voltage (start) 250V - (finish) 150V. To prevent the pH in the reservoirs from fluctuating, the solution was constantly circulated from the upper to the lower reservoirs.

7.14.3 Chromatography of RNA on oligo(dT) cellulose.

All operations were carried out at 0-4°C in the cold room and all glassware and reagents (except for oligo(dT)-cellulose) were sterilised. Crude ribosomal or supernatant plaice RNA ($300A_{260}$units) was dissolved in application buffer containing 0.01M Tris-HCl (pH 7.5)-0.5M KCl. The sample was applied to the oligo(dT)-cellulose (approx. 0.5g, dry wt.) X suspended in the application buffer (2ml) in centrifuge tubes. The sample was mixed thoroughly and incubated at 0°C for 30 min, then centrifuged at 30,000g for 5 min. The resulting supernatant was removed and the pellet was washed in two steps with buffers of reduced ionic strength. The first buffer contained 0.01M Tris-HCl (pH 7.5)-0.1M KCl, and the second contained 0.01M Tris-HCl (pH 7.5). Washing was continued at each of the three steps until no further material was eluted from the oligo(dT) cellulose. The washings from each of three steps were combined to produce three separate solutions. The fraction containing poly A (+) RNA was applied to the oligo(dT)-cellulose and rechromatographed. All RNA samples were then immediately precipitated by the addition of 0.2M NaCl and two vol. of ethanol, and the precipitate was collected and redissolved in sterile water. The oligo(dT)-cellulose was regenerated for repeated use by elution with 0.1M KOH. Poly(A) separation was attempted by using millipore filters and also Sepharose but oligo(dT) cellulose gave the best and most consistent results.

17.4.4 Sucrose gradient analysis of RNA.

8.5ml linear sucrose gradients (5-20% w/v) were prepared according to the method of Noll (159). The sucrose solutions were made up in 50mM Tris pH 7.5 and were previously sterilised by making them up to 0.1% diethyl pyrocarbonate, shaking overnight at 37°C, cooling to room temperature and readjusting the pH where necessary.

The RNA samples were dissolved in deionised formamide (50µl), 2.5 % sucrose. 50mM Tris, pH 7.5 was added to a final volume of 500µl. The samples were then layered on top of the gradients, which had previously been chilled for several hours. The gradients were centrifuged at 100,000g for 18h at 2°C and monitored for absorbance at 260nm on a Pye-Unicam SP 1800

spectrophotometer. 125μl fractions were collected. RNA was precipitated with 2.5 vol. of ethanol and 0.2M NaCl overnight at -20°C.

Two RNA preparations were used to define optimum centrifugation times for RNA analysis. Rat liver microsomal RNA was initially used. RNA ($25 \, A_{260}$ units) was layered on to a 8.5ml,(5-20%) sucrose gradient in the isolation buffer and centrifuged for 27h at 100,000g (2°C). The tube was pierced and the solution was monitored through a spectrophoto-meter at 260nm and fractions were collected and precipitated in ethanol and the RNA was examined on 2.6% polyacrylamide gels. These results gave a preliminary indication of the conditions required for sucrose-gradient centrifugation. A more extensive experiment was carried out with E. coli rRNA. Radioactive E. coli rRNA[^{3}H] -labelled uridine, 5000 cpm) was layered over 8.5ml 5-20% linear sucrose gradient (in 50mM Tris buffer, pH7.4) and centrifuged at 100,000g for 18h, 24h and 30h. The gradients were analysed and fractionated by counting in 6.6ml of Triton-toluene scintillant and 300μl of water. This method was used to dilute the sucrose so it could be counted in this specific scintillant. Centrifugation at 100,000g for 18h was found to give the best position of RNA in the gradient, whereas centrifugation for 24 and 30h caused the RNA to sediment too far down the gradient.

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