SOME STUDIES OF ASPECTS OF PROTEIN SYNTHESIS IN EMBRYONIC CHICK

by

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ABSTRACT

Studies were made of some transfer RNAs of chick embryo and of amino acid incorporation by the nuclear and mitochondrial fraction of chick embryo.

The possibility that a specific proline-accepting tRNA might regulate the onset of synthesis of collagen was investigated. Collagen synthesis begins at about 8 days of embryonic development in the chick. Transfer RNA was isolated from 4- and 8-day chick embryos and aminoacylated with radioactive proline. The labeled RNAs from each stage were chromatographed on methylated albumin kieselguhr and benzoylated diethylaminoethyl-cellulose columns, and the chromatographic profiles were examined for stage-specific differences. Initial observations of a quantitative difference in a prolyl-tRNA species from stage to stage could not be repeated, and the investigation was accordingly abandoned.

The second series of experiments was suggested by an observation of H.-M. Tsay, formerly of this laboratory (82). On chromatography of T1 ribonuclease digestion products of 3,4 -^{3}H- and uniformly-labeled ^{14}C-leucyl-tRNAs of chick embryo, a departure from unity in the ratio of labeled carbon to tritium was seen in one of the resulting peaks.

This work was repeated with tRNA preparations from several stages of chick embryo and adult chick liver. The carbon-enriched peak was observed in every case, and the hypothesis was made that a specific derivative of leucine, in which the 4,5-H atoms were lost, was formed during the tRNA aminoacylation or digestion reaction. No such phenomenon was observed on chromatography of leucine-labeled tRNA fragments obtained by digestion of rabbit liver and E. coli tRNA with T1 ribonuclease.

Various methods were used in attempts to isolate and characterize the proposed derivative, but none were successful.

It was thought at the time this work was done that a eukaryotic initiator tRNA might be N-blocked, as is the common prokaryotic initiator, N-formylmethionyl-$tRNA_F$. A labeled amino acid mixture was recovered from tRNA and tRNA digestion fragments by standard deacylation procedures. An ethyl acetate extraction method for N-blocked amino acid derivatives was found to yield no evidence for a carbon-enriched N-blocked derivative of leucine. It was therefore concluded that the possible derivative was of no significance in the initiation of protein synthesis in the chick, and the investigation was dropped.

The third investigation began as a continuation of

studies by Trevithick and Wainwright (144) on amino acid incorporation by chick embryo nuclear fraction in response to ATP and various inhibitors of protein synthesis. Trevithick and Wainwright observed great variability in the extent of incorporation of radioactive amino acid from fraction to fraction. Modification in the isolation and incubation procedures were made in this study, which reduced this variability to some extent. Nuclear fractions were assessed for purity by several means, including electron microscopy and enzymatic assays.

Amino acid incorporation by purified nuclear fraction was found to be linear with respect to time and to concentration of nuclear fraction. The incorporation of amino acid was shown to be sensitive to chloramphenicol and puromycin, and insensitive to cycloheximide, respiratory inhibitors, and ribonuclease.

The acidic protein components were extracted from nuclei after incubation with radioactive amino acid. This protein fraction was subjected to polyacrylamide gel electrophoresis. A number of protein bands were visualized by staining, of which two were found to be radioactively labeled.

In another experiment designed to demonstrate the incorporation of amino acid into protein by nuclear fraction, an extract of proteins was prepared from post-incubation nuclei. The kinetics of digestion of these proteins by

exopeptidases were shown to be similar to those obtained on digestion of internally-labeled E. coli protein. These results were interpreted to indicate that amino acid is incorporated into internal positions of proteins by a protein-synthesizing system within the nucleus.

A particulate fraction containing the nuclei and mitochondria ("nuclei plus mitochondria" fraction) of 3- to 5-day chick embryo was used extensively. The nuclear and mitochondrial fractions were purified after incubation and the level of radioactive amino acid incorporation determined. The effects of cycloheximide, puromycin, and chloramphenicol on amino acid incorporation by the components of this fraction were studied. The result were comparable to those of Trevithick and Wainwright with the exception of those experiments in which chloramphenicol was used. The nuclear moiety of the "nuclei plus mitochondria" fraction was found to be insensitive to chloramphenicol inhibition of amino acid incorporation.

Amino acid incorporation by the isolated nuclear fraction was shown to be inhibited by chloramphenicol, as was that of the mitochondrial fraction.

Mixtures of purified nuclear and mitochondrial fractions of 3- to 5-day chick embryo were made and incubated with ^{3}H-lysine in the presence and absence of chloramphenicol. As in the crude "nuclei plus mitochondria" fraction, the

incorporation of amino acid by the nuclear fraction was not inhibited by chloramphenicol.

The hypothesis was made that some component of the mitochondrial fraction protects the amino acid-incorporating system of the nuclear fraction from inhibition by chloramphenicol.

These experiments were repeated with "nuclei plus mitochondria" fraction of earlier embryos at the primitive streak and 10-somite stages of development. The nuclear fraction of embryos at these stages was found to be inhibited by chloramphenicol in the presence of the mitochondrial fractions.

Mixtures were made of purified nuclear and mitochondrial fractions of 2- and 5-day chick embryos. Mitochondrial fraction from 5-day, but not 2-day, chick embryo was found to prevent chloramphenicol inhibition of amino acid incorporation by the nuclear fractions of both stages.

List of abbreviations and symbols

aa	Aminoacyl- or amino acid
AMO Medium	Allfrey-Mirsky-Osawa Medium
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
B. subtilis	*Bacillus subtilis*
BD-cellulose	Benzoylated DEAE-cellulose
CoA	Coenzyme A
DNA	Deozyribonucleic acid
DEAE	Diethylaminoethyl
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetate
Fmet-tRNA$_F$	N-formylmethionyl-tRNA
glN	Glutamine
GTP	Guanosine triphosphate
his	Histidine
IPA	N^6-(Δ^2-isopentenyl)-adenosine
leu	Leucine
mRNA	Messenger ribonucleic acid
MAK	Methylated albumin kieselguhr
uCi	Microcuries
mCi	Millicuries
nm	Nanometers (10^{-9} meters)
phe	Phenylalanine

Poly AG	Synthetic polymer of adenine and guanine
POPOP	1, 4-bis-2-(4-methyl-5-phenyloxazoly)-benzene
PPO	2,5-diphenyloxazole
pro	Proline
PCA	Pyrrolidone carboxylic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
tRNA	Transfer ribonucleic acid
TCA	Trichloracetic acid
Tris	Tris-(hydroxymethyl)-aminomethane

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SECTION I - LITERATURE REVIEW

The participation of nucleic acids in the synthesis of proteins has been recognized for many years. A large body of evidence supports the present concept that deoxyribonucleic acid (DNA) serves as a template for the synthesis of ribonucleic acids, which in turn direct the synthesis of specific proteins.

DNA, the genetic material of the cell, is a helical structure composed of two strands of nucleotides. The nucleotides are linked by phosphodiester bonds, and the strands are joined by specific hydrogen bonding between adenine and thymine, and guanine and cytosine (1).

During transcription, the synthesis of ribonucleic acid (RNA), the bases of the coding strand of DNA form specific hydrogen bonds with the ribonucleotide bases. Adenine, guanine, and cytosine are common to DNA and RNA, and uracil replaces thymine in RNA. Polymerization of the ribonucleotides is effected by enzymic catalysis. Transcription has been reviewed extensively (2) and will not be discussed in detail.

The complementary RNA products of DNA structural genes are called messenger RNAs (mRNA). Each group of three bases along the mRNA strand specifies one amino acid in a

polypeptide chain. This group is called a triplet or codon, and 64 different triplet combinations of the four nucleotides are possible. Twenty amino acids are generally required for the synthesis of proteins, and it has been shown that one amino acid may be specified by more than one codon. Three of the triplet codons specify no amino acid in the normal system, and instead signal termination of amino acid addition to the polypeptide chain.

The first investigators to determine the nucleotide sequence specifying the incorporation of a particular amino acid were Nirenberg and Matthaei (3). Their later work and that of Ochoa, Garen and others established the specificity of each of the trinucleotide codons. This has been reviewed by Garen (4).

Messenger RNA does not interact directly with amino acids, but with specific transfer RNAs (tRNA) carrying covalently bound amino acids. This interaction takes place on the ribosome, a particle made up of two subunits composed of specific ribosomal RNAs and proteins. A group of three nucleotides of the tRNA, the anticodon, pairs, by specific hydrogen bonding, with the codon of the messenger RNA. Amino acids are brought sequentially into the order specified originally by the nucleotide sequence of DNA,

and polymerized into a protein chain. This process is termed translation of the genetic code. It has been demonstrated that the sequence of amino acid residues in a particular protein is colinear with the sequence of trinucleotide codons (5,6).

Ribosomes

The formation and structure of ribosomes of prokaryotes and eukaryotes have been reviewed recently (7,8). More recent data on the formation of ribosomal RNA in the nucleolus of eukaryotes are given by Weinberg and Penman (9), and Wellauer and Dawid (10).

The ribosome is composed of two subunits, one of which is larger than the other. The bacterial subunits have sedimentation values of 30S and 50S, and those of the mammalian, of 40S and 60S. During the process of protein synthesis, several ribosomes are associated with mRNA in a structure called a polyribosome, or polysome (11, 12). The subunits undergo cyclic association and dissociation in the course of protein synthesis (13,14,15,16). Messenger RNA binds to the 30S subunit of the ribosome. The 50S subunit bears two sites, the aminoacyl and the peptidyl sites, at which tRNA species are bound during protein synthesis.

Protein synthesis

The process of protein synthesis may be divided into three stages: initiation, chain elongation, and termination.

An initiation complex is formed by the association of messenger RNA, the two subunits of the ribosome, and a specific initiator tRNA. The formation of the complex is dependent upon initiation factors, which will be discussed. N-formylmethionyl-$tRNA_F$ is the initiating species in prokaryotes (17,18), and is also found in mitochondria (19,20) and chloroplasts (21). The similarities of bacterial and mitochondrial protein synthesizing systems will be discussed later in this review. The initiating tRNA species of higher organisms is a specific methionyl-tRNA (22,23,24,25,26).

Initiation factors

The formation of the initiation complex depends also upon several proteins, the initiation factors, and GTP. The initiation factors required for the binding of mRNA and tRNA to the ribosomes have been characterized in E. coli (27).

Some initiation factors of both prokaryotes and eukaryotes have been shown to effect specific binding and translation of particular mRNAs. Steitz, Dube, and Rudland (29) have shown that ribosomes from E. coli infected with phage T4 interact only weakly with the initiation region of one of the three cistrons of phage R17 RNA to form polypeptide chain initiation complexes. Ribosomes from uninfected E. coli bind strongly to the initiation sites of all three cistrons of R17 RNA. Binding tests of combinations of washed ribosomes and initiation factor fractions of infected

and uninfected cells showed this specificity to reside in the initiation factor fraction obtained by washing the ribosomes in solutions of high salt concentration.

Revel, et. al. and others have demonstrated that the F3 class of initiation factors is responsible for the specificity of binding of natural messengers by E. coli ribosomes (30,31,32). Although specific factors for the binding of particular messenger RNA are not always required, Heywood has presented evidence of specific initiation factors for the synthesis of the globins of muscle and erythrocytes in embryonic chicks (33). Ilan and Ilan have demonstrated the specificity of initiation factors as a function of the developmental stage of Tenebrio (34).

Initiation complex

The completed initiation complex of E. coli consists of the 70S ribosome to which is bound the mRNA and initiating aminoacyl tRNA species fMet-tRNA$_F$. The formation of this complex involves several intermediates. The first has been postulated to be an "active" 30S subunit carrying the three initiation factors IF-1, IF-2, and IF-3. (35). Messenger RNA is bound to the 30S subunit by the action of IF-3. (30) GTP and fMet-tRNA$_F$ are bound by IF-2 on the 30S subunit and the tRNA associates by specific hydrogen bonding with the AUG initiator codon of the MRNA. (36). IF-3 is released from the 30S complex at this point.

The 50S subunit is then bound with the hydrolysis of GTP. IF-1 and IF-2 are then released from the 70S intermediate. IF-1 is presumed to mediate the release of IF-2 from the complex, and may remove GDP from a postulated IF-2-GDP complex. It is certainly required for the recycling of IF-2 in the initiation process, rendering the IF-2 activity catalytic rather than stoichiometric. The 70S complex, with the fMet-$tRNA_F$ on the peptidyl or donor site of ribosome, is then complete. (37)

The sequence of events in the formation of the eukaryotic initiation complex has been postulated to differ significantly from that of prokaryotes (38). As in bacteria, the small subunit of the ribosome is first associated with messenger and initiator RNAs, but there is evidence that the first species to be bound to the 40S subunit is Met-$tRNA_F^*$, by $IF\text{-}E_2$ in association with GTP. $IF\text{-}E_3$ promotes this binding. A small RNA has been found to be associated with initiation factors, and may be involved in some way with the binding of MRNA, perhaps by specific hydrogen bonding (39). $IF\text{-}E_3$ catalyzes the binding of the messenger to the 40S subunit. The addition of the 60S subunit requires the factor $IF\text{-}E_4$. GTP is hydrolyzed on the addition of the 60S subunit.

Chain elongation

The sequential addition of amino acids to the peptide chain, proceeding from the amino terminus (40), is dependent upon soluble chain elongation, or transfer factors (41,42).

At the completion of the initiation complex of prokaryotes and eukaryotes, a second ribosomal binding site for aminoacyl-tRNA is made available. The tRNA carrying the amino acid specified by the second codon of the messenger is transferred to the bacterial acceptor site by a complex of elongation factor EF-Tu and GTP in a reaction involving hydrolysis of GTP. A peptide bond is formed between the formylated methionine and the second amino acid by peptidyl transferase. This activity resides in the ribosome and specific proteins of the large subunit have been identified as components (43). EF-Ts displaces GTP from EF-Tu, thereby regenerating the latter. Elongation factor G and GTP are involved in the release of the deacylated initiator tRNA from the peptidyl site and the translocation of the newly formed dipeptidyl-tRNA from the aminoacyl to the peptidyl site of the large subunit. Chain elongation in eukaryotes follows much the same pattern. Eukaryotic elongation factor 1 is analogous to EF-Ts and EF-Tu of prokaryotes, and EF-E2 serves the same function as EF-G.

The GTPase activity necessary to peptide chain formation is felt to reside in one ribosomal site. There is evidence from studies on B. stearothermophilus that a specific 5S RNA-protein complex from the 50S ribosomal subunit is the bearer of GTPase activity in initiation and elongation of peptide chains (44).

Chain termination

The mRNA codons UAA, UAG, and UGA are signals for termination of polypeptide synthesis in bacteria and mammals. Release factors specific for particular termination codons are required to free the polypeptide from the prokaryotic tRNA-mRNA-ribosome complex (45,46,47). In bacterial cells, N-formylmethionine release is stimulated by the S factor in the presence of GTP (48). A single release factor is present in eukaryotes and GTP is required for chain termination (49).

Recycling of ribosomal subunits

The ribosomal subunits dissociate on chain termination and in prokaryotes, the initiation factor IF-3 promotes this dissociation. (50). It is not yet known whether the corresponding IF-E3 eukaryotic factor is a dissociation or anti-association factor for eukaryotic ribosomes.

Transfer RNA

There are about 60 different tRNAs in the typical

cell (51). The complete nucleotide sequence of a purified tRNA was first determined by Holley and his coworkers (52). The structure of tRNA is at present being actively studied.

X-ray diffraction studies and Raman spectroscopy have confirmed early models of tRNA in which a cloverleaf secondary structure comprising single-stranded loops and double-stranded stems was postulated (53,54). A tertiary structure produced by folding of the cloverleaf is also indicated, and may be important in aminoacylsynthetase recognition (55).

Transfer RNA nucleotides are very highly modified, and all known modifications are effected at the polynucleotide level (50). Most of the modifications are methylations of either the base itself or the ribose moiety. Pseudouridine, in which ribose is bound to uracil by a carbon-glycosidic linkage, rather than by the usual nitrogen-glycosidic linkage, is extremely common in tRNAs. Thionucleosides (56) and nucleosides bearing large side chains have also been found. One of the most familiar of these is N^6-(2-isopentenyl)-adenosine (IPA), a cytokinin promoting plant growth and development. This substance has been found in several preparations of tRNAs from different sources. It is located at the 3' end of the anticodon in yeast seryl- and tyrosyl-tRNAs, in rat liver seryl-tRNA, and in two of the three species of E. coli Su^+_{III} tyrosyl-tRNAs. Species

3 contains the methylthio derivative of IPA, species 2 has IPA itself, and species 1 has an unmodified adenosine. Species 1 is unable to support the incorporation of amino acid in protein. The IPA-containing tRNAs have the common property of responding to a codon the first letter of which is U (57,58,59,60,61,62).

A similar situation has been observed in eukaryotic phenylalanine-tRNAs. One of the Y-type fluorescent bases is adjacent to the 3' end of the anticodon in several $tRNA^{Phe}$ species examined (63).

Transfer RNA in protein synthesis

Hoagland, et. al. made some of the first studies of the role of tRNA in protein synthesis (64). In 1955, they observed that an exchange reaction between ^{32}P-labeled pyrophosphate and ATP in the supernatant fraction of rat liver homogenate was stimulated by the addition of amino acids. If a second amino acid was added to a system that had been stimulated by a first amino acid, additional stimulation resulted (65). A form of amino acid activated for protein synthesis was suggested; an aminoacyl adenylate formed by the following sequence:

1. Enz + ATP $\rightleftharpoons$ Enz-AMP-PP
2. Enz-AMP-PP + amino acid $\rightleftharpoons$ Enz-AMP-aa + PP.

Specific activating enzymes for each amino acid were soon found (66,67,68).

The role of RNA of low molecular weight in the activation of amino acids was postulated by Crick (69) and demonstrated independently by Hoagland and coworkers (64) and Ogata and Nohara (70). Hoagland showed that when ^{14}C-leucine and ATP were incubated with the activating enzyme system present in a crude fraction, the "pH 5 fraction", of the supernatant from rat liver, a low molecular weight species of RNA present in the fraction became labeled. The radioactivity of the product was released either on incubation with hydroxylamine, yielding leucine hydroxamate, or into protein on incubation in the ribosomal protein-synthesizing system. GTP was required for the latter process, and a great excess of ^{12}C-leucine did not inhibit the incorporation significantly.

Ofengand and Berg demonstrated that activating enzymes are specific in the reaction of transfer of amino acid to RNA (71).

The 3'-OH end of each tRNA has the common sequence CCA. Amino acid is bound to the 2'-hydroxyl group of the terminal adenine nucleotide. The amino acid is then transferred to the 3'-OH site (72). The activating enzymes discussed above are responsible for both amino acid activation and transfer to tRNA, and are now referred to as aminoacyl-tRNA synthetases. The enzyme-bound aminoacyl-adenylate (reaction 2, above) reacts with a molecule of tRNA specific

for the amino acid and an ester linkage between the 2'-hydroxyl group of the ribose of the terminal adenosine and the activated carboxyl group of the amino acid is formed. As stated above, the aminoacyl group is transferred to the 3' position. AMP is liberated in the reaction. The precise mechanism is not yet determined (55). Multiple synthetases for the activation and transfer of one amino acid have been found (73,74,75,76).

Changes in chromatographic profile of isoaccepting tRNAs

More than one species of tRNA may accept the same amino acid (77). The complement of these isoaccepting tRNA species has been shown to change in differentiating cells, cells which become malignant, and cells infected with virus. Daniel, et. al. found differences in the arginyl-tRNAs of mammalian cells after infection by herpesvirus (78). Doi and his coworkers found differences in the relative proportions of two valyl-tRNAs during growth transitions caused by changes from minimal to enriched medium, and vice versa, and sporulation in B. subtilis (79). Differences in isoaccepting tRNA complements in higher organisms have been studied in sea urchins by Yang and Comb (80), in chick embryos and erythrocytes by Wainwright, et. al. (81), Tsay (82), and Lee and Ingram (83), and in insects by Ilan, Ilan, and Patel (84). Gallo and Pestka found differences among several tRNA species in normal and leukemic

lymphoblasts (85). Cells synthesizing different immunoglobulins have been shown by Yang and Novelli to have differences in tRNA patterns (86).

Some concepts of regulation of protein synthesis by RNA

Mechanisms by which tRNA may regulate protein synthesis have been proposed by Itano (87), Sueoka and Kano-Sueoka (88), and Ames and Hartman (89).

Itano has suggested that "modulating" triplets might code for tRNA species which affect the reading of the messenger RNA, perhaps causing the attachment of messenger to ribosome to be less stable, and thus limiting the rate of translation of the message containing this triplet.

The adaptor modification hypothesis of Sueoka and Kano-Sueoka does not invoke a mutation in the tRNA gene, but proposes that the codon recognition of a particular isoaccepting tRNA may be changed by structural modification; for example, methylation or conformational change. Messenger RNAs containing the codon to which such an altered tRNA responds might not be translated normally, but the translation of other messages would be unaffected.

Anderson concluded from his work on E. coli that rate-limiting tRNA species may regulate protein synthesis at the translational level (90). The rate of translation of poly AG in vitro by an S-30 protein-synthesizing system was shown to be limited by the amount of $tRNA^{Arg}$ recognizing the codons AGA and AGG.

Vaughn and Hansen have shown some evidence for a role of uncharged tRNA in the control of the initiation complex (91).

Aminoacyl-tRNA synthetases are also implicated in the control of protein synthesis. Strehler and his coworkers have found they may control the relative amounts of free and acylated forms of cognate tRNAs (92). Kanabus and Cherry have found differences in leucyl-tRNA synthetases from two tissues of soybean (76). Ilan, Ilan, and Patel showed the necessity for both a new tRNA and a new synthetase for the metamorphosis of Tenebrio larvae (84).

Initiator tRNA

As mentioned previously, N-formylmethionyl-tRNA has been shown to be the initiating species in E. coli (93), as well as in mitochondria and chloroplasts. Marcker, Clark, and Sanger (17,18) found two species of methionine-accepting tRNA in E. coli and showed that one Met-tRNA could be formylated by reaction with formyltetrahydrofolate, catalyzed by the E. coli enzyme Met-tRNA transformylase. This species, Met-$tRNA_F$, responds to the codons AUG, GUG, and UUG at the beginning of a message, so that N-formylmethionine is incorporated at the N-terminal of the peptide chain. The other species, Met-$tRNA_M$, responds to the codon AUG, and methionine is incorporated into internal positions of the polypeptide chain. Met-$tRNA_F$ associates in the usual case with initiation factors (22). Exceptions have been found

in in vitro experiments. Met-tRNA$_M$ may be an initiating species at high Mg^{++} concentrations, and Met-tRNA$_F$ may be incorporated at internal positions in the absence of Met-tRNA$_M$.

Accumulating evidence indicates that N-formylmethionyl-tRNA is normally the universal initiating species of tRNA in prokaryotes, although exceptions to this general rule have been noted. Streptococcus faecalis can grow in the presence of the folate antagonists aminopterin and trimethoprim if end-products of the biosynthetic pathways requiring folate as a cofactor are added to the medium, which suggests that N-formylmethionyl-tRNA$_F$ may not be involved in initiation in this species (94).

Protein synthesis in the cytoplasm of eukaryotic cells is not initiated with N-formylmethionyl-tRNA. In the past few years, much work has been done to determine the initiating tRNA species of eukaryotic systems.

Pyrrolidone carboxylic acid-tRNA (PCA-tRNA), formed by cyclization of the glutamine moiety of glN-tRNA, was suggested as an initiator of immunoglobulins by Moav and Harris (95). Baglioni later failed to find PCA-tRNA in mouse myeloma cells after labeling with ^{14}C-glutamine, and found that tRNA could not be acylated with pyrrolidone carboxylic acid. He showed that glutamine end terminals of proteins can cyclize spontaneously or by enzymatic

action, and concluded that pyrrolidone carboxylic acid is probably not an initiator (96).

In 1968, Arnstein and Rahaminoff suggested that an N-blocked valyl-tRNA is the initiator of rabbit hemoglobin (97). More recent work on the rabbit reticulocyte system has shown that an unsubstituted methionyl-tRNA is the initiating species (22,23,24,98,99). As in prokaryotes, two species of Met-tRNA are present in the reticulocyte. Neither is formylated in vivo, but it is possible to formylate one of them with the bacterial transformylase. This species is referred to as Met-$tRNA_F^*$ and is the initiating species in rabbit reticulocytes. It transfers methionine to the N-terminus only. Methionine is removed from the nascent polypeptide chain after 15-20 residues have been added.

The other species, Met-$tRNA_M^*$, transfersmethionine to internal positions of the chain. Met-$tRNA_F^*$ binds to ribosomes at low magnesium ion concentration in response to M1 and M2, eukaryotic initiation factors, but binds to only a slight extent, if at all, in response to the transfer factor T1. The converse is true of Met-$tRNA_M^*$.

Met-$tRNA_F^*$ is also the initiating species in the synthesis of protamine in trout testis (100) and in the synthesis of adenovirus proteins by cultured human KB cells which have been infected by this virus (101).

There is, however, some conflicting evidence which may indicate the involvement of other tRNA initiating species in eukaryotes. The N-terminus of rat liver f2a histones is N-acetylseryl-glycyl-arginyl--, and Liew, Haslett, and Allfrey found N-acetylseryl-tRNA in rat liver. On the basis of the finding that tRNA can be sequentially charged with ^{3}H-serine and ^{14}C-acetate, the authors concluded that N-acetylseryl-tRNA is involved in the initiation of histone biosynthesis (102).

Better evidence for the involvement of other initiating species was provided by Narita and his coworkers, who studied ovalbumin synthesis by hen oviduct minces incubated with ^{14}C-acetate and amino acids. Digestion products of the incubation mixture included ^{14}C-acetylglycyl-serine. Puromycin inhibited both the synthesis of radioactively labeled ovalbumin and ^{14}C-acetylglycyl-serine, which suggested that N-terminal acetylation was closely linked with ovalbumin synthesis. N-acetylglycyl-puromycin was not found in the course of these experiments (103), but later work did demonstrate its formation by the minces and provided strong evidence for the conclusion that protein synthesis by hen oviduct minces is initiated with N-acetylated glycyl-tRNA (104).

Melcher has, however, raised serious questions about the significance of Narita's results (105). Melcher has fouhd that yeast cells form acylaminoacylpuromycins and

aminoacylpuromycins in the presence of high concentrations of puromycin; that is, under conditions in which it was expected that only methionyl-puromycin would be found. Several amino acids formed such derivatives with puromycin and methionyl derivatives did not predominate. The yeast cells were preincubated with chloramphenicol to inhibit mitochondrial protein synthesis, and added cycloheximide did not inhibit the formation of derivatives by more than 10%, though protein synthesis was inhibited by more than 90%. It therefore seems that the formation of aminoacyl derivatives of puromycin is not necessarily related to protein synthesis.

Such anomalous reactions have been seen in both prokaryotes and eukaryotes. In the presence of methanol, Fmet-tRNA$_F$ of E. coli can react with puromycin in the absence of template and the 30S ribosomal subunit (106). This phenomenon seemingly differs from the formation of aminoacyl derivatives of puromycin by yeast in that contribution of the larger ribosomal subunit to the reaction is evidently absent in the latter. Acetylphenylalanyl-tRNA of rat liver can react with puromycin in the presence of transfer factor T2 and GTP (107). The bulk of the evidence, however, seems to show that initiation of protein synthesis in prokaryotes is effected by Fmet-tRNA$_F$, and, in eukaryotes, by Met-tRNA$_F^*$.

It has recently been shown that eukaryotic initiator tRNAs differ from other tRNAs in internal structure. All other tRNAs active in protein synthesis, including the E. coli initiator tRNA, have a common sequence G-T-Ψ-C-G(A)-. Initiator tRNAs from yeast, wheat germ, rabbit liver and sheep mammary gland have been shown to lack this sequence (108,109).

Suppressor tRNA

Some strains of E. coli have been shown to have nonsense mutations in the phosphatase structural gene, as a result of which the "amber", UAG, or "ochre", UAA nonsense codon has appeared in the messenger RNA. Incomplete polypeptides are produced as a result of these mutations. Further mutation may occur in another part of the genome, which leads to suppression of the effect of the primary nonsense mutation. An amino acid is inserted in response to the nonsense codon, and chain elongation continues. Three suppressor genes have been characterized by the amino acid which is inserted: serine by Su-1$^+$, glutamine by Su-2$^+$; and tyrosine by Su-3$^+$(110).

The Su-3$^+$ suppressor gene has been shown to code for a tyrosine-specific tRNA that differs from Su-3$^-$ tyrosine-specific tRNA in one nucleotide of the anticodon, a change that allows recognition of the amber codon UAG. The ochre suppressor genes also exert their effects through transfer RNAs and permit a low level of amino acid incorporation in

response to the codons UAG and UAA (110). Other suppressors have been identified with tRNAs since the above work.

Other functions of tRNA

The first step in the histidine biosynthetic pathway of Salmonella typhimurium is catalyzed by the enzyme phosphoribosyltransferase. The synthesis of this enzyme, and the rest of the enzymes in the histidine pathway, may be repressed by histidyl-tRNA. Phosphoribosyltransferase itself has a high affinity for tRNA, and the binding of his-tRNA is favored over that of other species of tRNA. All five of the histidine regulatory genes seem to be involved in the synthesis or aminoacylation of tRNA (111). A complex ofleucyl-tRNA and threonine deaminase has been proposed as an autoregulatory repressor of synthesis of the enzyme (112).

Tryptophan pyrrolase is present in both wild-type and vermilion mutant Drosophila melanogaster, but the enzyme is inactive in the mutant. Treatment of homogenates of the mutant with ribonuclease T1 activates the mutant enzyme. Wild-type tRNA was added to the activated enzyme preparation, and was shown to reverse the activation. The inhibition was found to be due to a tyrosine-specific tRNA in the uncharged state (113).

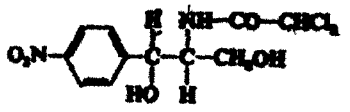
Transfer RNA is involved in the synthesis of aminoacyl phosphatidylglycerols, such as lysylphosphatidylglycerol (114). It also participates in the synthesis of bacterial cell wall peptidoglycans. In Staphylococcus epidermidis, one each of the isoaccepting glycyl- and seryl-tRNA species functions only in peptidoglycan synthesis, and does not participate in general protein synthesis (115).

Inhibitors of protein synthesis

Much of the work on protein synthesis in nuclei and mitochondria that will be discussed in this survey includes experiments in which inhibitors of protein synthesis were used.

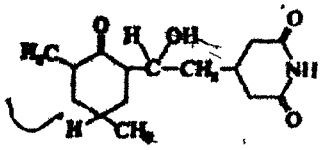
Chloramphenicol is an inhibitor of protein synthesis on the ribosomes of bacteria, and those of yeast, rat liver, and locust mitochondria (116,117,118).

The structure of chloramphenicol is shown below

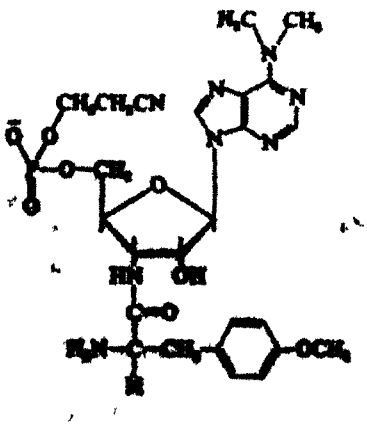


Chloramphenicol interacts specifically with the large subunit of bacterial, mitochondrial and chloroplast ribosomes at, or near, the active site of peptidyl transferase. (43). At high concentrations, chloramphenicol has some effect on energy production by inhibiting NADH oxidase (119). The work discussed here is limited to studies at low concentrations.

Cycloheximide is an inhibitor of protein synthesis in the cytoplasm of eukaryotes, and is analogous to chloramphenicol in activity, interacting with peptidyl transferase of 60S ribosomal subunits.
The structure of cycloheximide is



Puromycin acts as a competitor of aminoacyl-tRNA on all types of ribosomes in the chain elongation reaction.
The nascent peptide chain



is transferred by peptidyl transferase to puromycin rather than to the aminoacyl-tRNA next specified by the message (120).

Protein synthesis in mitochondria

Mitochondria have been shown to possess unique types of DNA, RNA, and ribosomes, which differ from those present in other parts of the cell (121,122,123).

Ribosomes isolated from the mitochondria of yeast (124), Aspergillus nidulans (125), and chick cells (126) differ in size from those in the cytoplasm, and contain different species of RNA. The RNAs of chick mitochondrial ribosomes are products of mitochondrial genes (126).

Specific transfer RNAs of mitochondria from rat liver, Neurospora, and HeLa cells can be distinguished from cytoplasmic tRNAs (127,128,129).

Isolated mitochondria have been shown to be able to incorporate amino acids into protein (130,131). Following the initial demonstrations, the location and characterization of the proteins synthesized by mitochondria were studied. Beattie and her coworkers showed that rat liver mitochondria incorporate amino acid predominantly into the structural proteins of the inner membrane (132). The same is true of the mitochondria of Neurospora crassa. None of the proteins of the outer mitochondrial membrane are made in the mitochondria (133). The soluble proteins of the

matrix, the proteins of the outer membrane, and some proteins of the cristae membranes are synthesized outside the mitochondrion on cytoplasmic ribosomes. Some of the components of cytochrome oxidase, for example, have been shown to be coded by a nuclear gene and synthesized in the cytoplasm (134). The proteins of the mitochondrial ribosome are probably made on cytoplasmic ribosomes (135).

Studies with inhibitors of protein synthesis in mitochondria and the cell cytoplasm have shown the interdependence of cytoplasmic and mitochondrial protein synthesis in the production of functional mitochondrial enzymes.

Some studies of protein synthesis by locust flight muscle mitochondria in the presence of cycloheximide seem to validate the assumption that cycloheximide may be used to inhibit all non-mitochondrial protein synthesis without effect on the mitochondrial system. The proteins synthesized in the presence of cycloheximide by isolated locust mitochondria are comparable in electrophoretic pattern to those synthesized in the absence of cycloheximide. In vivo studies showed that 80-85% of the labeled proteins found in the mitochondrial fraction of the control samples after incubation with labeled amino acid did not appear in the presence of cycloheximide (136).

Oxidative metabolism in yeast may be repressed by anaerobiosis and growth on glucose. During derepression, functional mitochondrial enzymes appear (137). The synthesis and function of proteins containing heme (cytochromes) were assayed during derepression in Saccharomyces, and both the chloramphenicol-sensitive and cycloheximide-sensitive protein synthesizing systems were found to be necessary for the production of functional enzyme.

In both these cases, mitochondrial protein synthesis is evidently necessary for integration of enzyme molecules into the mitochondrial structure.

The role of non-cytoplasmic protein-synthesizing systems during the course of embryonic development is an important field of interest. Temporal differences in the effect of inhibitors of protein synthesis on developing embryos have been observed. The participation of the mitochondria in the early development of the mouse embryo, from the two- or four-cell stage to the blastocyst, has been assessed by Piko and Chase. Mitochondrial RNA and protein synthesis of embryos cultured in vitro was specifically inhibited with ethidium bromide and chloramphenicol. Development to the blastocyst stage was essentially unaffected by these inhibitors, although the morphogenesis of the mitochondrial genome is evidently not required for normal embryonic development to the blastocyst stage (138).

Protein synthesis in nuclei

Amino acid incorporation by cell nuclei has been investigated in many species. This is a subject of interest because of the possibility that observed incorporation might represent synthesis of proteins that serve specialized functions within the nucleus. These proteins might include controllers of nucleic acid synthesis, for example.

Important early work on amino acid incorporation by nuclei includes that of Allfrey and coworkers with isolated calf thymus nuclei. They were able to show that amino acid was incorporated into lipoproteins and strongly-associated chromosomal proteins (139). Zimmermann (140) isolated nuclei and nucleoli from HeLa cells with careful attention to the removal of cytoplasmic contamination, incubated them with radioactive amino acid, and demonstrated in vitro labeling of specific proteins. Similar work has been done on isolated nuclei of plants, fish, birds, and other mammals (141,142,143,144). Protein synthesis within cell nuclei remains, however, a controversial topic. Several valid objections can be raised to much of the earlier work in particular. One of the most serious of these is that removal of cytoplasmic contaminants, particularly perinuclear ribosomes, was not demonstrated in many investigations. Allfrey's work on calf thymus nuclei has been criticized

on these grounds, as has that of Birnsteil with pea nuclei (143). Bach and Johnson emphasized the importance of removing ribosomes from the outer nuclear membrane (145). These ribosomes are capable of carrying out protein synthesis.

Cytoplasmic contribution to observed levels of amino acid incorporation into nuclei is particularly difficult to assess in in vivo experiments. It has been shown that many proteins found in the nucleus are synthesized on cytoplasmic ribosomes and subsequently transported into the nucleoli of HeLa cells. Some ribosomal proteins have been shown to be synthesized in the cytoplasm (140,146). Robbins and Borun have shown that some, if not all, of the histones of rat nuclei are synthesized in the cytoplasm and then move to the nucleus (147). An increasing amount of labeled protein is transferred from the cytoplasm to the nucleus of sea urchin embryos as early development progresses (148). Goldstein has described two classes of nuclear proteins in Amoeba proteus, one of which migrates rapidly back and forth between the nucleus and the cytoplasm (149).

This phenomenon has been so well established that Goldstein (150) has been extremely critical of observers who conclude, from in vivo studies on the kinetics of uptake of labeled amino acid into nucleus and cytoplasm, that de novo protein synthesis occurs within the nucleus. He states that within the shortest initial time period

yet established by such studies, labeled protein could be synthesized in the cytoplasm and transported to the nucleus.

A specific protein destined for transport to the nucleus might not necessarily be synthesized by contaminating cytoplasmic components on in vitro incubation of incompletely purified nuclear fraction. Any protein synthesis by contaminating cytoribosomes would, however, lead to erroneous conclusions regarding the level of amino acid incorporation by nuclear fraction.

Detergents have been used to remove the outer nuclear envelope and attached ribosomes (140). Strict criteria for the demonstration of purity of the nuclear fraction have been established, and most of the later workers have characterized their preparations by chemical, enzymatic and microscopic methods (151,152,153).

Highly contradictory results have been observed in work with the nuclear fraction of various species with respect to the requirements for amino acid incorporation. Allfrey showed a requirement for Na^+ ions for amino acid uptake by calf thymus nuclei, and an inhibition by elevated levels of K^+ (141). Other results do not indicate such strict requirements (154). The hypertonic sucrose solutions used in most procedures to isolate nuclei have been variously reported to have an inhibitory or no effect on amino acid

incorporation by the purified fraction (141,155).

Observations on the effect of various inhibitors of protein synthesis on the nuclei vary widely. This is yet another reason why the validity of observations of nuclear amino acid incorporation has been questioned.

Some of the results obtained by various workers are summarized below:

		Effect of inhibitors		
Source of nuclei		chloram-phenicol	cyclo-heximide	puromycin
Calf thymus	(141)	none	-	inhibition (75-80%)
HeLa cell	(140)	none	none	none
Rat liver	(147)	inhibition	-	-
	(146)	inhibition	none	none
	(157)	inhibition	-	-
	(158)	inhibition	inhibition	inhibition
Chick	(144)	inhibition	inconsistent	none

Observations of qualitative differences in response to inhibitors between the cytoplasmic and nuclear fractions of cells of particular species have been invoked to support the hypothesis that nuclei have a discrete protein-synthetic machinery. It is particularly important in such cases to demonstrate that a pure preparation has been achieved. Anderson has noted that purified nuclear preparations may be impermeable to inhibitors, and that very high concentrations

of such agents may be required to effect observable inhibition (159). The variation of methods of isolation of nuclei may lead to contradictory results. The developmental stage of the organism from which nuclei are isolated may also have an influence on the observed results. Trevithick (160) has observed that the response of embryonic trout testis nuclei to cycloheximide varies with the developmental stage.

The absence of microbial contamination must also be demonstrated. Recent work by Dravid and Wong (153) on amino acid incorporation by isolated rat brain nuclei gives detailed evidence of erroneous results that may be obtained due to microbial contamination. Their initial observations on the effect of chloramphenicol on amino acid incorporation gave widely conflicting results, and led them to suspect that the inhibitory effect of chloramphenical was exerted on non-nuclear protein synthesis. Culture of the incubation mixture did indeed demonstrate microbial contamination. Nuclei prepared and incubated under sterile conditions were insensitive to chloramphenicol.

It is generally agreed that nuclei have an endogenous energy metabolism (141,140,143,154). Most of the nuclear amino acid incorporating systems studied do not require an exogenous energy source, yet are inhibited by respiratory poisons (154,161). Dravid and Wong did not find stimulation of amino acid incorporation by isolated rat brain nuclei

on the addition of ATP (1 mM), but excess ATP with an energy-generating system was found to be inhibitory (153).

A direct relationship between the level of amino acid incorporation and the amount of nuclear fraction incubated must be shown to prevail if it is to be concluded that the nuclear fraction does indeed perform this function. This has been found difficult to achieve in many systems (140, 144,153).

There does seem to be good evidence, however, that nuclei are capable of incorporating amino acids *in vitro*. Much careful work has been done in which contributions from bacterial or cytoplasmic sources have been carefully eliminated.

Does this amino acid incorporation, however, represent protein synthesis? Much of the earlier work does not demonstrate this adequately. Amino acid incorporation was expressed merely in terms of counts in hot TCA-insoluble material, and no attempt was made to characterize this fraction.

The prevention by puromycin of amino acid incorporation is an accepted demonstration of protein synthesis, due to the fact that it has been shown to inhibit synthesis in all systems; prokaryotic, eukaryotic, and those of organelles. This has not been obtained in many investigations of nuclear amino acid incorporation (as noted above), but the failure may be due to impermeability of the nuclei (159).

More direct methods to demonstrate internal label in the polypeptide product have been attempted, but it may be argued in one case that there is evidence of bacterial contamination (154). Zimmermann found specific labeled proteins on gel electrophoresis of material extracted from HeLa cell nuclei after incubation, and excluded the possibility of bacterial origin, partly on the basis of lack of inhibition by chloramphenicol (140). In contrast, Dravid and Wong do not conclude that they have demonstrated protein synthesis by sterile preparations of nuclei because they could not observe an inhibitory effect of puromycin (153).

A report of the synthesis of a specific protein, asparagine synthetase, by isolated potato bud nuclei has been presented recently (163). The method of isolation of the nuclei does not, however, appear to be sufficiently rigorous to exclude the possibility of cytoplasmic contamination.

Lamkin and coworkers have recently shown that nucleoli from rat tumor nuclei have endogenous tRNAs for each amino acid (163). "Ribosomes", which closely resemble those in the cytoplasm have also been reported (157).

There is, in summary, a great deal of circumstantial evidence for the synthesis of protein by nuclei, although there have not been many investigations in which every criterion of purity and proof of protein synthesis has been met.

SECTION II - A STUDY OF PROLYL-tRNAs OF CHICK EMBRYO

Introduction

The hypothesis that a particular isoaccepting species of tRNA may be involved in the regulation of protein synthesis has been discussed in the "Literature Review".

The prolyl-tRNAs of 4-day and 8-day chick embryos were investigated in order to determine whether a new species of $tRNA^{Pro}$, or a change in the relative proportions of the proline-accepting tRNAs of chick embryos became apparent at the time of the onset of the synthesis of collagen (approximately 7 days).

Collagen contains equal amounts of proline and hydroxproline, which together account for 20% of the mass of collagen (164). The precursor of collagen hydroxyproline has been shown to be proline and hydroxyproline itself is generally considered not to be incorporated into the protein (164,165). Some evidence has been found for the hydroxylation of prolyl-tRNA (166,167), but the overwhelming evidence indicates that the hydroxyproline (and hydroxylysine) of collagen is formed by the action of a hydroxylase at the polypeptide level (163,168).

On the basis of these results, no attempt was made to

find a hydroxyproline-accepting tRNA. Because of the high proportion of proline and hydroxyproline in collagen, and their relative scarcity in other proteins, it was felt that a prolyl-tRNA would be the most probable regulator of collagen synthesis, if indeed this synthesis is regulated by tRNA.

The investigation was abandoned when no differences were found in the prolyl-tRNA complements of 4- and 8-day chick embryos.

Methods

Chicks

Eggs of a standard line of white Leghorn were incubated at 38° C for 4 and 8 days. Embryos were dissected free of the embryonic membranes, rinsed with cold 0.9% saline, and blotted on filter paper. The embryos were used immediately in further procedures, or were frozen in liquid nitrogen and stored at -15° C until use.

Isolation of tRNA from chick embryo

Chick embryo tRNA was isolated by a modification (169) of the method of Brunngraber (170). One hundred grams of fresh or frozen embryo were homogenized in an ice-cold mixture of 150 ml water-saturated phenol plus 150 ml of 1.0 M NaCl, 0.005 M EDTA in 0.1 M Tris-HCl buffer, pH 7.5. The homogenate was centrifuged for 10 min at 20,000 x g in the Servall RC-2B centrifuge, the upper (aqueous) layer removed, and an equal volume of water-saturated phenol added. The mixture was mechanically shaken at 4° C for 30 min, and centrifuged as above. The phenol extraction was repeated. Three volumes of cold 95% ethanol were added to the aqueous layer, and the resultant precipitate was collected by centrifugation and resuspended in 250 ml of 0.1 M Tris-HCl buffer, pH 7.5. The solution was then passed

through a column of 2 g of DEAE-cellulose equilibrated with cold 0.1 M Tris-HCl buffer, pH 7.5, at a flow rate of 1 ml/min. The column was washed with this buffer until the optical density of the effluent was less than 0.1 at 260 nm. The RNA was eluted with 1.0 M NaCl in 0.1 M Tris-HCl buffer, pH 7.5, until the optical density at 260 nm was negligible. The RNA solution was extracted twice with phenol in order to remove possible bacterial contamination, and twice with ether. Three volumes of cold 95% ethanol were added to the aqueous solution and it was allowed to stand overnight at -15° C. The precipitate was collected, washed twice with 95% ethanol, resuspended in 3 ml of 1.8 M Tris-HCl buffer, pH 8.0, and incubated at 37° C for 45 min to deacylate tRNA (171). Three volumes of 95% ethanol and a volume of 20% potassium acetate buffer, pH 5.2, sufficient to yield a final concentration of 2%, were added to precipitate tRNA. The precipitate was collected by centrifugation and dissolved in distilled water. The solution was then dialyzed against three changes of distilled water for 3 hr at 4° C.

Preparation of enzyme extracts

Enzyme extracts were prepared at 4° C and used immediately. One g of 4- or 8-day chick embryo was homogenized in a Potter-Elvehjem homogenizer in 2 ml of Medium AM (172), a modification of Medium A of Keller and Zamecnik (173).

Medium AM contained 0.35 M sucrose, 0.05 M Tris-HCl buffer, pH 7.5, 0.025 M KCl, 0.04 M $MgCl_2$, 2.0×10^{-4} M EDTA, and 6.0×10^{-3} M 2-mercaptoethanol. The homogenate was centrifuged at 37,000 x g for 30 min. The supernatant fraction obtained is termed the "crude enzyme extract." This extract was then dialyzed 3 hr against three changes of 100 ml of Medium AM with continuous stirring.

Aminoacylation of tRNA

Radioactive aminoacyl-tRNA was prepared by the method of Kano-Sueoka and Sueoka (174). The reaction mixture contained 2-10 $O.D._{260}$ units of tRNA, 0.5 ml of the dialyzed enzyme extract, 100 μmoles of Tris-HCl buffer, pH 7.5, 5 μmoles of magnesium acetate, 3 μmoles of ATP, 4 μmoles of reduced glutathione, 4 μmoles of isotopically labeled proline at a specific activity of 1.5-2 mCi/μmole and 1 μmole each of the remaining 19 amino acids. After incubation of the mixture at 37° C for 20 min. 1 ml. of Medium AM containing 20 mg of non-radioactive proline was added. The mixture was deproteinized twice with equal volumes of Medium AM-saturated phenol, and residual phenol extracted with ether. Aminoacyl-tRNA was precipitated at -15° C with 0.1 vol 2% potassium acetate buffer, pH 5.2, and 2.5 vol absolute ethanol. The precipitate was dissolved in 2 ml of 0.05 M sodium phosphate buffer, pH 6.3, containing 0.1 M NaCl in preparation for chromatography on columns

of methylated albumin Kieselguhr (MAK). A sample of 0.2 ml was taken to determine the extent of aminoacylation.

Precipitation of product and determination of radioactivity

One ml of cold 25% trichloracetic acid (TCA) containing carrier proline was added to precipitate tRNA from the sample. The precipitate was collected on a Whatman GF/A glass fiber disc, washed four times with 5 ml volumes of cold 5% TCA, once with 5 ml of absolute ethanol, and once with ether. This disc was dried under an infrared lamp and added to a scintillation vial containing 5 ml of scintillation fluid. The background radioactivity of the vial containing scintillation fluid was determined before addition of the disc. The fluid contained 5 g of 2,5-diphenyloxazole (PPO) and 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (Dimethyl POPOP) in 1 liter of toluene.

Column chromatography

Methylated albumin kieselguhr column chromatography

Methylated bovine serum albumin was prepared by the method of Mandell and Hershey (175). The modifications of Sueoka and Cheng (176) were followed in the preparation of the column. The column was loaded with the solution of aminoacyl-tRNA, described above and washed with 100 ml of 0.1 M NaCl in 0.05 M sodium phosphate buffer, pH 6.3. It was then eluted with a linear salt gradient made up of 110 ml each of two solutions of 0.3 M and 0.8 M NaCl in phosphate buffer, and generated by a commercial gradient

mixer (LKB Varigrad). Two ml fractions were collected, and the optical density at 260 nm and the refractive index were determined. To each fraction was added 0.05 ml of bovine serum albumin solution as carrier and 1 ml of 25% TCA. The precipitates were collected on glass fiber discs, washed as previously described, and the radioactivity determined.

Benzoylated DEAE-cellulose column chromatography

Benzoylated DEAE-cellulose (BD-cellulose) was prepared according to the method of Gillam, et. al. (177), and a commercial preparation was also used. A column of BD-cellulose was prepared (1 x 20 cm) and loaded with 10 $O.D._{260}$ units of aminoacyl-tRNA dissolved in 0.45 M NaCl plus 0.01 M $MgCl_2$ in 0.05 M sodium acetate buffer, pH 5.0. The column was washed with 30 ml of the buffer and eluted at 21° C at a flow rate of 1 ml/min with a linear salt gradient made up of 150 ml each of solutions of 0.45 M and 1.2 M NaCl in the sodium acetate-magnesium buffer. Fractions of 2 ml were collected, chilled to 4° C and treated according to the methods described in the section on MAK column chromatography.

Results

Acylation was performed by the procedure described in "Methods". Table 1 shows the results of a representative acylation reaction. The charging conditions developed by Tsay (82) were felt to be adequate, and the determination of optimal conditions for prolyl-tRNA formation was not considered to be of primary importance at this stage of the experiment. This assumption seems now to have been unwarranted. The conditions of acylation were not varied after the attainment of satisfactory levels of acylation became routine.

No differences were found in the extent to which a given tRNA sample could be acylated by 4- or 8-day chick embryo enzyme preparations. Transfer RNA was not removed from the enzyme preparations, so all samples of tRNA aminoacylated in preparation for column chromatography were incubated with a homologous enzyme extract; i.e. 4-day chick embryo tRNA with 4-day enzyme extract, in order to avoid possible ambiguity in the results due to endogenous tRNA in the enzyme preparations. High concentrations of nucleases are present in chick homogenates and may have affected the results, uniformly or not. The investigation was abandoned before a proposed study of the effects of nucleases was made.

TABLE 1

Incorporation of ^{3}H-proline (cpm) into 8-day chick embryo tRNA*

Sample	cpm
Blank	1,914
Reaction mixture	253,620

* Each reaction mixture contained 10.0 O.D.$_{260}$ units of tRNA, 0.5 ml dialyzed enzyme extracts, and other components as described in "Methods".

Chromatography of prolyl-tRNAs on MAK and BD-cellulose columns

Samples of 4-day chick embryo ^{14}C-prolyl-tRNA and 8-day chick embryo ^{3}H-prolyl-tRNA were chromatographed separately on MAK columns. The labels were reversed, and these experiments repeated. Mixtures of tRNA from the two stages were co-chromatographed in later experiments in the same manner.

Initial experiments indicated quantitative differences in the amount of a species of prolyl-tRNA of 4-and 8-day embryos, appearing as a minor peak preceding the main peak eluted from the MAK column. Figure 1 shows the results of an experiment in which tRNAs from both stages were co-chromatographed. Samples in the first peak which showed low radioactivity were counted for 20 min to reduce error.

These results could not be repeated, however, and later experiments showed no stage-specific differences in the relative amounts of the different species of prolyl-tRNA. Figure 2 shows the results that were uniformly obtained in later experiments. The reasons for the initial observation of differences between 4-day and 8-day chick embryo prolyl-tRNAs are not known. They may have been due to differential effects of nucleases within early preparations

Figure 1 Elution profiles of 4-day and 8-day chick embryo prolyl-tRNAs on a MAK column

_____, Optical density at 260 nm

- . -, ^{3}H-prolyl-tRNA of 8-day chick embryo

-----, ^{14}C-prolyl-tRNA of 4-day chick embryo

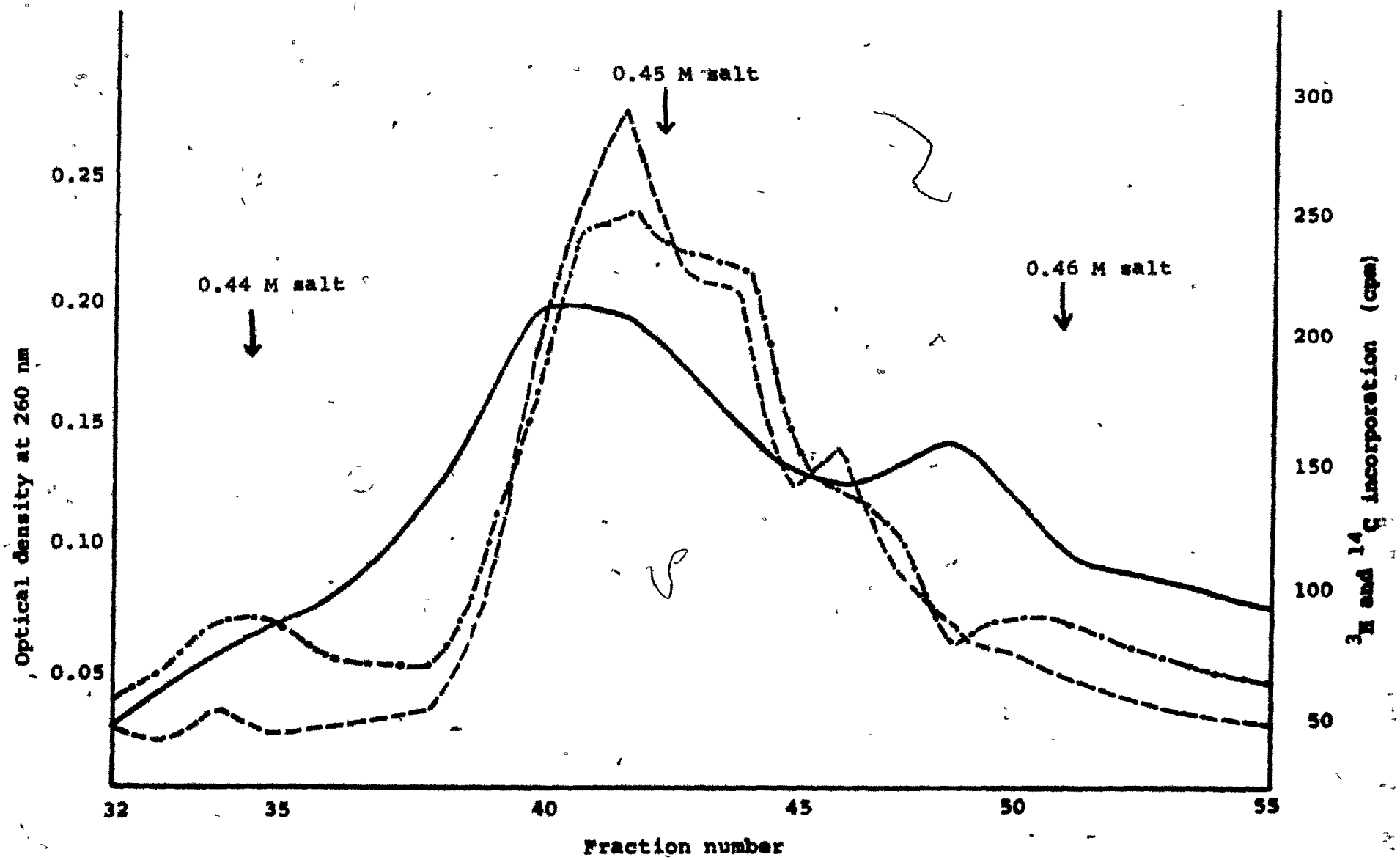
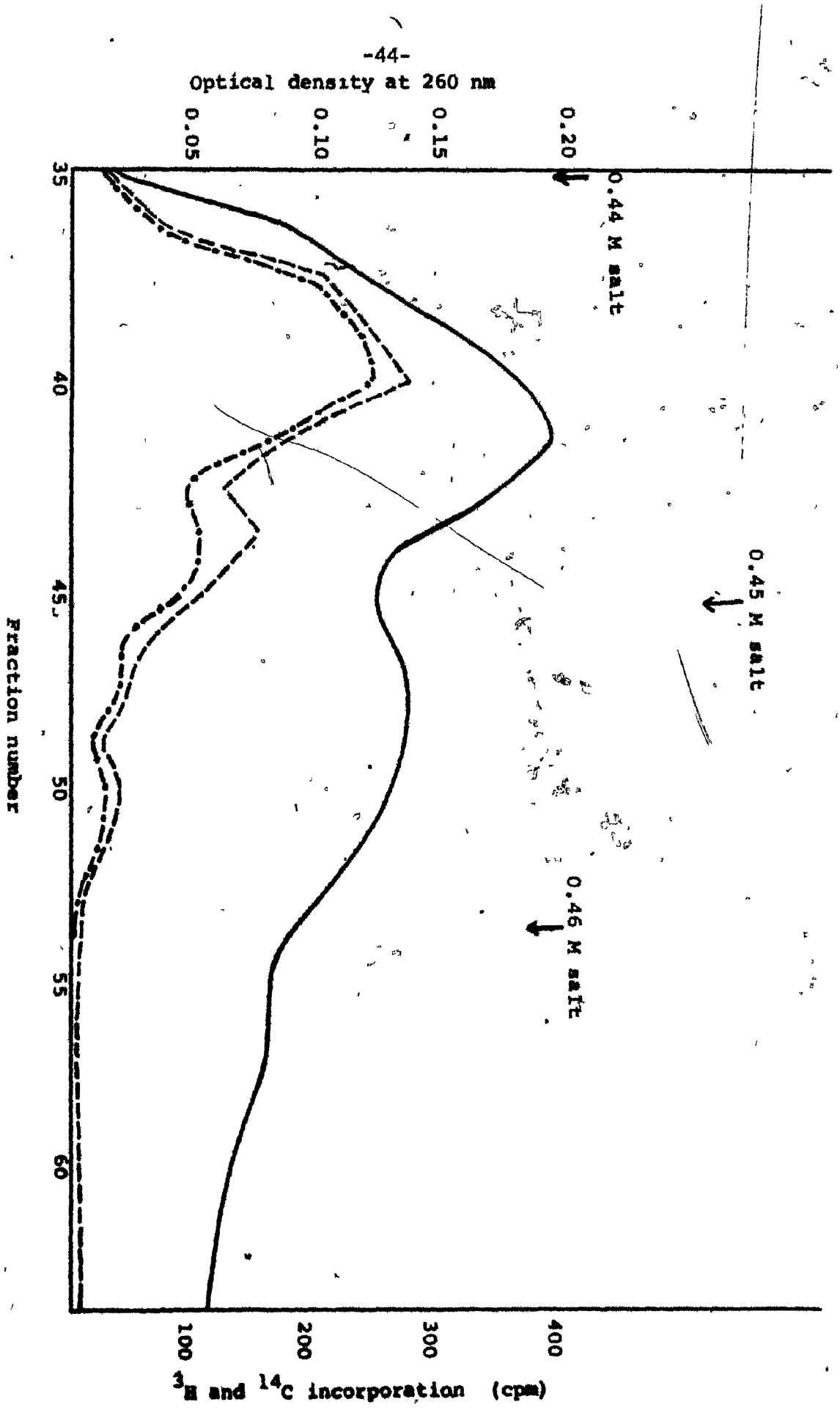

0.45 M salt
0.44 M salt
0.46 M salt
Optical density at 260 nm
0.25
0.20
0.15
0.10
0.05
300
250
200
150
100
50
^{3}H and ^{14}C incorporation (cpm)
32
35
40
45
50
55
Fraction number

Figure 2 Elution profiles of 4-day and 8-day chick embryo prolyl-tRNAs on a MAK column

———, Optical density at 260 nm

-.-.-., ^{3}H-prolyl-tRNA of 8-day chick embryo

------, ^{14}C-prolyl-tRNA of 4-day chick embryo


Optical density at 260 nm
0.05
0.10
0.15
0.20
0.44 M salt
0.45 M salt
0.46 M salt
Fraction number
35
40
45
50
55
60
100
200
300
400
^{3}H and ^{14}C incorporation (cpm)

of 4-day and 8-day chick embryo tRNA.

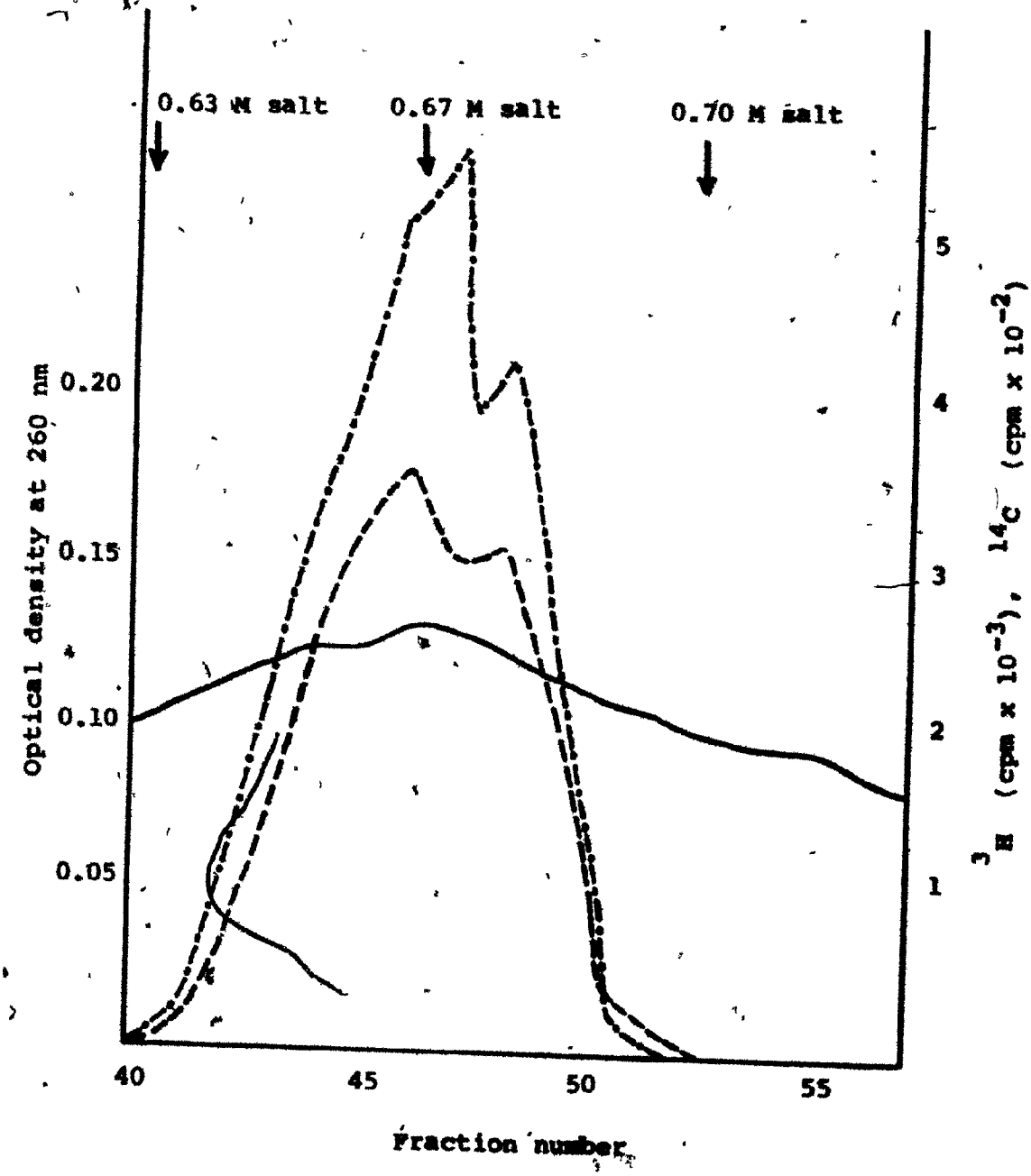
Chromatography on BD-cellulose columns also failed to show any differences in the prolyl-tRNA complements of the two developmental stages (Figure 3).

Figure 3 Elution profiles of 4-day and 8-day chick embryo prolyl-tRNAs on a BD-cellulose column

_____, Optical density at 260 nm

-.-.-., ^{3}H-prolyl-tRNA of 8-day chick embryo

------, ^{14}C-prolyl-tRNA of 4-day chick embryo


0.63 M salt
0.67 M salt
0.70 M salt
Optical density at 260 nm
0.20
0.15
0.10
0.05
^{3}H (cpm x 10^{-3}), ^{14}C (cpm x 10^{-2})
5
4
3
2
1
40
45
50
55
Fraction number

Discussion

The studies reported here do not show any support for the hypothesis that a novel proline-accepting tRNA, or a change in the relative proportions of previously existing proline-specific tRNAs, is involved in the onset of synthesis of collagen in the chick embryo.

Two major improvements could have been made in the experiment, the first being a kinetic study of the course of the acylation process, to determine that maximum acylation was being achieved. Later work in this laboratory by Wainwright, et. al. (81) showed that levels of acylation of an order of magnitude greater than those achieved in this work were vital to the demonstration of a small difference in alanyl-tRNA complements of chick embryos and extra-embryonic membranes.

Alternative methods for the fractionation of tRNAs, such as the reverse phase chromatographic method described by Kelmers, Novelli, and Stulberg (178), had been developed at this time, and a more exhaustive search for differences in the tRNA complements of 4- and 8-day chick embryos could have been made by the use of such methods.

These procedures did not seem profitable at the time, as there was no compelling reason to suppose that the

synthesis or the regulation of synthesis of collagen depended upon the presence or the increased amount of a particular prolyl-tRNA. In all cases in which differences in a particular tRNA of the complement of a species have been found to correlate with differentiation, viral infection, or the like, many tRNA species have not been found to show any change. Tsay (82), and Lee and Ingram (83) compared the chromatographic profiles of several tRNA isoaccepting species in chick tissues at different stages of development, and found most types to be identical, qualitatively and quantitatively, at each stage.

Studies made since that time that have shown similar results include those of Yang and Comb (80), who found differences in only two of nine tRNA species during development of sea urchin embryos. Shearn and Horowitz (179) concluded that observed quantitative changes in isoaccepting tRNAs of Neurospora crassa on derepression of tyrosinase were due to differential ribonuclease activity.

Later work in this laboratory has provided support for the results reported here. BD-cellulose chromatography of chick prolyl-tRNAs of embryonic and adult stages has failed to show differences in tRNA complement between the two stages (169).

The search for a change in prolyl-tRNAs during the course of chick development was accordingly abandoned, and a new investigation begun.

SECTION III - A STUDY OF CHICK EMBRYO LEUCYL-tRNAs

Introduction

This series of experiments arose as a result of an observation by Tsay, then of this laboratory, that the chromatographic profiles of T1 ribonuclease-treated 4,5,-^{3}H-leucyl-tRNA and uniformly-labeled ^{14}C-leucyl-tRNA of the same stage of chick embryo differed greatly in one peak (82). He attributed this difference to contamination of the ^{14}C-leucine used in his experiments, but it seemed worthwhile to investigate this phenomenon more fully at the time. The results seemed to indicate that the 4,5-^{3}H-leucine bound to one of the tRNA fragments was modified in such a way that the radioactive atoms were lost. Uniformly labeled ^{14}C-leucine retained radioactivity and thus predominated in the peak of interest.

The compound β-hydroxy-β-methyl-glutaryl-SCoA is an intermediate both in the synthesis of the cytokinin N^{5}-(Δ^{2}-isopentenyl)-adenosine and the degradation of leucine. The possibility thus existed that label introduced *via* leucine appeared in cytokinin.

Another possible derivative, a pyrroline carboxylic acid formed by loss of tritium and cyclization of leucine was also considered. At this time the initiating species

of eukaryotes was unknown, and the work of Moav and Harris (95) mentioned in the "Literature Review", lent some support to the hypothesis that the observed derivative might be an N-blocked initiating species.

The preliminary observations of Tsay (82) were repeated and comparative studies were done with other species. Attempts were made to isolate and characterize the leucine derivative and to assess its biological importance.

This investigation was also eventually abandoned.

Methods

Methods described previously for incubation of chicks, isolation of transfer RNA, aminoacylation of tRNA, precipitation of radioactive aminoacyl-tRNA, and determination of radioactivity were used in the present experiment.

Biological material

Fertilized eggs were incubated at 38° C for 5 days. Adult chicken liver was obtained commercially. Fresh rabbit liver was a gift of Dr. Longley of the Physiology and Biophysics Department of this University.

Isolation of tRNA from chick embryo

In most experiments, the method described previously was followed. The alternate procedure was a modification (180) of the method of Silbert, et. al. (181). Twenty g of chick embryo was homogenized with 3 volumes of Medium AM. The homogenate was centrifuged at 37,000 x g for 30 min. and an equal volume of 90% (v/v) phenol was added to the supernatant fraction. The mixture was shaken mechanically for 1 hr at 4° C and centrifuged at 20,000 x g for 15 min. The phenol extraction was repeated once. The aqueous phase was applied directly to a DEAE-cellulose column (bed volume 20 ml) previously equilibrated with buffer B, pH 7.5; consisting of 3×10^{-4} M EDTA, 4×10^{-3} M $MgCl_2$.

and 0.1 M Tris-HCl. The column was washed with a solution of 0.1 M LiCl in buffer B until the optical density at 260 nm was negligible. Transfer RNA was eluted from the column with 1.0 M LiCl in buffer B. Fractions having an optical density of greater than 0.1 at 260 nm were pooled. The solution was adjusted to 2% in potassium acetate and tRNA was precipitated with 2 volumes of ethanol at -15° C. The precipitate was collected by centrifugation, dissolved in 3 ml of 1.8 M Tris-HCl buffer, pH 8.0, and incubated at 37° C for 45 min to deacylate tRNA. Potassium acetate and ethanol were added to precipitate tRNA, which was collected by centrifugation, dissolved in buffer B, and dialyzed for 4 hr against 3 changes of 200 ml of buffer B.

Preparation of enzyme extracts

Dialyzed extracts of chick embryo, chicken liver, and rabbit liver enzyme preparations were prepared. The extracts were partially purified to remove endogenous tRNA. Dialyzed extract obtained from 1 g of tissue was diluted to 10 ml in Medium AC; consisting of 0.117 M sucrose, 0.017 M Tris-HCl buffer, pH 7.5, 0.0083 M KCl, 0.013 M $MgCl_2$, 2×10^{-4} M EDTA, and 6×10^{-3} M 2-mercaptoethanol. The sample was applied to a pre-equilibrated DEAE-cellulose column (1 x 5 cm) at 4° C. The column was washed with Medium AC and

eluted with 0.1 M NaCl in Medium AC. Fractions of 1.5 ml were collected and the optical density at 280 nm determined. The contents of two or three tubes around the peak optical density were pooled as "partially purified enzyme extract" (172).

A partially purified preparation of E. coli B amino-acyl-tRNA synthetases was prepared by the method of Kano-Sueoka and Sueoka (174).

Three g of E. coli B were ground at 4° C with 9 g of alumina for 10 min and suspended in 9 ml of 0.01 M Tris-HCl buffer, pH 7.3, containing 1 mM $MgCl_2$. The suspension was centrifuged at 10,000 x g for 15 min and the supernatant fraction was then centrifuged at 105,000 x g for 2.5 hr. The upper two-thirds of the supernatant was dialyzed for 3 hr against 2 changes of 1 liter of 0.01 M Tris-HCl buffer, pH 7.3, containing 1 mM $MgCl_2$ plus 6 mM 2-mercaptoethanol. The dialyzed fraction was adsorbed onto a DEAE-cellulose column previously equilibrated with 0.02 M potassium phosphate buffer, pH 7.7. The column was washed with 0.02 M phosphate buffer, pH 7.3, until the optical density at 280 nm was negligible. The enzyme fraction was then eluted with 0.35 M NaCl in potassium phosphate buffer, pH 7.3, containing 6 mM 2-mercaptoethanol. Collection of fractions and selection of those to be pooled were carried out as above.

Aminoacylation of tRNA

Transfer RNA was acylated as described previously in a reaction mixture containing 10 nmole 4,5-^{3}H leucine or uniformly labeled ^{14}C-leucine of specific activity of 5-15 mCi/mM.

T_1 ribonuclease digestion of aminoacyl-tRNA

T_1 RNase digestion of aminoacyl-tRNA was carried out by the method of Subak-Sharpe, et. al. (182).

Ten $O.D._{260}$ units of aminoacyl-tRNA alcohol precipitate were collected by centrifugation and dissolved in 0.5 ml of 0.1 M sodium acetate buffer, pH 5.5, containing 3 mM EDTA and 600 or more units of T_1 RNase. The mixture of tRNA and RNase was incubated for 45 min or 4 hr at 37° C, and diluted with 10 ml of cold 0.01 M ammonium formate buffer, pH 5.5. In experiments in which doubly labeled samples were to be chromatographed, suitable amounts of the two isotopically labeled aminoacyl-tRNAs were mixed to provide approximately equivalent activities of ^{14}C and ^{3}H.

DEAE-cellulose column chromatography

Fractionation of aminoacyl-tRNA oligonucleotides was performed by the method of Ishida and Miura (183). The diluted digest was applied to a DEAE-cellulose columns (1 x 5 cm) previously equilibrated with 0.01 M ammonium formate buffer, pH 5.5. The column was washed with 30-45 ml of 0.01 M ammonium formate buffer, pH 5.5, and eluted with a

linear gradient made up of 120 ml each of 0.01 M and 0.1 M formate buffer, pH 5.5. Fractions of 2 ml were collected, the optical density at 260 nm and the refractive index determined, and transferred to scintillation vials. The formate was evaporated in an oven at 80° C, and 5 ml of Bray's (184) scintillation fluid were added to the vials, and the radioactivity determined.

Deacylation of aminoacyl-tRNA and aminoacyl-tRNA fragments

An alternative to incubation in concentrated Tris buffer to deacylate aminoacyl tRNAs, or fragments thereof, was suggested by Dr. J. A. Verpoorte (185). Aminoacyl-tRNA was dissolved in 10^{-4} M NaOH, incubated at 37° C for 30 min, and re-precipitated.

Preparation of supernatant fraction of deacylated tRNA for chromatography

The supernatant fraction obtained after deacylation of tRNA or tRNA fragments was evaporated in a rotary evaporator to remove alcohol. The residue, containing amino acids, NaOH and potassium acetate, was treated with perchloric acid to precipitate K^+ as the perchlorate. After centrifugation, the supernatant fraction was adjusted to the pH suitable for the relevant chromatographic method.

Chromatography

Fractionation of the amino acid mixture by copper Sephadex column chromatography was carried out by the method

of Fazakerley and Best (186) and the modifications of this method by Buist and O'Brien (187).

Sixteen g of Sephadex G-25, medium grade, were stirred slowly into 30 ml of 0.16 M $CuSO_4$ and made alkaline by the addition of 60 ml of 0.5 N NaOH with stirring. Fifty ml of 0.05 M sodium tetraborate buffer, pH 11, was added, the solid allowed to settle, and the supernatant poured off. This procedure was repeated twice.

A column (1.7 x 38 cm) was prepared and washed with 50 ml of the tetraborate buffer. A sample of 1-2 ml containing less than 20 mg of amino acids was washed on to the column with three 1 ml portions of buffer. Elution was continued at a flow rate of 0.5 ml/min for 1 hr. The eluting solution was then changed to 0.2 N HCl and elution continued at the same flow rate for 6 hr.

Fractions of 2 ml were collected and aliquots of 0.2 ml were added to Bray's scintillation fluid for determination of radioactivity.

In a modification of this method, Chelex 100 in the sodium form, mesh size 50-100, was stirred with 2 volumes of saturated $CuSO_4$ at 4° C for 24 hr. The supernatant was decanted and the solid washed with deionized water until no free copper was shown on addition of sodium diethyldithiocarbamate. The resin was suspended in sodium tetraborate buffer, pH 11, and the suspension brought to pH 11

with 1 N NaOH. A column (1.3 x 12 cm) was prepared at 4° C and washed with 25 ml of tetraborate buffer. The sample of amino acids to be chromatographed was brought to pH 11 with 0.1 N NaOH and applied to the column, which was eluted with 50 ml of the tetraborate buffer. Fractions of 1 ml were collected at 0° C in tubes containing 1 N HCl to neutralize the effluent. Aliquots were taken and counted as above.

Paper chromatography

Two-dimensional paper chromatography was performed using a solvent system designed to separate leucine and its isomers (188). The prepared supernatant fraction containing radioactive amino acid was spotted 1 cm from the corner of a 14 x 14 in square of Whatman #1 filter paper. The chromatogram was developed in the first direction by a solvent consisting of 1-butanol-benzyl alcohol (1:1) (v/v) saturated with pH 8.4 buffer, made up of 50 ml 0.067 M boric acid and 8.55 ml 0.067 M NaOH. After drying at room temperature the chromatogram was developed at right angles with 1 M ammonium acetate, pH 7.5, -95% ethanol (3:7) (v/v). The developed chromatogram was dried and cut into 1 cm squares (total number, 400), which were added to vials containing scintillation fluid for determination of radioactivity.

Thin layer chromatography

The method of Haworth and Heathcote (189) was used to

separate amino acids by thin layer chromatography on cellulose. Fifty g of MN-300 (Macherey Nagel) cellulose powder was slurried with 200 ml of 80% methanol and poured into a Buchner funnel. The solid was washed with the following solutions:

300 ml 2-propanol-acetic acid-water (60:20:20) (v/v)
200 ml methanol-water (25:75) (v/v)
200 ml methanol-1 N HCl (60:40) (v/v)
200 ml water
200 ml methanol

and dried overnight *in vacuo*. To 15 g of the washed cellulose was added 70 ml of water plus 10 ml of absolute ethanol, with stirring. The suspension was homogenized for 60 sec with a propeller-type stirrer. Glass plates were coated by means of the Shandon apparatus to give a thickness of 150 microns of cellulose. The amino acid sample was spotted in a corner 1 cm from the edges of the plate and the chromatogram was developed in the first direction by 2-propanol-butanone-1 N HCl (60:15:25) (v/v). Solvent from the previous day's run was allowed to stand in the tank until replaced by fresh solvent, to achieve tank saturation. When the solvent front had reached 13 cm from the origin, the plate was removed, dried for 15 min in a stream of cool air and then for 15 min in a 60° C oven to remove HCl. The cellulose layer was broken with a spatula to isolate the yellow band at the solvent front. The chromatogram was then developed at right angles in 2-methylpropanol-2-butanone-propanone-methanol-water-(0.88 M) ammonia (40:20:20:1:14:5) (v/v) until the

solvent front was again 13 cm from the origin. The plate was dried in a stream of cool air. Squares of 1 cm were scrapped off the plate and added to Bray's solution in scintillation vials.

A ninhydrin-cadmium acetate chromogenic reagent described by the same authors was used to indicate areas of the plate which contained amino acids. A solution of 0.5 g of cadmium acetate in 50 ml of water plus 10 ml of glacial acetic acid was prepared. Ninhydrin was added to 0.2% (w/v) just before use. This solution was sprayed onto the plate, and the plate was dried with warm air. Pink spots indicated the presence of amino acids. The reagent is said to be sensitive enough to detect 0.5 nanomoles of amino acid (189).

Amino acid analysis

Automatic amino acid analysis according to the method of Spackman, Stein, and Moore (190) was performed by Mrs. Rita G. Breckon. In some experiments, radioactive samples of amino acids were chromatographed, and the effluent was collected in 2 ml fractions rather than being mixed automatically with ninhydrin.

Ethyl acetate extraction

Aminoacyl-tRNA was prepared, an aliquot of 0.01 ml was precipitated on a Whatman 3 MM filter paper disc by the addition of 1 ml of ice-cold 10% TCA, and the disc was

washed with TCA, ethanol-diethyl ether (1:1) (v/v), and ether. The radioactivity was determined. An aliquot of 0.1 ml was put on another filter paper disc, and the disc was put into a conical centrifuge tube containing 1 ml of 0.5 M NH_4OH. Hydrolysis of aminoacyl-tRNA was carried out at 37° C for 30 min, the tube was chilled, and 0.2 ml of formic acid and 1.5 ml of ethyl acetate were added. The contents were mixed with a vortex mixer, centrifuged, and 1.0 ml of the ethyl acetate phase added directly to 10 ml of Bray's scintillation fluid for determination of radioactivity (191).

Results

DEAE-cellulose column chromatography

Samples of 4- to 5-day chick embryo tRNA were aminoacylated with ^{3}H- and ^{14}C-leucine in separate reaction mixtures and digested with T1 ribonuclease at 37° C for 4 hr. Figure 4 shows superimposed graphs of the optical density and radioactivity of 4-day ^{14}C- and ^{3}H-leucyl-tRNA fragments eluted from DEAE-cellulose columns in two separate experiments. The ratio of labeled carbon to tritium in the first peak is seen to be much greater than that of the other peaks. This result was checked by digesting a doubly-labeled sample of 10 $O.D._{260}$ units of aminoacyl-tRNA and subjecting the digest to chromatography on DEAE-cellulose. A large quantitative difference in the first peak was observed in this experiment, but qualitative results were similar. The results are shown in Figure 5. The differences in profiles observed between the two experiments shown in Figures 4 and 5 are considerable, but were not further investigated.

Variation of developmental stage of chick

Transfer RNA preparations were isolated from chicks at other stages of development and studied in the same manner. No stage-specific qualitative differences in the first peak were observed.

Variation of mode of preparation of synthetase

The extent of aminoacylation of a given tRNA preparation varied with the degree of purity of the enzyme preparation, but no differences were noted in the elution profiles of the fragments of digested tRNA. Purified enzyme preparations as described in "Methods" were approximately 20% more active than dialyzed preparations in the acylation reaction.

Variation of the length of T1 RNase digestion period

T1 ribonuclease digestion was carried out at 37° C for either 45 min or 4 hr. The results show no consistent relationship between the length of the digestion period and the carbon to tritium ratio in the first peak to be eluted from the column, or in the extent of recovery of radioactivity after chromatography of the chick embryo tRNA fragments.

Table 2 shows the effect of variation of the T1 RNase digestion period on the total recovery of radioactive label and the percentage of each label appearing in peak 1 on chromatography of 5-day chick embryo tRNA fragments.

Table 3 shows the results of chromatography of tRNA fragments of different developmental stages and some effects of variation of the mode of preparation of enzyme. Further data on the effect of varying the digestion period are also shown. The data presented on adult liver was of particular interest, indicating no effect of digestion period.

Figure 4 Elution profiles of 4-day ^{3}H- and ^{14}C-leucyl-tRNA T1 RNase digestion products on a DEAE-cellulose column

....., Optical density at 260 nm

_____, ^{14}C-leucyl-tRNA fragments of 4-day chick embryo

-----, ^{3}H-leucyl-tRNA fragments of 4-day chick embryo

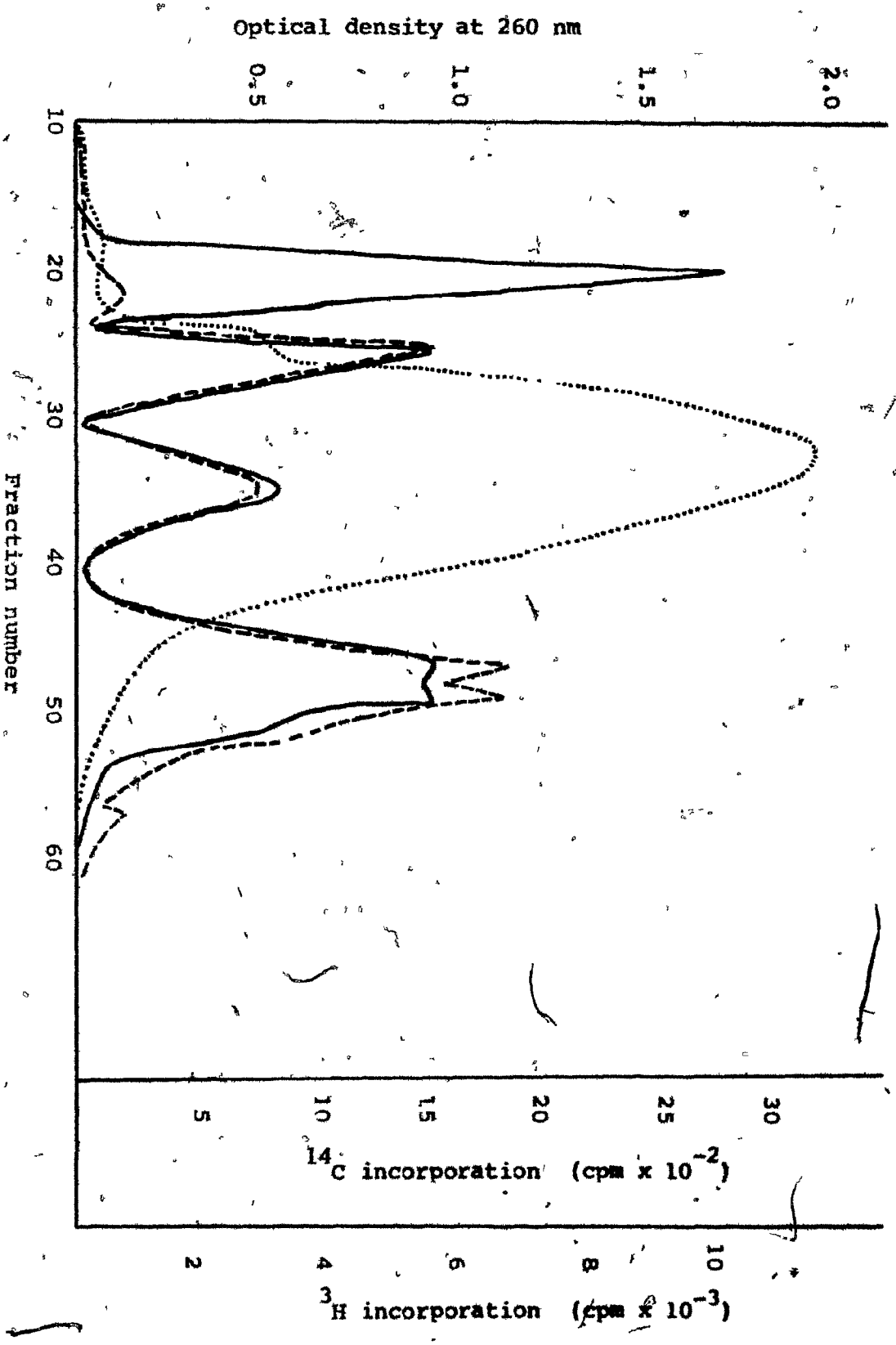

Optical density at 260 nm
0.5
1.0
1.5
2.0
10
20
30
40
50
60
Fraction number
5
10
15
20
25
30
^{14}C incorporation (cpm x 10^{-2})
2
4
6
8
10
^{3}H incorporation (cpm x 10^{-3})

TABLE 2

The effect of variation of the length of the T1 RNase digestion period on recovery of radioactivity and distribution of label on chromatography chick embryo aminoacyl-tRNA fragments*

	Digestion period 45 min	4 hr
Total radioactivity applied to column (cpm)		
^{3}H	207,592	184,672
^{14}C	160,694	149,727
Recovery of radioactivity as a percentage of the total radioactivity applied to the column		
^{3}H	38.6	25.0
^{14}C	32.0	27.0
Radioactivity of first peak as a percentage of that recovered		
^{3}H	11.2	3.6
^{14}C	6.0	10.6

* 10 $O.D._{260}$ units of tRNA were used in each experiment. Aminoacylation, T1 RNase digestion, and chromatography on DEAE-cellulose were performed as described in "Methods".

Figure 5. Elution profiles of doubly-labeled sample of 4-day chick embryo tRNA T1 RNase digestion products on a DEAE-cellulose column

....., Optical density at 260 nm

_____, ^{14}C-leucyl-tRNA fragments of 4-day chick embryo

------, ^{3}H-leucyl-tRNA fragments of 4-day chick embryo

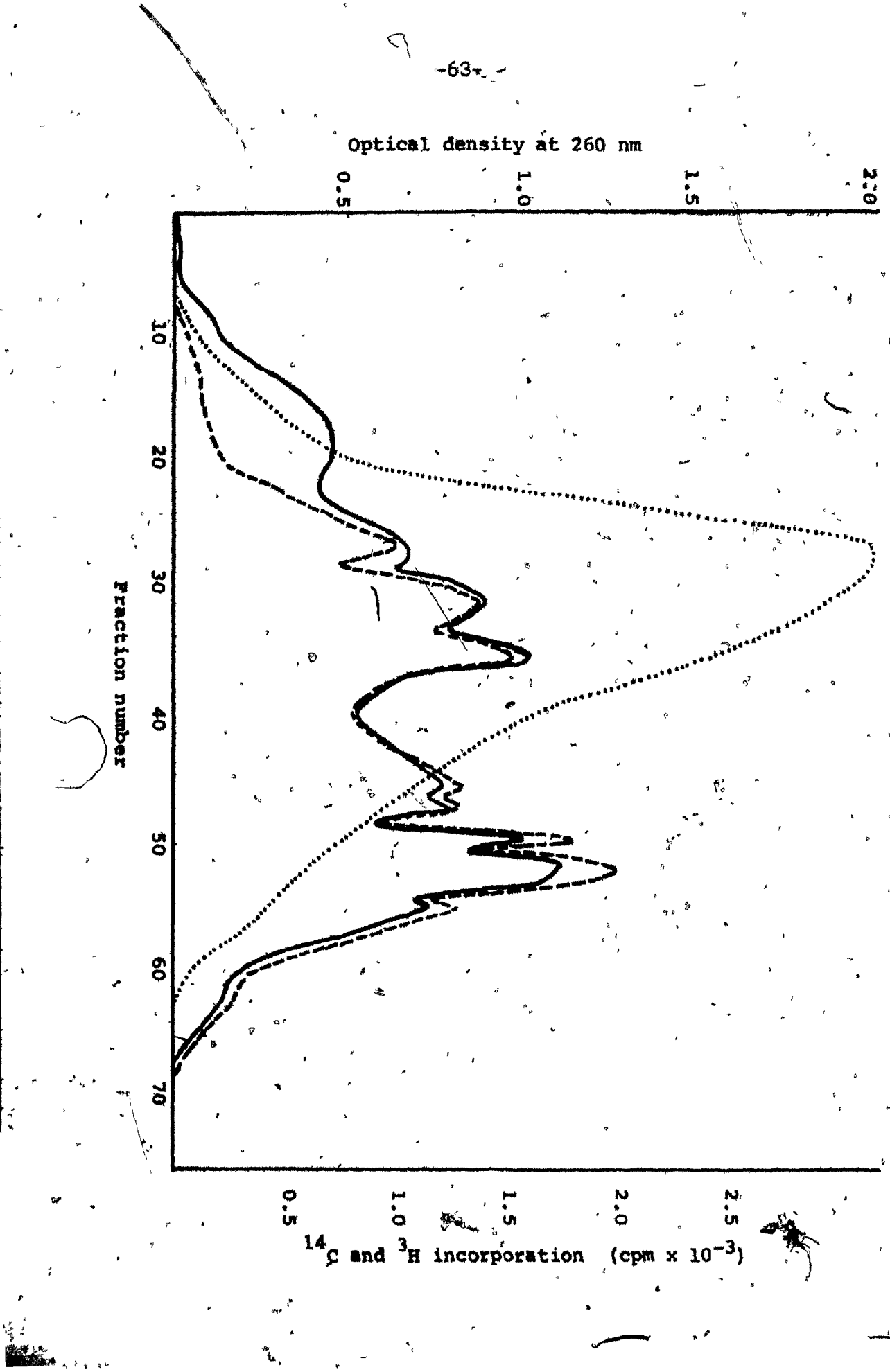

Optical density at 260 nm
0.5
1.0
1.5
2.0
10
20
30
40
50
60
70
Fraction number
0.5
1.0
1.5
2.0
2.5
^{14}C and ^{3}H incorporation (cpm x 10^{-3})

TABLE 3

The effect of variation of mode of preparation of enzyme and length of Tl R period on recovery of radioactivity and distribution of label on chroma chick aminoacyl-tRNA fragments from several developmental stage

Source of tRNA*	Aminoacylation enzyme preparation	Length of digestion period	Total radi of t ^{3}H
4-day chick embryo	dialyzed	45 min	161,132
	dialyzed	4 hr	160,120
8-day chick embryo	crude extract	45 min	36,877
adult chick liver	purified	45 min	121,877
	dialyzed	4 hr	90,795

* 10 $O.D._{260}$ units of tRNA were used in each experiment. Aminoacylation, T digestion, and chromatography on DEAE-cellulose were performed as described

gth of T1 RNase digestion
l on chromatography of
ental stages

Total radioactivity (cpm) of tRNA digest		Recovery of radioactivity as a percentage of the total applied to column		Radioactivity of first peak as a percentage of that recovered	
^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
161,132	179,323	14.2	15.8	2.7	13.9
160,120	161,133	24.0	24.0	0.69	7.5
36,877	34,292	50.0	54.0	10.6	38.5
121,877	121,400	9.9	11.5	10.0	29.0
90,795	108,495	7.6	7.6	10.0	30.0

cylation, T1 RNase
described in "Methods".

T1 RNase digestion of tRNAs of other species

The same type of experiments were carried out with tRNAs isolated from other species.

Ten $O.D._{260}$ units of a commercial preparation of rabbit liver tRNA was incubated with ^{14}C- and ^{3}H-leucine, and other components of the reaction mixture as described in "Methods". A dialyzed enzyme preparation was made from fresh rabbit liver. The acylated tRNA product was digested with T1 RNase at 37° C for 45 min and chromatographed on DEAE-cellulose. No differences were seen in the relative proportions of each label in the fractions obtained. These results are shown in Figure 6.

E. coli tRNA was aminoacylated with homologous purified enzyme and digested in the same manner. Again, no differences were found in the radioactive carbon and tritium chromatographic profiles, as is seen in Figure 7.

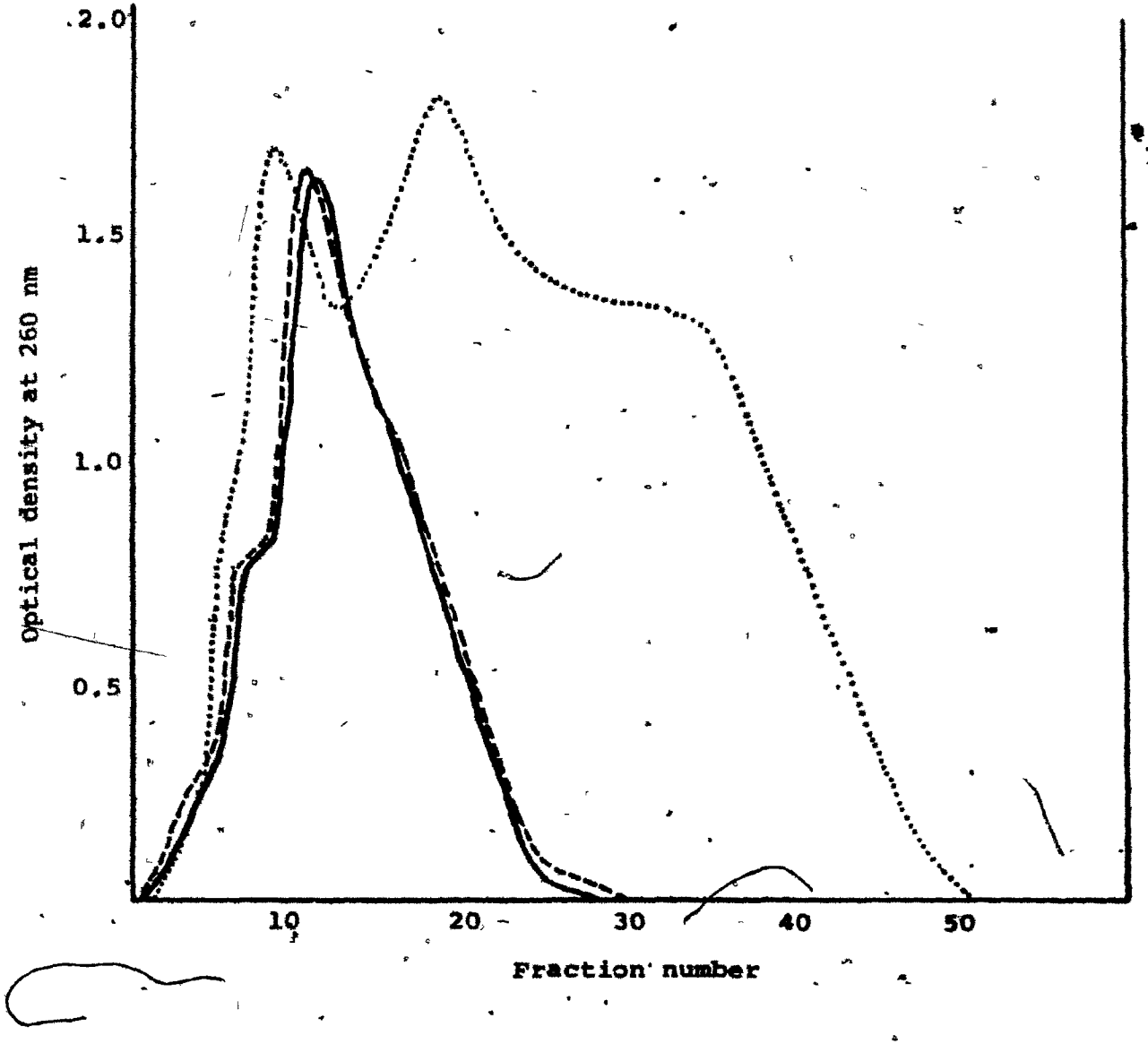
Chick liver purified enzyme preparation was used to acylate a sample of E. coli tRNA. The extent of acylation was 10.5% of that achieved with the homologous enzyme, and the chromatographic profile of the digestion products show some suggestive evidence for quantitative differences due to enzyme source in the extent of labeling of the various species of E. coli $tRNA^{Leu}$. No marked differences in the pattern of carbon- and tritium-labeled fragments were observed (Figure 8).

Figure 6. Elution profiles of rabbit liver ^{3}H- and ^{14}C-leucyl-tRNA T1 RNase digestion products on a DEAE-cellulose column

....., Optical density at 260 nm

———, ^{14}C-leucyl-tRNA fragments of rabbit liver

------, ^{3}H-leucyl-tRNA fragments of rabbit liver


2.0
1.5
1.0
0.5
Optical density at 260 nm
10
8
6
4
2
0
^{14}C and ^{3}H incorporation (cpm x 10^{-2})
10
20
30
40
50
Fraction number

[redacted]

Figure 7 Elution profiles of T1 RNase digestion products of E. coli ^{3}H- and ^{14}C-leucyl-tRNA, aminoacylated with E. coli enzyme, and chromatographed on DEAE-cellulose

....., Optical density at 260 nm

_____, ^{14}C-leucyl-tRNA fragments

------, ^{3}H-leucyl-tRNA fragments

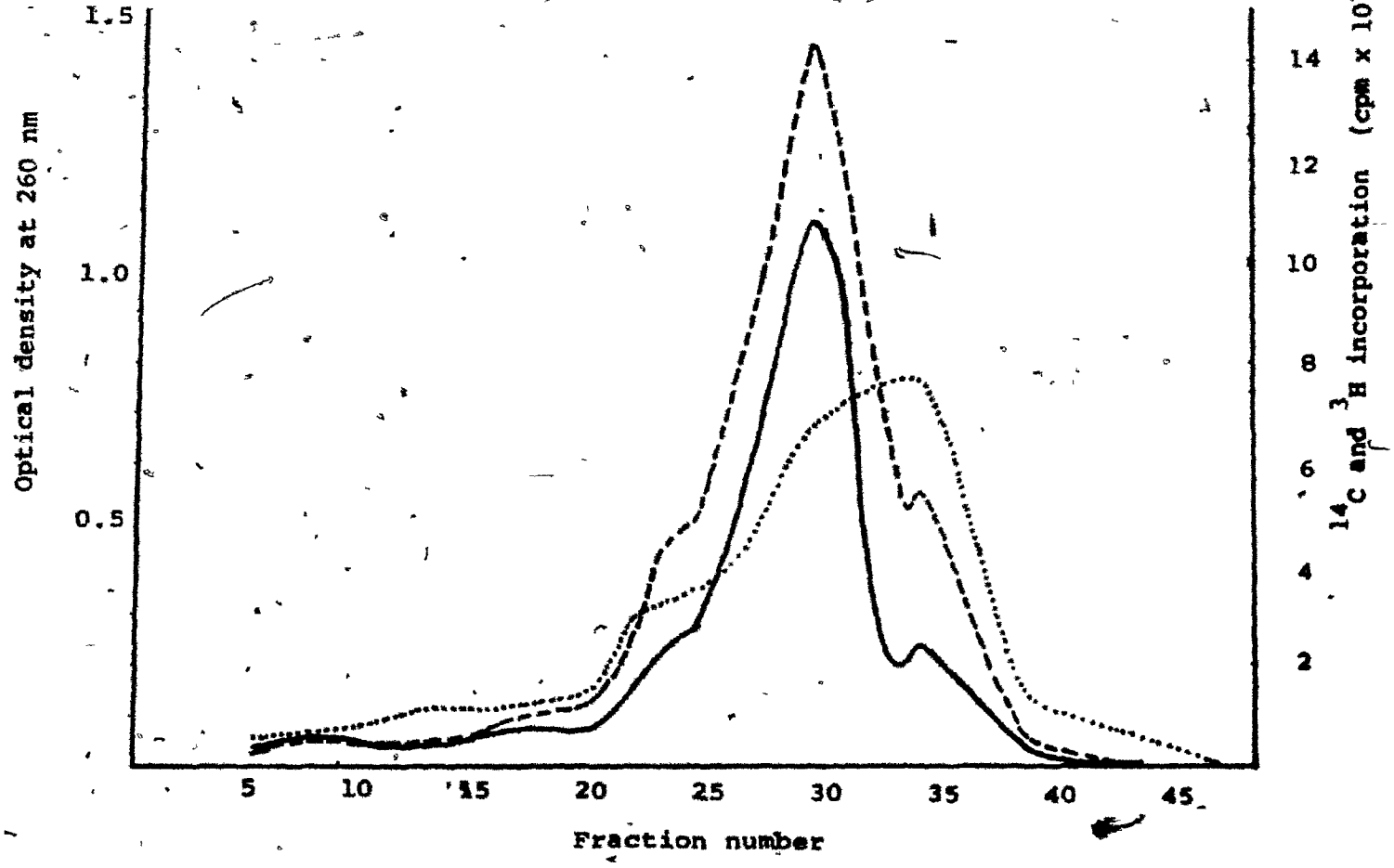
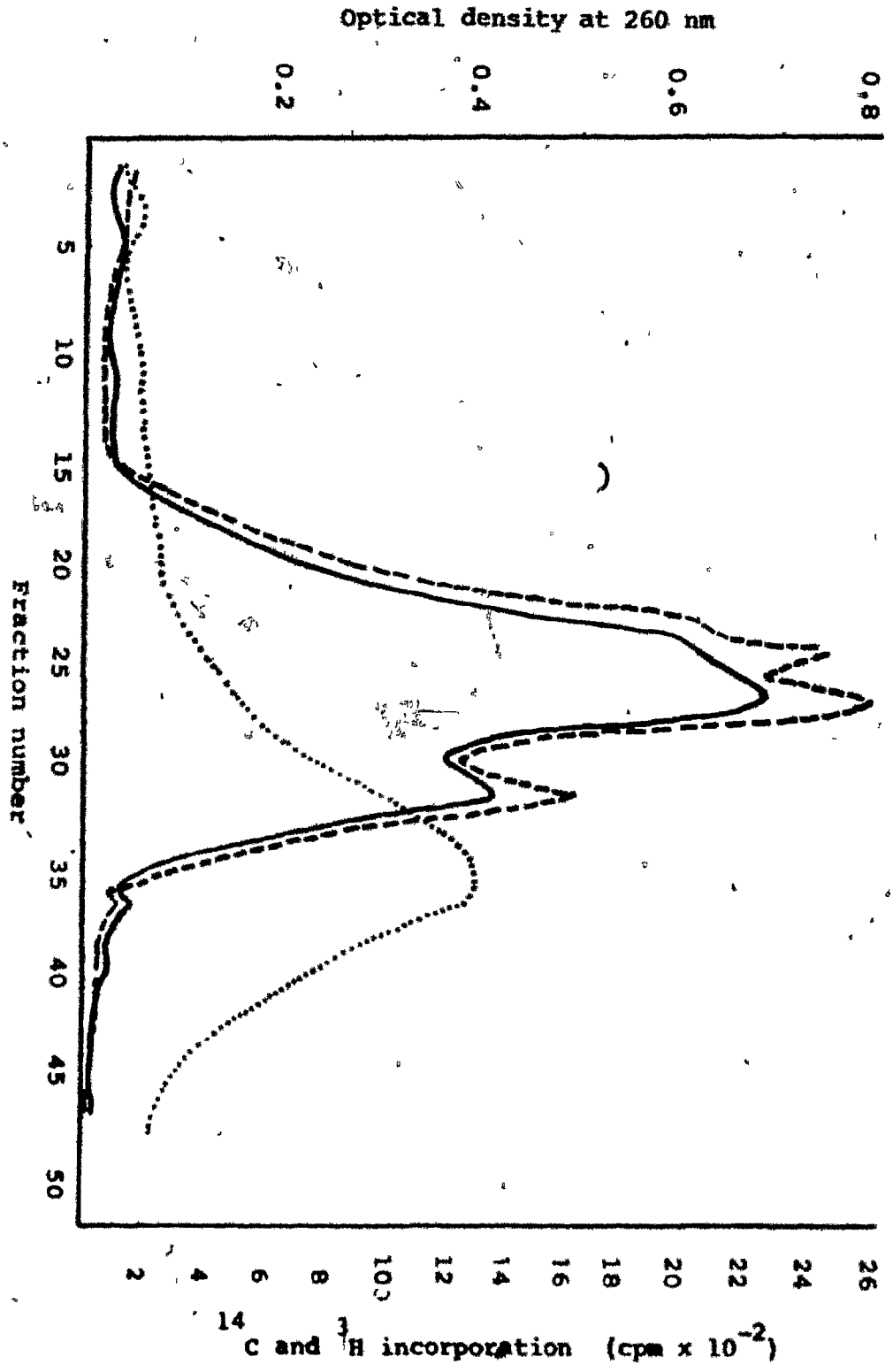

Optical density at 260 nm
1.5
1.0
0.5
5
10
15
20
25
30
35
40
45
Fraction number
14
12
10
8
6
4
2
^{14}C and ^{3}H incorporation (cpm x 10^{-3})

Figure 8 Elution profiles of T1 RNase digestion products of *E. coli* ^{3}H- and ^{14}C-leucyl-tRNA, aminoacylated with chick enzyme, and chromatographed on DEAE-cellulose

....., Optical density at 260 nm

_____, ^{14}C-leucyl-tRNA fragments

------, ^{3}H-leucyl-tRNA fragments


Optical density at 260 nm
0.2
0.4
0.6
0.8
Fraction number
5
10
15
20
25
30
35
40
45
50
2
4
6
8
10
12
14
16
18
20
22
24
26
14C and 3H incorporation (cpm x 10-2)

Chick liver tRNA could not be acylated to a sufficient extent by E. coli enzyme to allow the converse experiment.

The results of the above experiments, notably the data of Table 3, seemed to indicate that a derivative of leucine was being formed at some time during the amino-acylation or T1 ribonuclease digestion of chick tRNA. Various methods of separation of amino acids and their derivatives were used in attempts to effect purification of this proposed derivative. A great enrichment of carbon label, relative to tritium, was sought in a compound that could be separated from leucine.

Paper chromatography

Paper chromatography of the amino acids recovered from deacylated ^{14}C- and ^{3}H-leucyl-tRNAs, and from the T1 RNase digestion products of these tRNAs, failed to separate compounds differing in the relative amounts of ^{14}C and ^{3}H. In most cases, two areas of the chromatogram were found to be radioactive, but consistent results were not obtained. The ratios of labeled carbon to tritium were the same in both spots. Non-radioactive leucine added as carrier always coincided with one of the spots, as observed by ninhydrin visualization.

A mixture of non-radioactive, ^{14}C- and ^{3}H-leucine was chromatographed separately as a control, and only one area of the chromatogram was shown to be radioactive, and to yield a lavender color on treatment with ninhydrin.

Thin layer chromatography

Thin layer chromatography yielded more consistent results. Two radioactive areas were found and the R_f of each determined. They are presented below with control values for leucine:

	R_{f1}	R_{f2}
Spot 1	59	29
Spot 2	88	66
Leucine	91	66
Leucine, value from literature (189)	90	69

Spot 1 stained yellow with the ninhydrin reagent, and Spot 2 and leucine stained pinkish-purple. The two spots did not differ in relative amounts of ^{14}C and ^{3}H.

Ligand exchange chromatography

Chromatography on copper-Sephadex columns failed to separate radioactive species. All label was recovered in one or two fractions as the elution front.

Amino acid analysis

The amino acid mixtures recovered from whole tRNA and tRNA fragments were chromatographed on the Beckman automatic amino acid analyzer. Early results indicated two ninhydrin-positive peaks, but one was later shown to be due to the Tris buffer used in deacylation of tRNA. The other compound

was judged to be leucine by elution characteristics, and co-chromatography with a known leucine standard confirmed the identity.

Radioactive samples of material hydrolyzed from tRNA were chromatographed and fractions collected. One radioactive peak was found at the standard elution volume of leucine.

Radioactive leucine was heated to 37° C for 20 min to simulate the conditions of acylation and applied to the column. Fractions were collected, and only one fraction of the effluent was found to be radioactive. Background radioactivity throughout the fractions was found to be no greater than 0.1% of that of the major peak.

Methallylglycine, $CH_2=\overset{CH_3}{\overset{|}{C}}-CH_2-\underset{NH_3}{\underset{|}{CH}}-COOH$, was co-chromatographed with leucine to determine whether a compound identical to that which would be formed by the loss of two hydrogen atoms from the 4,5-carbons of leucine could be distinguished from leucine on amino acid analysis. No separation of these two compounds was effected by the analyzer.

Ethyl acetate extraction

A mixture of ^{14}C- and ^{3}H-leucyl tRNAs was subjected to hydrolysis with 0.5 M NH_4OH at 37° C for 30 min, and a

control sample of a mixture of both radioactive amino acids was similarly treated. Ethyl acetate extraction of these preparations was carried out as described in "Methods". The results showed no significant amount of ethyl acetate-extractable material in the experimental sample.

Sample*	Radioactivity of ethyl acetate phase as a percentage of the total radioactivity of each sample
tRNA hydrolysate	0.9 ± 0.6
leucine standard	0.7 ± 0.3

* Each value is the average of three determinations.

Discussion

The phenomenon studied in these experiments, a preponderance of labeled carbon relative to tritium in a portion of the chromatographic profile of doubly-labeled leucyl-tRNA fragments, has been noted by Tsay (82), and by Shearn and Horowitz in their investigations of amino-acylation of Neurospora crassa tRNAs (179). In both cases it was attributed to contamination of the ^{14}C-leucine used. Attempts were made in this work to determine if the phenomenon might instead indicate the formation of a biologically significant derivative of the leucine moiety of a leucyl-tRNA species.

Transfer RNA was isolated from chick embryos at various stages of development, and from adult chick liver. The tRNA preparations were charged with ^{3}H- and ^{14}C-leucine by homologous enzyme preparations of varying degrees of purification. It should be noted that at this time, it was generally believed that there was only one activating enzyme for each amino acid. The acylated tRNA preparations were then digested with T1 ribonuclease and the resulting fragments chromatographed on DEAE-cellulose columns. The chromatographic profiles of each of these tRNA preparations were found to be very similar (Figs. 4,5). This observation

provided presumptive evidence that the anomalous carbon peak indicated the formation in the chick of a distinct derivative of leucine rather than nonspecific degradation of the isotopically labeled amino acid. The failure to find any area of preponderance of carbon label on chromatography of doubly-labeled tRNA fragments of rabbit liver (Fig. 6) or E. coli (Figs. 7,8) also strengthened this view, although good resolution of the TRNA fragments of these species was not achieved.

Four different batches of ^{14}C-leucine were used in the early experiments, and no differences were noted in the results. This also supported the hypothesis that a specific derivative of leucine was formed.

The possibility that the label of leucine was incorporated into a cytokinin group was eliminated indirectly. All label could be removed from chick tRNA by standard deacylation procedures, which indicated that the leucine label was not incorporated into an isopentenyl group (62).

Comparisons of recovered amino acids from whole tRNAs and tRNA fragments were attempted in order to determine whether the proposed leucine derivative was formed during the aminoacylation reaction or during digestion with T1 RNase. The amino acid mixtures that were recovered from

tRNA and tRNA fragments were chromatographed separately on paper and thin layer plates. As has been shown in "Results", no further increase in labeled carbon relative to tritium was found in either case, but more than one radioactive area was found on chromatography of each of the amino acid mixtures. This result strengthened the tentative conclusion, made on the basis of results of variation of the time period of T1 RNase digestion (Tables 2,3), that the leucine derivative was formed during the aminoacylation reaction.

None of the methods used in attempts to isolate a specific derivative of leucine were successful. Thin layer chromatography gave the best separation of radioactive compounds, but the results were inconsistent.

The final experiment, extraction of N-blocked amino acid derivatives by ethyl acetate, was undertaken to assess the biological significance of the proposed derivative. At the time this work was done, it was generally felt that an initiator aminoacyl-tRNA of a eukaryotic system would be found to have a blocked amino group. Relevant experiments by Moav and Harris (95) and by Arnstein and Rahaminoff (97) have been discussed in the "Literature Review". Rosenberg and Elson (191) found an N-substituted seryl-tRNA (hydroxypyruvyl-tRNA) in Myxococcus xanthus by the ethyl acetate extraction method, and suggested that it might be an initiator in this system.

Herve and Chapeville showed that the alkaline stability of an aminoacyl-tRNA is greater if the amino group is blocked (192). Some indications were found in these experiments that the tritium label was more labile than the carbon in tRNA deacylation reactions, although attempts to make use of this difference in an isolation procedure for the carbon-labeled derivative were unsuccessful.

No further evidence for an N-blocked derivative was found on ethyl acetate extraction of the amino acid mixture recovered from aminoacyl-tRNA after deacylation. This result was taken to indicate that the possible derivative of leucine was not involved in the initiation of protein synthesis in the chick.

It was concluded that the anomalous carbon peak seen on chromatography of T1 RNase fragments of radioactive leucyl-tRNA was due to degradation of radioactive leucine which was accelerated in the aminoacylation reaction. The inconsistent behavior of the derivative in deacylation reactions and on chromatography indicated that more than one derivative might be formed, which would be consistent with progressive degradation. This conclusion was supported in a personal communication from E. Anthony Evans, of the Amersham-Searle Company (193).

SECTION IV - AMINO ACID INCORPORATION BY CHICK EMBRYO NUCLEAR AND MITOCHONDRIAL FRACTIONS

Introduction

The present investigation was undertaken in order to extend the observations of Trevithick and Wainwright (144) on the effects of some inhibitors of protein synthesis on the incorporation of amino acids by the nuclear fraction of chick embryo. The work of Wainwright (169) on the effect of inhibitors of protein synthesis on the onset of synthesis of hemoglobin in chick blastodiscs had indicated the involvement of a non-cytoplasmic, bacterial-like protein synthesizing system. Mitochondrial involvement in the synthesis of heme, the prosthetic group of hemoglobin has been documented. The emymes involved in the synthesis of heme are distributed between the mitochondria and the cytoplasm (194). The incorporation of amino acids by chick embryo mitochondrial fraction under various conditions of incubation was, therefore, also of interest.

The extent of incorporation of amino acid by the nuclear fraction varied greatly from fraction to fraction in the experiments of Trevithick and Wainwright, both in control samples and those treated with inhibitors, although variability of replicate samples was acceptably low. The objectives of the present experiment were to devise a standard method for

the purification of the nuclear fraction and to determine optimal conditions for amino acid incorporation by this fraction, with the aim of reducing variability among fractions. More consistent results on the effect of inhibitors might then be obtained. Some experiments on amino acid incorporation by the mitochondrial fraction were also planned.

It should be mentioned that studies on a mixed "nuclei plus mitochondria" fraction were done prior to many of those on purified nuclear fractions, and it was later found that the results of this series of experiments might not be directly comparable to those obtained with adequately purified fractions.

Methods

Chicks

Fertilized eggs were incubated at 37° C in a warm room or an automatic incubator. Fresh material was used exclusively in this series of experiments. In those experiments in which early embryos were used, blastodiscs were explanted onto a solid minimal medium by the procedure described by Wainwright and Wainwright (195). Descriptions of the procedures used in individual experiments will be given.

Homogenization

Embryos were homogenized in 4 volumes of liquid medium in a Tri-R homogenizer with 1½ passes of the pestle at 750 rpm. Two homogenization media were used in the course of these experiments, and will be described later in this work.

Isolation of nuclear fraction

Crude nuclear fraction

The homogenate was centrifuged at about 1,000 x g for 10 min. The supernatant was saved for isolation of mitochondria or discarded. In the first experiments, the pellet was resuspended in 0.5% Triton-X-100 in homogenization medium, except as otherwise noted, centrifuged at about 1,000 x g for 5 min, washed once in homogenization medium and taken up in 2 ml homogenization medium. The concentration of Triton-X-100 was increased to 1.0% later in the

series of experiments on purified nuclear fraction of 5-day chick embroyo.

Purified nuclear fraction

The crude nuclear fraction was layered onto a discontinuous gradient made up of 1 ml each of 1.3 M, 1.75 M, and 2.3 M sucrose in 3×10^{-3} M $CaCl_2$, and the sample was centrifuged for 30 min at 40,000 rpm in the Spinco SW 50 rotor or at 35,000 rpm in the Spince SW 56 rotor. The resulting pellets were rinsed and resuspended by gentle manual homogenization in homogenization medium.

Isolation of mitochondrial fraction

Mitochondria were isolated by a modification (169) of the method of Mitra and Bernstein (196). The homogenate was centrifuged at 1,000 x g for 10 min to remove nuclei and cell debris. The supernatant was centrifuged at 5,000 x g and the pellet discarded or pooled with the first for isolation of nuclear fraction. The supernatant was centrifuged at 20,000 x g for 20 min, and the pellet washed 5 times in homogenization medium by centrifugation at 18,000 x g for 10 min. The pellet was resuspended in 5 ml homogenization medium by manual homogenization.

Preparation of "nuclei plus mitochondria" fraction

The whole embryo homogenate was centrifuged at 20,000 x g for 30 min. The supernatant was discarded and the pellet resuspended and washed twice in homogenization medium

at 20,000 x g for 10 min. The pellet was resuspended in 3-4 volumes of medium.

Incubation

In some experiments the crude nuclear fraction was incubated with ^{3}H-lysine and the gradient purification was carried out after incubation. Purified nuclear fraction was used in other studies. Similarly, in some experiments on the "nuclei plus mitochondria" fraction, that preparation described above was incubated; in others, the nuclei and mitochondria were purified as described above and a 1/1 (by cell equivalent) mixture of the two purified fractions was incubated. In all experiments in which mitochondria alone were incubated, the complete isolation procedure was carried out prior to incubation.

Minimal medium was used in early experiments as the homogenization and incubation medium. The medium of Allfrey, Mirsky, and Osawa (139) (AMO Medium) was substituted for minimal medium in later experiments. The homogenization medium in later cases was 0.25 M sucrose in 3 x 10^{-3} M $CaCl_2$. The incubation medium consisted of 0.5 vol homogenization medium containing cellular fraction to which was added buffer, salts and glucose as energy source. The components of 1 ml of incubation mixture are shown below for each medium:

Component	μmoles of component in medium	
	Minimal Medium	AMO Medium
NaCl	147.0	13.5
KCl	4.1	-----
$CaCl_2$	1.8	1.0
$MgCl_2$	0.5	0.5
KH_2PO_4	3.0	1.7
K_2HPO_4	-----	5.7
Na_2HPO_4	4.5	-----
glucose	11.0	20.0
sucrose	-----	167.0
	pH 6.85	pH 7.25

Incubation procedure - crude nuclear fraction and "nuclei plus mitochondria" fraction

Each sample contained 0.5 ml of a suspension of the cell fraction(s) used in homogenization medium, other components of AMO Medium when used, 10.3 μCi ^{3}H-lysine of specific activity 3.91 C/mM, and 2 μmoles of each of the other 19 non-radioactive amino acids. Supplements were added as described in individual experiments, and the volume was brought to 1 ml by the addition of distilled water. Background samples were prepared in the same manner, but without radioactive lysine. The samples were incubated aerobically with shaking in the Dubnoff metabolic incubator at 37° C for 1 hr. A 200-fold excess of non-radioactive lysine was added to all tubes, and 10.3 μCi of ^{3}H-lysine to the background samples. After further incubation for 5 min, all tubes were chilled in an ice bath.

The incubation procedure used in the series of experiments on purified nuclear fraction differed from the above

in that nuclear fraction containing 0.5 mg as protein was used in all cases, and non-radioactive amino acids were omitted. Background samples were prepared with nuclear fraction which had been boiled for 5 min. The incubation mixtures were preincubated for 5 min prior to the addition of radioactive amino acid. Incubation was then continued for 30 min. Experimental samples were then boiled for 5 min, after which an equal volume of 20% TCA - 0.1 M non-radioactive amino acid (that corresponding to the labeled amino acid used) was added. The samples were then placed in a boiling water bath for 20 min to discharge tRNA.

Background samples prepared with boiled fraction usually gave higher counts than those prepared at the end of the incubation period which contained radioactive and non-radioactive amino acid. They were felt, however, to provide a better value for non-specific association of labeled amino acid.

Determination of radioactivity

The samples were washed onto Whatman GF/A glass fiber disks, and washed four times with a cold solution of 5×10^{-2} M non-radioactive amino acid in 5% TCA, once with absolute ethanol, and once with ether. The disks were dried and transferred to precounted scintillation vials containing 15 ml Bray's scintillation fluid (184) or toluene-"Omnifluor" (New England Nuclear) for the determination of radioactivity.

Purification of fractions after incubation of "Nuclei plus mitochondria"

Incubation mixtures containing both nuclear and mitochondrial fractions were centrifuged at 1,000 x g for 10 min. The supernatant was centrifuged at 1,000 x g for 10 min, and the two pellets containing nuclear fraction were pooled. The supernatant was centrifuged at 20,000 x g for 20 min, and the pellet containing mitochondrial fraction washed 5 times as described above.

Assays of marker enzymes

Assays of lactate dehydrogenase and glucose-6-phosphatase and the mitochondrial enzyme cytochrome oxidase were performed to determine the degree of purification of subcellular fractions.

Lactate dehydrogenase catalyzes the reaction pyruvate + NADH + H^+ ⇌ lactate + NAD^+ and is a soluble enzyme. Lactate dehydrogenase was determined by following the decrease in absorbance at 340 nm, due to the disappearance of NADH, of an incubation mixture containing 0.1 ml 0.01 M Na pyruvate, 0.1 ml 0.002 M NADH, a quantity of whole embryo homogenate or cell fraction, and 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.4. The volume was adjusted to 3.0 ml with distilled water (197).

Cytochrome oxidase, an enzyme of the mitochondrial electron transport system which oxidizes cytochrome c and

is itself oxidized by oxygen, was assayed by measuring the decrease in optical density at 550 nm of an incubation mixture containing 0.5 ml of a fresh solution of 200 μM cytochrome c in 0.01 M phosphate buffer, pH 7.0, a quantity of homogenate or other fraction, and 0.1 M phosphate buffer, pH 7.0 to the final volume of 3.0 ml (198). Determination of cytochrome oxidase activity in the series of experiments on purified nuclear fraction was done according to the method of Cooperstein and Lazarow (199). A solution of 1.7×10^{-5} M cytochrome c in 0.03 M phosphate buffer was prepared and was reduced by the addition of 1/300 vol freshly prepared 1.2 M sodium hydrosulfite. The solution was aerated gently for 15 min. An aliquot of the fraction to be assayed was added to 3.0 ml reduced cytochrome c in a cuvette and the decrease in absorption at 550 nm was monitored. A few grains of potassium ferricyanide were added after 3 min to oxidize remaining cytochrome c.

Glucose-6-phosphatase is a microsomal enzyme which hydrolyzes the phosphate group from glucose-6-phosphate. It was determined by incubating 0.5 ml of homogenate or cell fraction in an equal volume of a 1/1 mixture of 0.2 M citrate buffer, pH 6.5 and a solution of the sodium salt of glucose-6-phosphate at a concentration of 24.5 mg/ml.

The mixture was incubated at 37° C for 30 min and 1.0 ml 10% TCA was added. The reaction mixture was centrifuged and the concentration of inorganic phosphate in the supernatant determined (200).

Electron microscopy

Fractions of purified nuclear fraction were fixed in Millonig's-phosphate buffered 2% glutaraldehyde pH 7.2 at 4° C for 24 - 72 hr. They were subsequently washed in buffer solution overnight. Pieces of the fraction were then post-fixed in phosphate buffered OsO_4 for two hours, then washed twice through buffer and dehydrated through concentration-graded ethanol to absolute ethanol. The absolute ethanol was washed out with several changes of propylene oxide. The tissue fractions were then infiltrated with Spurr's epon by passing them through increasing concentrations of this polymer dissolved in propylene oxide. They were then embedded in the same epoxy inside capsules and were left to polymerize at 60° C for 12 hr. The fractions were cut with a diamond knife on an LKB Ultramicrotome at 600 - 1000 Å, placed on copper grids and stained with uranyl acetate for 10 min, washed and stained in Pb citrate for 3 min, washed, dried, and observed and photographed with a Zeiss EM 95.

DNA, RNA, and protein contents of the homogenate and purified nuclear fraction

DNA and RNA were determined by the method of Santen and Agranoff (201). This method depends upon the differential solubilities of DNA and RNA in perchloric acid. A series of lipid extractions was followed by an extraction with cold 5 % TCA. The residue was dissolved in 1 N KOH at 37° C for 16 hr. Concentrated perchloric acid was then added to precipitate DNA and protein. The residue was washed once with concentrated perchloric acid, the supernatant fractions pooled, and the RNA content determined spectrophotometrically. DNA was then extracted from the residue by a series of washes with 1 N $HClO_4$ and also determined spectrophotometrically.

Protein was determined by the method of Lowry (202).

Separation of the different classes of nuclear proteins

The saline-soluble proteins, the histones, and the acidic proteins of the purified nuclear fraction were separated by the method of Teng, Teng, and Allfrey (203).

The purified nuclear pellet was first extracted twice with 25 vol 0.14 M NaCl to yield saline-soluble proteins. The residue was recovered by centrifugation at 3,000 x g for 5 min and further extracted with 25 vol 0.25 N HCl twice. After centrifugation at 3,000 x g 5 min, the supernatants containing the histones were pooled and the residue was extracted once with 10 vol 1:1 $CHCl_3$:methanol—0.2 N HCl

and once with 10 vol 1:1 $CHCl_3$:methanol-0.2 N HCl to remove lipids. The pellet recovered after centrifugation was dried with ether, suspended in 5 vol cold buffer consisting of 0.1 M Tris-HCl pH 8.4 containing 0.01 M EDTA and 0.14 M 2-mercaptoethanol. An equal volume of phenol saturated with this buffer was added and the suspension was allowed to stand at 2^0 C 14 hr. After brief homogenization, the suspension was centrifuged at 12,000 x g 10 min. The aqueous phase was re-extracted with phenol and the phenol phases were then combined and dialyzed against 100 vol 0.1 M acetic acid - 0.14 M 2-mercaptoethanol for 3 hr. The dialysis solution was changed and dialysis was continued until the phenol phase was reduced to one-fifth its original volume. The aqueous layer was removed and the remaining phenol was dialyzed against 0.05 M acetic acid - 9.0 M urea - 0.14 M 2-mercaptoethanol for 24 hr. The dialysis solution was then changed to 0.1 M Tris-HCl pH 8.4 - 8.6 M urea - 0.01 M EDTA - 0.14 M 2-mercaptoethanol and dialysis was continued 2 hr. The phenol-soluble acidic proteins are restored to the aqueous phase by this treatment.

Labeling and extraction of E. coli proteins

An E coli methionine auxotroph was grown in Davis Minimal Medium (204) containing 0.5 mCi ^{35}S-methionine. The organisms were harvested at the end of the log phase by centrifugation. A 1/1 mixture of phenol and 0.1 M sodium

phosphate buffer. pH 7.4 was added and the suspension agitated. After being allowed to stand overnight, the suspension was centrifuged and the phenol layer reserved. This layer was dialyzed against 0.1M Na phosphate buffer until the phenol was replaced. The aqueous solution of protein was lyophilized.

Digestion of protein fractions

Leucine aminopeptidase and carboxypeptidase A (Sigma) were used to digest labeled nuclear and bacterial proteins. The protein was dissolved in 0.1 M sodium phosphate buffer, pH 7.9, and an appropriate amount of either enzyme (as determinted in preliminary experiments) was added. The reaction mixture was incubated at 25° C or at 37° C. The reaction was followed by determining the radioactivity remaining in the cold TCA-insoluble fraction of aliquots removed at different time intervals throughout the course of the incubation.

Electrophoresis

Electrophoresis of labeled nuclear protein was carried out on 10% polyacrylamide gels containing 0.1 % SDS by Dr. C. B. Lazier of this University, by the method of K. Weber and M. Osborn, Journal of Biological Chemistry 244: 4406, 1969.

Results

The work described below may be roughly divided into three sections: preliminary studies with total homogenates and crude nuclear fractions, work with a purified nuclear fraction, and experiments with a fraction containing nuclei and mitochondria.

A. Incorporation of amino acid by fresh whole embryo homogenates

Preliminary studies were made of amino acid incorporation by freshly dissected whole embryo homogenates. The earlier work by Trevithick and Wainwright (144) had been done on embryos which had been explanted 16-24 hours before use, and it was felt that embryos which had not been depleted of intracellular yolk might not incorporate a high level of radioactive amino acid.

Table 4 shows the incorporation of ^{14}C-synthetic algal hydrolyzate, ^{3}H-lysine by fresh whole embryo homogenates. The effect of ATP on incorporation was also studied.

Background incorporation was found to be very high, particularly when the synthetic hydrolyzate was used. The addition of 10^{-3} M ATP usually decreased both the background and experimental incorporation levels. Some precedent for this finding exists, and will be discussed. In Experiment I, no decrease in background was observed, and, in Experiment II, an increase in the net incorporation level was found. The nature of the apparent inhibition of incorporation in Experiments I, III, and IV was not investigated.

TABLE 4

Incorporation of radioactive amino acids by whole embryo homogenates*

Sample	Incorporation of radioactive amino acid (cpm/aliquot) ± S.D.	(net)	Incorporation in the presence of 10^{-3} M ATP (cpm/aliquot) ± S.D.	(net)
Experiment I				
Blank	4,488 ± 471		5,313 ± 547	
Control	7,488 ± 308	3,000	8,066 ± 715	2,753
Experiment II				
Blank	1,097 ± 217		635 ± 40	
Control	1,585 ± 102	489	2,078 ± 140	1,443
Experiment III				
Blank	3,602 ± 1,012		1,837 ± 282	
Control	9,083 ± 640	5,481	5,052 ± 319	3,215
Experiment IV				
Blank	4,259 ± 1,144		2,221 ± 285	
Control	9,869 ± 735	5,610	5,765 ± 372	3,544

* Experiment I was done with 10 μCi of ^{14}C-synthetic algal hydrolyzate, Experiment II, with 25 μCi of ^{3}H-leucine, and III and IV, with 25 μCi of ^{3}H-lysine. Each reaction mixture contained 0.3 g embryo homogenate on the basis of wet weight, in minimal medium supplemented with non-radioactive amino acids.

Each value represents the average of two determinations.

B. Determination of the degree of purification of subcellular fractions

It is important in all studies of isolated subcellular fractions that the degree of purification of the fraction be determined. In most of the later experiments done in this study, this was assessed by determining the activities of marker enzymes in the whole embryo homogenates and the subcellular fractions isolated from them.

The activities of three enzymes were assayed in the course of purification of the nuclear and mitochondrial fractions; lactate dehydrogenase, glucose-6-phosphatase, and cytochrome oxidase. Lactate dehydrogenase is a soluble enzyme, glucose-6-phosphatase is associated with the microsomal fraction, and cytochrome oxidase is present in the mitochondria.

Homogenates of 5 g of fresh chick embryo were prepared and assays for each enzyme were performed. After purification of the nuclear and mitochondrial fractions as described in "Methods" the assays were repeated. The range of enzyme activities of the fractions is shown below:

	Activity of enzyme as a percentage of that of whole homogenate		
Fraction	Lactate dehydrogenase	Glucose-6-phosphatase	Cytochrome oxidase
Homogenate	100	100	100
Nuclear fraction	0 - 0.02	0.02 - 0.07	1.2 - 1.5
Mitochondrial fraction	0.03 - 0.05	0.08 - 0.22	60 - 80

C. Amino acid incorporation by chick embryo crude nuclear fraction

The preliminary experiments of Trevithick and Wainwright (144) on incorporation of radioactive lysine by crude nuclear fraction of chick embryo were repeated. Radioactive lysine was used in studies of incorporation in order to avoid non-specific association of hydrophobic amino acid side chains with the lipid components of the nuclear membrane.

Crude nuclear fraction was prepared as described in "Methods" but without treatment with Triton-X-100. Incubation was performed as described above in minimal medium.

The results of the initial experiments were not satisfactory. The level of incorporation was low (600-800 cpm) and the background samples showed high counts relative to the experimental samples (300-400 cpm).

It was felt that the minimal medium used might be unsuitable for incorporation of amino acid by the nuclear fraction. The incubation medium was accordingly changed from minimal to AMO Medium as described in "Methods", and a supplement of non-radioactive amino acids was added. Incorporation of ^{3}H-lysine by crude nuclear fraction was generally much greater in this medium, and attempts to lower the background uptake of amino acid were ultimately successful. The results of a representative experiment are shown in Table 5.

The effects of ATP and GTP on the incorporation of ^{3}H-lysine by crude nuclear fraction.

Isolated nuclei of various organisms have been found to differ in requirements for an exogenous energy source (see "Literature Review"). The response of chick embryo crude nuclear fraction to ATP and GTP was studied in early experiments.

The effect of ATP on incorporation of lysine into the crude nuclear fraction varied widely. ATP at a concentration of 2×10^{-3} M had an inhibitory effect on lysine incorporation in each experiment. In Experiment II, ATP was inhibitory at all concentrations used. ATP at concentrations of 10^{-3} M and 5×10^{-4} M was shown to stimulate amino acid incorporation in Experiments I, III, and IV. The addition of GTP at a concentration of 5×10^{-4} M did not enhance the stimulatory effect of ATP. These results are shown in Table 6.

The effect of increased Mg^{++} concentration on ^{3}H-lysine incorporation by crude nuclear fraction

Variation of the concentration of magnesium ion in *in vitro* experiments on protein synthesis can have a profound effect on the reactions involved in this process.

A ten-fold increase in the concentration of Mg^{++}, from 5×10^{-4} M to 5×10^{-3} M, assayed for effect on amino acid incorporation by crude nuclear fraction. The increased concentration seemed to contribute to unacceptably high variability between duplicate samples, and increased incorporation

TABLE 5

Incorporation of ^{3}H-lysine by the crude nuclear fraction of fresh 3-day chick embryos*

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot)	
Blank		
1	95.0	
2	96.0	av. 95.5
Control		
1	4,079	
2	3,480	
3	4,777	av. 4,112 ± 443

* Each sample contained the crude nuclear fraction of 0.25 g (wet weight) embryo, 10.3 μCi of ^{3}H-lysine and non-radioactive amino acids in AMO Medium as described in "Methods".

only to 110-115% of that of control samples. This change was not adopted in later experiments.

D. Amino acid incorporation by chick embryo mitochondrial fraction

As has been noted, the activity of the mitochondrial fraction was a secondary area of interest in these studies. One group of experiments was done to determine the effect of chloramphenicol on amino acid incorporation by purified mitochondrial fraction of 3-day chick embryo. The mitochondrial fraction was isolated as described in "Methods" and incubated with 103 μCi of ^{3}H-lysine and supplements of the other 19 non-radioactive amino acids in AMO Medium in the presence and absence of chloramphenicol. The incorporation of ^{3}H-lysine by the mitochondrial fraction was found to be inhibited by chloramphenicol by an average value of 72%. These results are shown in Table 7.

The lack of complete inhibition of the mitochondrial fraction by chloramphenicol may be thought to indicate incomplete purification of the fraction. Electron micrographs of mitochonchrial fraction prepared in the same way were made later in the course of this work and showed extensive contamination of the fraction by other cytoplasmic components.

TABLE 6

The effects of ATP and GTP on amino acid incorporation by crude nuclear fraction of 4-day chick embryo*

ATP concentration (M)	Incorporation of ^{3}H-lysine (cpm) as a percentage of the control value without ATP
Experiment I	
0	100
5×10^{-4}	172
5×10^{-4} + 5×10^{-4} GTP	170
10^{-3}	127
2×10^{-3}	77
Experiment II	
0	100
5×10^{-4}	89
10^{-3}	44
2×10^{-3}	16
Experiment III	
0	100
5×10^{-4}	181
10^{-3}	206
2×10^{-3}	99
Experiment IV	
0	100
5×10^{-4}	164
5×10^{-4} + 5×10^{-4} GTP	165

* Each sample contained Triton-X-100-treated crude nuclear fraction of 0.28 g wet weight embryo, 10.3 μCi ^{3}H-lysine, non-radioactive amino acids, and additions in AMO Medium as described in "Methods".

TABLE 7

The effect of chloramphenicol on ^{3}H-lysine incorporation by purified mitochondri[illegible]

TABLE 7

The effect of chloramphenicol on ^{3}H-lysine incorporation by purified mitochondrial fraction of 3-day chick embryo*

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot) ± S.D.	(net) cpm	Incorporation as a percentage of control	(net) cpm
Experiment I				
Blank	37 ± 3		2.2	
Control	1,681 ± 261	1,644	100.0	
100 µg/ml Chloramphenicol	389 ± 163	352	23.1	21.4
Experiment II				
Blank	261 ± 43		19.8	
Control	1,313 ± 113	1,052	100.0	
100 µg/ml Chloramphenicol	634 ± 59	373	48.2	35.4
Experiment III				
Blank	1,809 ± 9		5.4	
Control	33,506 ± 3,281	31,697	100.0	
100 µg/ml Chloramphenicol	10,462 ± 617	8,653	31.2	27.2

* Each reaction mixture contained purified mitochondrial fraction of 0.83 g wet weight of 3-day chick embryo, 103 µCi ^{3}H-lysine, and other components as described in "Methods". Each value represents the average of two determinations.

E Amino acid incorporation by purified nuclear fraction of 5-day chick embryo

This series of experiments was done with nuclear fraction of 5-day chick embryo which had been prepared with 1.0% Triton-X-100. Assessments of the degree of purity of the fraction were made initially.

The activities of the non-nuclear marker enzymes lactate dehydrogenase, glucose-6-phosphatase, and cytochrome oxidase were determined using the whole homogenate and the nuclear fraction. Very little activity of these enzymes was found in the purified nuclear fraction. The data are given in Table 8.

Electron microscopy of the purified nuclear fraction was done by Mr. Bora Merdsoy of this university, and Dr. M. Hansell confirmed that cytoplasmic ribosomes were absent from the purified preparation. Figure 9 is a typical electronmicrograph of the nuclear fraction.

The RNA/DNA, DNA/protein, and RNA/protein ratios determined for the purified nuclear fraction are given below:

RNA/DNA 0.286

DNA/protein 0.121

RNA/protein 0.035

The recovery of DNA in the nuclear fraction averaged 40% (relative to the DNA content of whole homogenate).

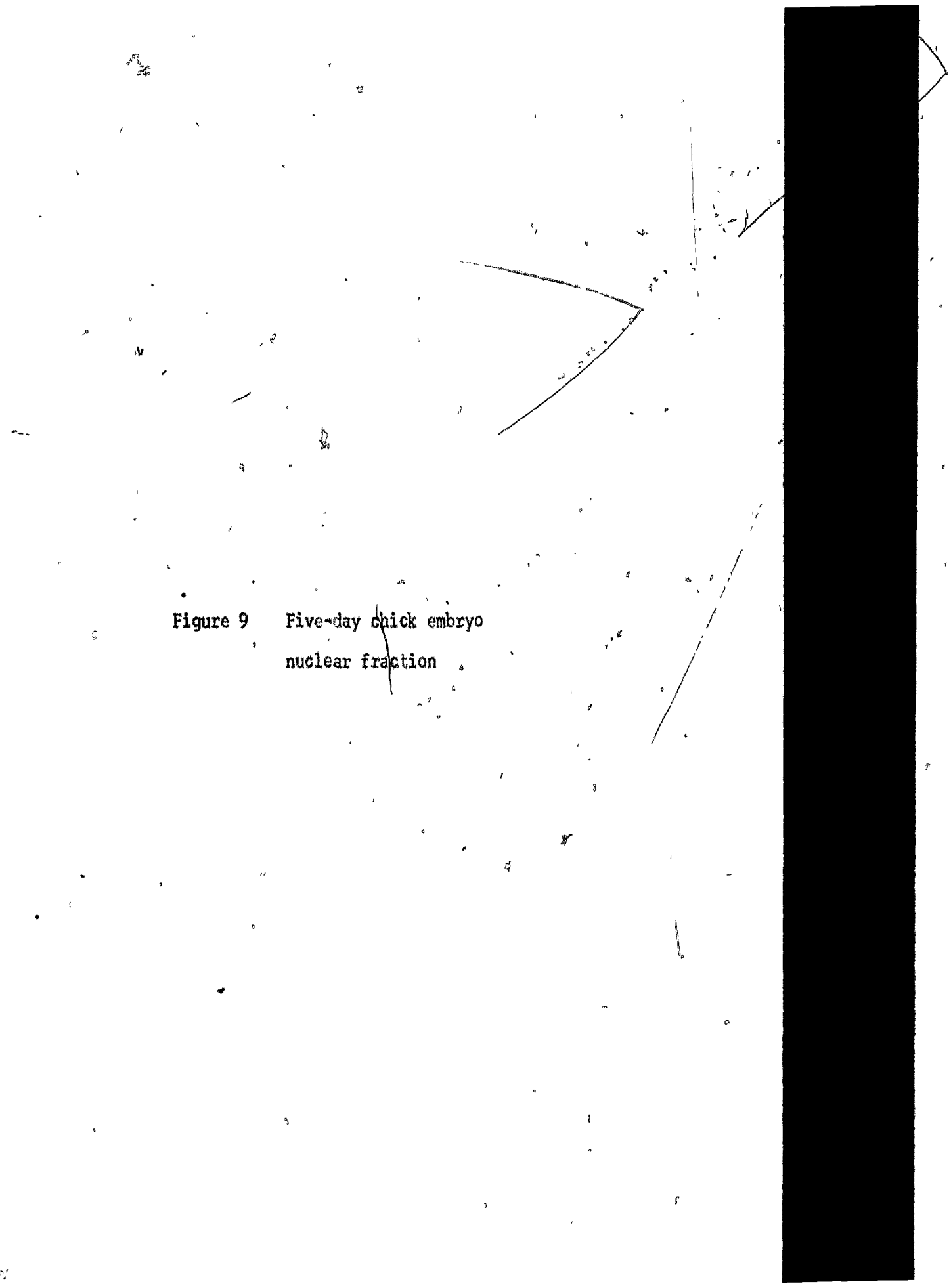


Figure 9 Five-day chick embryo nuclear fraction

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Checks for bacterial contamination in the nuclear fraction and in the incubation mixtures were made in the course of several experiments. Aliquots of the nuclear fraction and of the components of the incubation mixture, and of the complete mixture at the beginning and end of the incubation period were plated on blood agar. The plates were incubated at 37° C for 24-48 hr. It was found that if fresh or frozen components were used, colonies were very rarely found. The highest degree of contamination observed in mixtures at the end of the incubation period was four colony-forming units/ 0.1 vol of the mixture.

The effect of cytoplasmic contamination on the incorporation of radioactive amino acid by purified nuclear fraction

Experiments were done in which the nuclear fraction was deliberately contaminated with cytoplasm in order to assess the possible contribution of cytoplasm to the level of incorporation of radioactive amino acid. The supernatant fraction obtained after the initial centrifugation of the whole homogenate was reserved and portions were added to the incubation mixture containing purified nuclear fraction. Control samples consisted of nuclear and cytoplasmic fractions alone. Two experiments were done, and the results are given in Table 9. In the first experiment, cytoplasmic and nuclear fractions were incubated

alone and separately by the usual procedures as controls. Blank samples were prepared with boiled fractions. In the second experiment, additional samples were prepared in which cytoplasmic fraction was added to incubation mixtures containing nuclear fraction just prior to termination of the incubation period. In both experiments the cytoplasmic fraction was found not to incorporate amino acid. It is highly probable that endogenous nucleases destroyed the protein-synthesizing capacity of the cytoplasmic fraction during the 1.5 hr period in which it was reserved at 0° C, while nuclear purification was completed. There also appeared to be an inhibitory effect of cytoplasmic fraction on incorporation by the nuclear fraction.

The results of the above experiments, however, lent some weight to the assumption that observed incorporation of amino acid into "purified nuclear fraction" was due to the nuclear fraction itself, and further experiments to characterize this incorporation were performed. Some changes were made in the incubation procedure, as has been noted in "Methods", on the basis of results obtained in preliminary experiments.

The uptake of radioactive amino acid by purified nuclear fraction as a function of time and of amount of fraction

Saturation curves

Saturation curves were established for the uptake of ^{3}H-lysine and ^{3}H-leucine by a given quantity of nuclear

TABLE 8

The activities of lactate dehydrogenase, glucose-6-phosphates and cytochrome oxidase in the whole homogenate and purified nuclear fraction of 5-day-chick embryo*

	Glucose-6-phosphatase moles/m/mg protein	Cytochrome oxidase O.D/min/mg protein	Lactate dehydrogenase
Homogenate	4.73×10^{-4} $\pm 0.56 \times 10^{-4}$	14.2 ± 2.1	10.8 ± 3.7
Nuclear fraction	6.2×10^{-6} $\pm 0.5 \times 10^{-6}$	1.5 ± 0.7	0.28 ± 0.15
% total activity of homogenate found in nuclear fraction	1.3	10	2.6

* Methods of assay of the enzymes are given in "Methods".

TABLE 9

The incorporation of ^{3}H-leucine by purified nuclear fraction in the presence and absence of cytoplasmic components*

Sample	Incorporation of ^{3}H-leucine (cpm/aliquot) ± S.D.	(Net)
Experiment I.		
Blank	1,190 ± 360	
Nuclear fraction	4,370 ± 66	3,180
Blank	7,688 ± 243	
Cytoplasmic fraction	7,483 ± 310	0
Nuclear fraction + cytoplasmic fraction		
1.0/0.5	6,634 ± 104	0
1.0/1.0	6,737 ± 2,032	0
1.0/2.0	5,798 ± 247	0
Experiment II		
Blank	1,374 ± 98	
Nuclear fraction	3,795 ± 124	2,421
Blank	7,747 ± 659	
Cytoplasmic fraction	7,240 ± 142	0
Nuclear fraction + cytoplasmic fraction		
1.0/1.0	9,777 ± 2,526	656

* Each incubation mixture contained nuclear fraction, 0.5 mg as protein, 10 µCi ^{3}H-leucine, specific activity 5.5 Ci/ mole, AMO Medium and other components as described in "Methods". Cytoplasmic fraction was added in 0.5, 1.0, or 2.0 cell equivalents. Blank values obtained with individual fractions have been combined, and the total subtracted from experimental values for the mixed incubations.

fraction before studies of uptake as a function of time and of amount of nuclear fraction were attempted. Both amino acids were found to be maximally incorporated by 0.5 mg nuclear fraction as protein at a concentration of 2 mM, specific activity 2-5 Ci/mmole (Figure 10).

The time-course of incorporation of radioactive amino acid by purified nuclear fraction of 5-day chick embryo

The uptake of radioactive amino acid as a function of time was studied. A large-scale incubation mixture was prepared, containing 5 mg purified nuclear fraction, other components in the proportions given in "Methods", and 100 μCi ^{3}H-leucine, specific activity 5.5 Ci/mmole. An aliquot of 1 ml was removed to a boiling water bath immediately after addition of the radioactive amino acid as a zero-time sample. Further aliquots were taken at specified intervals throughout the course of incubation and boiled for 5 min. The samples were precipitated and prepared for determination of radioactivity by the usual methods. Figure 11 shows the results of this experiment.

The incorporation of ^{3}H-leucine by increasing amounts of 5-day chick embryo purified nuclear fraction

A linear relationship of uptake of radioactive amino acid to amount of nuclear fraction was found, within the limits shown in Figure 12. In each of the expetiments reported, separate samples were prepared containing increasing amounts of nuclear fraction and of ^{3}H-leucine.

Figure 10 The uptake of ^{3}H-leucine by purified nuclear fraction as a function of leucine concentration.*

* Each incubation mixture contained 0.5 mg nuclear fraction as protein, 10 μCi ^{3}H-leucine, sp. act. 55 Ci/mmole (1.8×10^{-10} mole), AMO Medium and other components as described in "Methods". Non-radioactive leucine was added to yield higher concentrations shown.

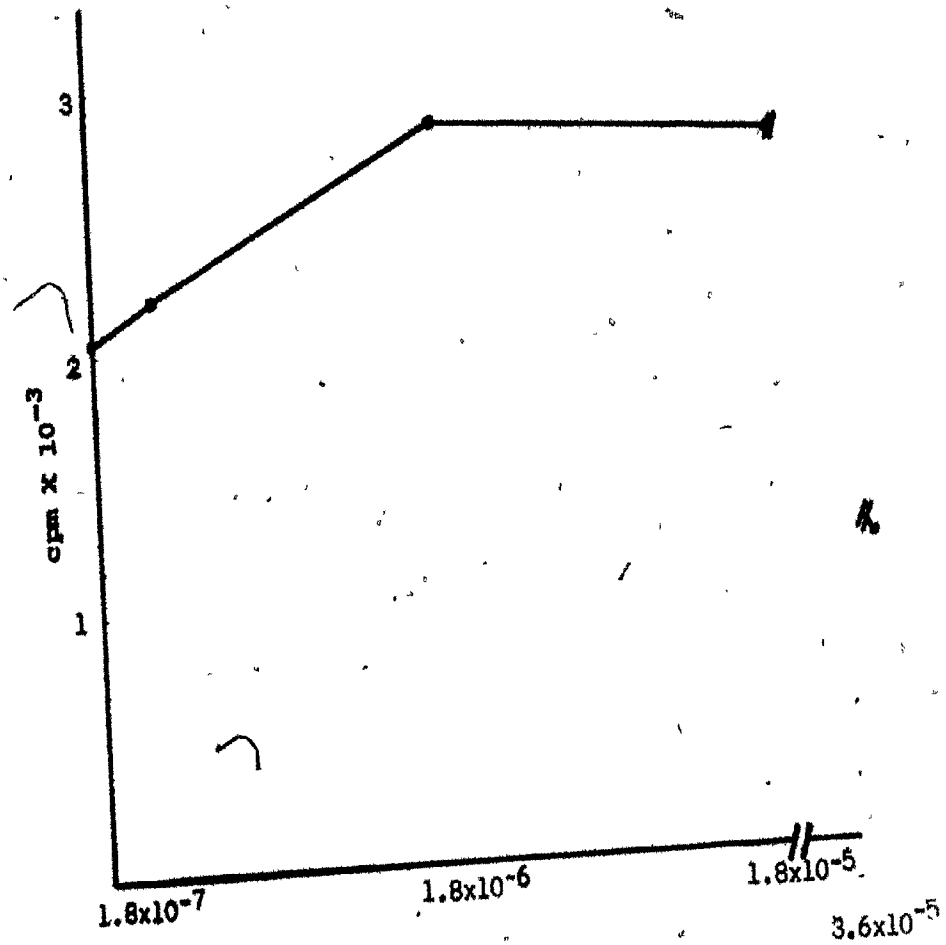

3
2
1
cpm x 10^{-3}
1.8×10^{-7}
1.8×10^{-6}
1.8×10^{-5}
3.6×10^{-5}
leucine concentration, M

Figure 11 The time-course of ^{3}H-leucine incorporation by purified nuclear fraction *

* The incubation mixture contained 5 mg purified nuclear fraction, as protein, AMO Medium, and 100 μCi ^{3}H-leucine sp. act. 5.5 Ci/mmole. Aliquots were removed to boiling water at specified time intervals. Radioactivity was determined as described in "Methods".

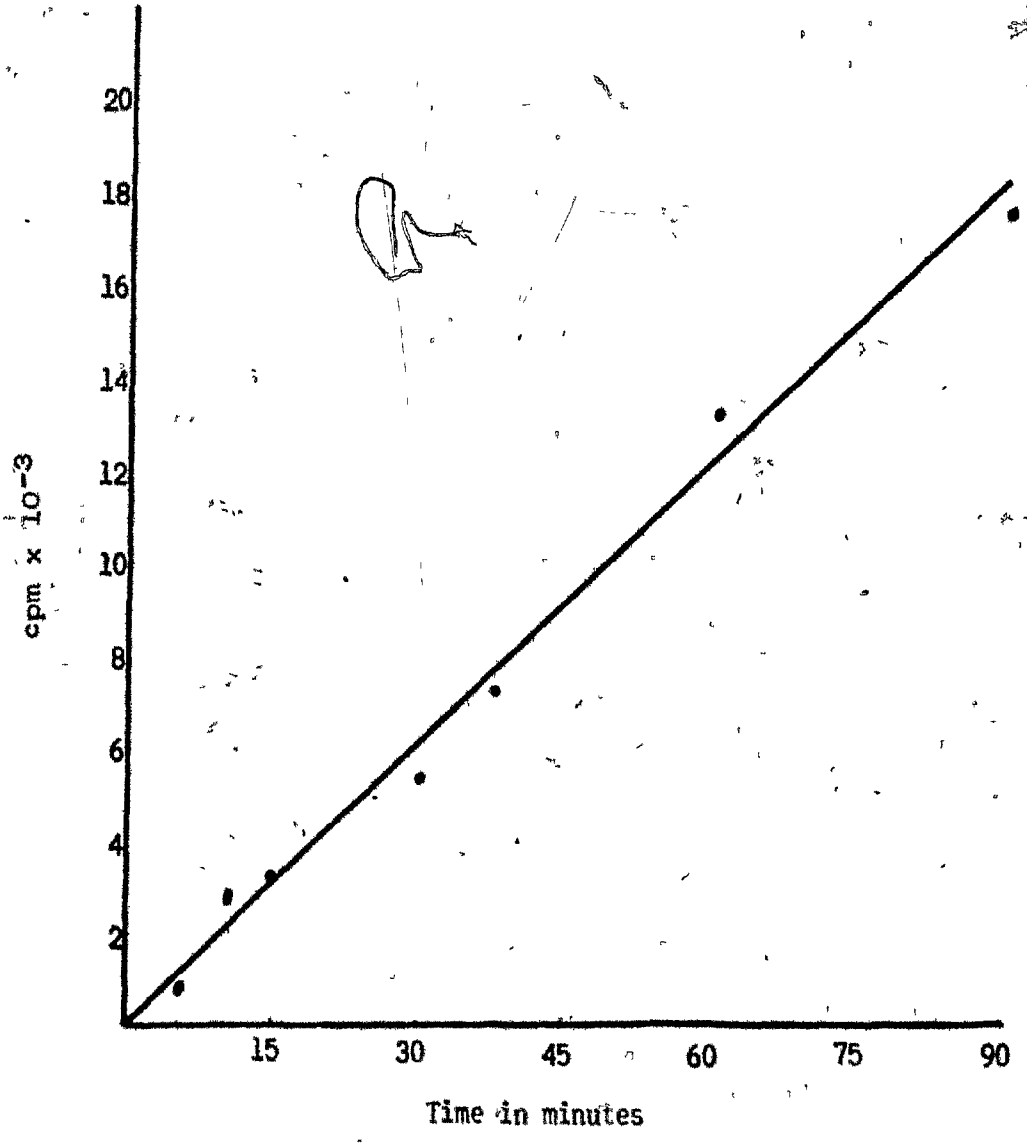

20
18
16
14
12
10
8
6
4
2
cpm x 10-3
15
30
45
60
75
90
Time in minutes

Figure 12 The incorporation of ^{3}H-leucine by increasing amounts of purified nuclear fraction *

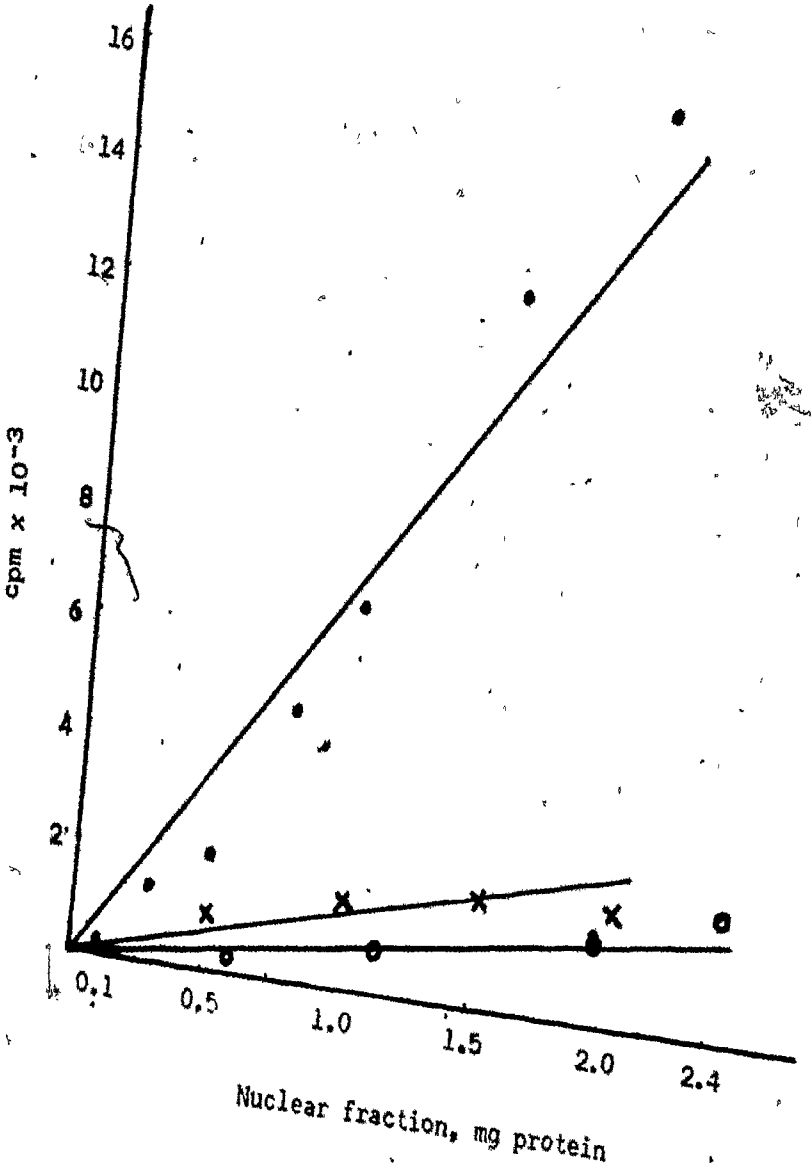
x——x Experiment I

O——O Experiment II

●——● Experiment III

* Each incubation mixture contained a quantity of 1.0 % Triton-X-100-purified nuclear fraction as specified, 50 μCi of ^{3}H-leucine /0.5 mg protein, and AMO Medium. Blank samples were initially boiled. The incubation period was 30 min, after which experimental samples were boiled for 5 min, TCA was added and radioactivity determined as described in "Methods".

cpm x 10^{-3}


16
14
12
10
8
6
4
2
cpm x 10-3
0.1
0.5
1.0
1.5
2.0
2.4
Nuclear fraction, mg protein

[redacted]

The blank samples consisted of boiled nuclear fraction in quantities equal to those in the experimental samples, and other components of the incubation mixture.

The variability of uptake of amino acid by a given amount of nuclear fraction from experiment to experiment may be noted here, as well as in other experiments. It is felt that this may reflect the physiological state of the embryo. Preparations of nuclear fractions from batches of embryos that showed gross evidence of being retarded in development were often found to be less active in incorporation studies. It has also been possible to correlate gross variations in uptake of amino acid by nuclear fraction with changes in the parent chicken flock at the supplier's. Other investigators in this department have observed variations in incorporation of nucleic acid and protein precursors and of enzymatic activities from season to season.

The effect of inhibitors of protein synthesis and of respiration on amino acid incorporation by purified nuclear fraction

The incorporation of radioactive amino acid by nuclear fraction in the presence of chloramphenicol, cycloheximide, puromycin, cyanide, dinitrophenol, and ribonuclease was measured. The usual incubation procedures were followed in most of these experiments and any exceptions will be noted.

Chloramphenicol

The effect of 100 µg chloramphenicol on amino acid incorporation by nuclear fraction was studied. The results of early experiments with 3-day chick embryo nuclear fraction, prepared with 0.5% Triton-X-100, are presented in Table 10. The incorporation by this fraction was almost completely inhibited by chloramphenicol. This result agrees with those of Trevithick and Wainwright (144).

Later experiments on 5-day chick embryo nuclear fraction prepared with the use of 1.0% Triton-X-100 gave the results shown in Table 11. Better and more consistent incorporation among experiments was obtained in the later work. The degree of inhibition by chloramphenicol, however, was not found to be as marked. Less nuclear material was used in incubation mixtures in this second series of experiments, but within the range used, there was no proportionate effect of chloramphenicol.

Cycloheximide

Cycloheximide at a concentration of up to 200 µg/0.5 mg nuclear fraction as protein was found to have no effect on the incorporation of amino acid by purified nuclear fraction. This result contrasts with some to be discussed later, which were obtained with "nuclei plus mitochondria" fraction, and suggests that in the latter case, cytoplasmic amino acid incorporation might have contributed to the results.

Cycloheximide has been shown to inhibit cytoplasmic protein synthesis in chick embryo (144).

Puromycin

The effect of several concentrations of puromycin on purified nuclear fraction and on sonicated nuclear fraction was investigated. High concentrations of puromycin were required to produce marked inhibition of the nuclear fraction. The results of several experiments are combined in Figure 13. The sonicated nuclear fraction incorporated ^{3}H-leucine very poorly (approximately 600 cpm/mg protein) and this incorporation was completely inhibited by puromycin at a concentration of 10^{-2} M.

Respiratory inhibitors

The inhibitors of respiration, cyanide and dinitrophenol, did not inhibit amino acid incorporation by nuclear fraction. On the contrary, stimulation of incorporation was observed in the presence of both compounds. The results of one such experiment are given in Table 12.

Ribonuclease A

Experiments to determine the effect of ribonuclease on amino acid incorporation by the purified nuclear fraction yielded unexpected and highly variable results. A seven-fold stimulation of incorporation was found in the presence of 200 µg RNase A in the first experiment. A second

TABLE 10

The effect of chloramphenicol on ^{3}H-lysine incorporation by purified nuclear fraction of 3-day chick embryo*

Sample	Incorporation of 3H-lysine (cpm/aliquot (net) ± S.D.		Incorporation as a percentage of control corrected for blank	
Experiment I				
Blank	152 ±	48		1.2
Control	11,859 ±	2,756	11,707	100.0
100 µg/ml Chloramphenicol	553 ±	222	401	4.6	3.4
Experiment II				
Blank	127 ±	31		15.1
Control	837 ±	34	710	100.0
100 µg/ml Chloramphenicol	195 ±	12	68	23.2	9.5

* Each reaction mixture contained nuclear fraction, isolated with 0.5 % Triton-X-100, of 0.83 g wet weight of 3-day chick embryo, 103 µCi ^{3}H-lysine AMO Medium and other components as described in "Methods". Each value represents the average of 2 determinations.

TABLE 11

The effect of chloramphenicol on ^{3}H-lysine incorporation by purified nuclear fraction of 5-day chick embryo*

Sample	Incorporation of ^{3}H-leucine (cpm/aliquot ± S.D.)	Incorporation as a percentage of control corrected for blank
Experiment I		
0.1mg Nuclear fraction	966 ± 102	100
0.1mg Nuclear fraction + 100 µg chloramphenicol	293 ± 5	30
Experiment II		
0.25 mg Nuclear fraction	887 ± 27	100
0.25 mg Nuclear fraction + 100 µg chloramphenicol	343 ± 19	39
Experiment III		
0.5 mg Nuclear fraction	1,154 ± 264	100
0.5 mg Nuclear fraction + 100 µg chloramphenicol	173 ± 51	15

* Each reaction mixture contained nuclear fraction expressed as protein, purified with 1.0% Triton-X-100, 10 µCi ^{3}H-lysine, AMO Medium and other componenets as described in "Methods". Each value represents the average of three determinations.

Figure 13 The effect of puromycin on the incorporation of ^{3}H-leucine by purified nuclear fraction *

* Each reaction mixture contained 0.5 mg nuclear fraction as protein, 10 µCi ^{3}H-leucine sp. act. 5.5 Ci/mmole, AMO Medium and other components as described in "Methods". The combined results of three experiments are shown.

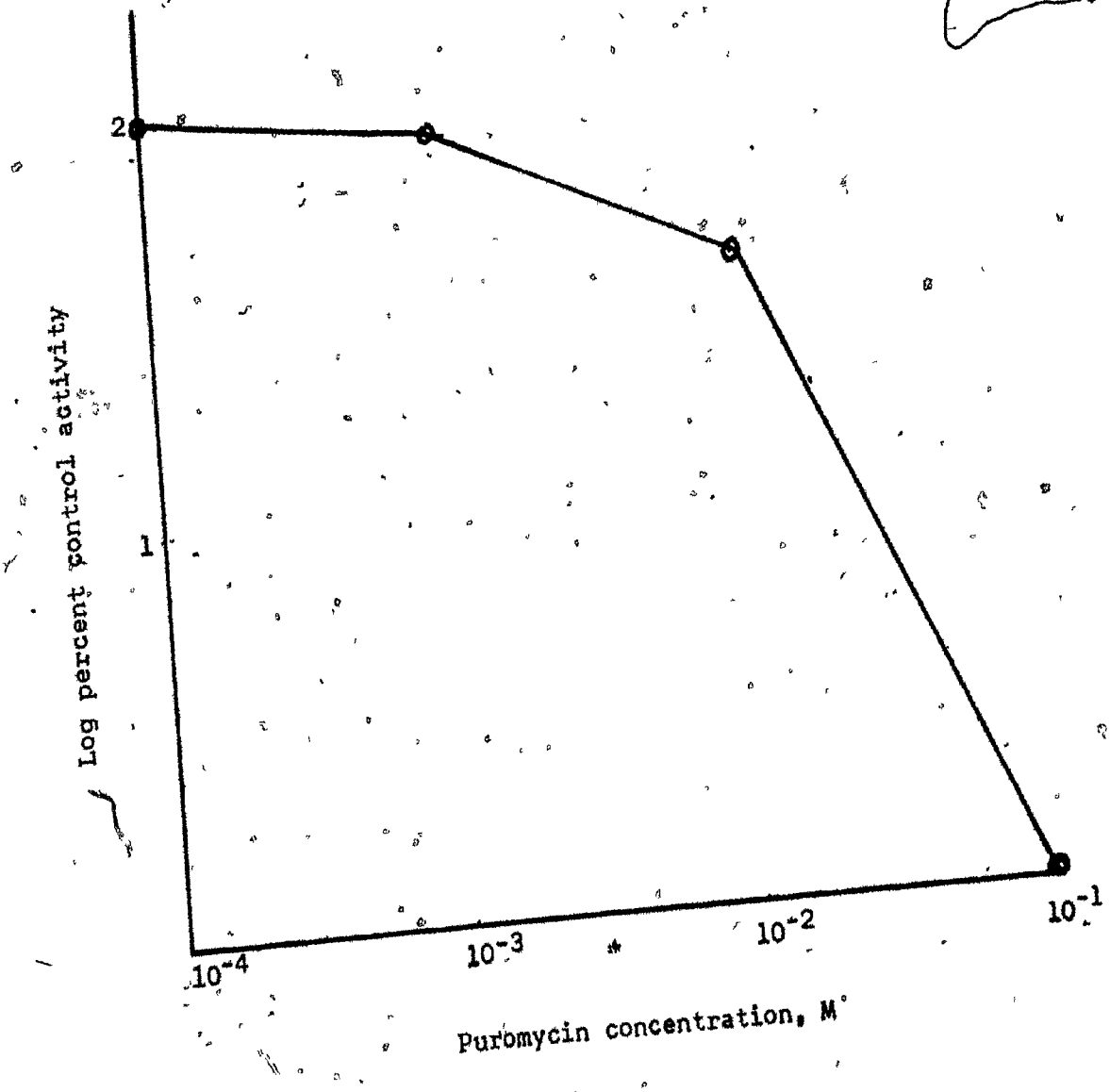

2
1
Log percent control activity
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Puromycin concentration, M

TABLE 12

The effect of cyanide and dinitrophenol on the incorporation of ^{3}H-leucine by purified nuclear tractor of 5-day chick embryo*

Sample	Incorporation of ^{3}H-leucine (cpm/aliquot ± S.D.)	Incorporation as a percentage of control
Nuclear fraction	786 ± 53	100
Nuclear fraction + 10^{-4} M cyanide	1,236 ± 340	158
Nuclear fraction + 10^{-3} M dinitrophenol	949 ± 466	121

* Each reaction mixture contained nuclear fraction, 0.5 mg as protein, 5 μCi ^{3}H-leucine and other components as described in "Methods". (Refer to Figure 11.) Each value is the average of three determinations and is corrected for blank values

experiment was done in which additional control samples were unsed. Blank samples were prepared with boiled nuclear fraction alone, and boiled nuclear fraction plus ribonuclease. Other controls were prepared in which RNase was added at the end of the incubation period. Even if the difference obtained by subtracting the level of incorporation of nuclear fraction alone from that obtained from the latter controls is subtracted from the level of incorporation of nuclear fraction plus RNase, a stimulation of incorporation in the presence of ribonuclease is indicated.

A further experiment showed no effect of ribonuclease on incorporation. These results are presented in Table 13.

The effect of chloramphenicol and ATP on amino acid incorporation by purified nuclear fraction

Experiments were done in which the effect of chloramphenicol and ATP, alone and in combination, on amino acid incorporation by the nuclear fraction was studied. These were carried out to determine whether the inhibitory effect of chloramphenicol on nuclear fraction might be due to interference with energy production. It was also felt these experiments might give some indication as to whether results to be reported below, in which an alleviation of inhibition by chloramphenicol of amino acid incorporation by the nuclear fraction in the "nuclei plus mitochondria" preparation were due simply to the possible function of mitochondria as an energy-generating source. Incubation mixtures were prepared in the usual way, including 0.5 mg nuclear fraction as protein

TABLE 13

The effect of Ribonuclease A on the incorporation of ^{3}H-leucine by 5-day chick embryo purified nuclear fraction*

	Incorporation of ^{3}H-leucine (cpm/aliquot ± S.D.)	Incorporation as a percentage of control
Experiment I		
Nuclear fraction	662 ± 78	100
Nuclear fraction + 200 µg RNase A	5,107 ± 70	770
Experiment II		
Nuclear fraction	2,442 ± 119	100
Nuclear fraction + 200 µg RNase A	4,870 ± 100	200
Nuclear fraction + 200 µg RNase A added at end of incubation	3,906 ± 197	160
Experiment III		
Nuclear fraction	3,072 ± 213	100
Nuclear fraction + 200 µg RNase A	2,990 ± 47	98

* Each reaction mixture contained nuclear fraction, 0.5 mg as protein, 5 µCi ^{3}H-leucine sp. act. 5.5 ci/mmole, and other components as described in "Methods" (Refer to Figure 11). Values are the average of three determinations and are corrected for blank values.

alone and nuclear fraction plus 100 µg chloramphenicol or 10^{-3} M ATP alone or in combination. The relevant blank samples were prepared with boiled nuclear fraction. ATP was found to have a slight stimulatory effect alone, but did not relieve inhibition by chloramphenicol. On the contrary, this inhibition was more pronounced in the presence of ATP. These results are shown in Table 14.

The final group of experiments was concerned with the characterization of the product of amino acid incorporation into the nuclear fraction. The possibility that the labeled amino acid was simply added to the termini of pre-formed proteins, rather than being incorporated into internal positions of proteins synthesized de novo, was investigated.

The kinetics of release of label from a preparation of labeled nuclear protein by exopeptidases was investigated in order to distinguish between these possibilities. If amino acid were incorporated internally, one would expect to find a gradual release of label in the course of exopeptidase digestion.

As a source of a control internally labeled protein preparation, an E. coli methionine auxotroph was grown in medium containing ^{35}S-methionine. A crude phenol extract of protein was prepared and dialyzed against buffer to remove phenol.

Nuclear proteins were separated from a labeled preparation of nuclei as described in "Methods".

TABLE 14

The effect of chloramphenicol and ATP, alone and in combination, on the incorporation of ^{3}H-lysine by purified nuclear fraction of 5-day chick embryo*

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot ± S.D.)	Incorporation as a percentage of control value
Nuclear fraction	1,636 ± 37	100
Nuclear fraction + 10^{-3} M ATP	1,808 ± 462	110
Nuclear fraction + 100 µg chloramphenicol	1,171 ± 148	71
Nuclear fraction + ATP + chloramphenicol	971 ± 104	59

* Each reaction mixture contained nuclear fraction, 0.5 mg as protein, 10 µCi ^{3}H-lysine, and AMO Medium. Procedures were as described in "Methods" and the legend to Figure 11. Each value represents the average of three determinations.

Both E. coli and nuclear proteins were subjected to digestion by leucine aminopeptidase and carboxypeptidase A. The results shown in Figure 14 are those of an experiment in which total ^{3}H-labeled nuclear protein and total E. coli ^{35}S-methionine labeled protein were digested with both leucine aminopeptidase and carboxypeptidase A. Earlier experiments in which classes of nuclear proteins were isolated and digested separately gave much the same pattern of results, though the level of radioactivity was considered too low, particularly in the histone fraction, to adequately demonstrate the process of digestion.

Methionine remains the N-terminal amino acid of many completed E. coli proteins, and, as might be expected, the largest initial drop in radioactivity remaining in acid-insoluble material took place in the ^{35}S-methionine E. coli labeled protein + leucine aminopeptidase mixture. All the curves indicate, however, that the label in both preparations of protein is found in internal positions. The nuclear material, as well as the E. coli protein, is shown to have a "resistant core", from which the label is released at a reduced rate.

Electrophoresis of nuclear protein

Polyacrylamide gel electrophoresis of total nuclear protein was performed on 10% polyacrylamide-0.1% SDS gels.

The gels were stained with Coomassie Blue and were sliced into 0.25 cm sections, which were placed into separate scintillation vials containing 10 ml toluene-"Omnifluor"-3% Protosol. Protein staining revealed material excluded from the gel, a slight band at 1.0 cm, and a broad band at 3.75 - 4.5 cm. The radioactivity essentially paralled the stain, as may be seen in Figure 15.

Acidic nuclear proteins were extracted from labeled preparations and subjected to SDS gel electrophoresis. A scan of the stained gel incorporating results yielded by determination of radioactivity of slices of an identical gel is shown in Figure 16.

In summary, the nuclear fraction has been isolated and characterized. The purified fraction is inhibited in amino acid incorporation by puromycin and chloramphenicol and unaffected by cycloheximide. The incorporation is also resistant to inhibitors of respiration and ribonuclease. Gel electrophoresis of the acidic protein fraction of the labeled nuclear preparation indicates two radioactive proteins.

The results of a series of experiments on a subcellular fraction containing nuclei and mitochondria will now be reported. We were primarily interested in the characteristics of the nuclear fraction, but felt that, in view of the involvement of the mitochondria in the synthesis of heme, some studies of incorporation of amino acid by mitochondria should be done.

Figure 14 The digestion of E. coli and chick embryo nuclear proteins by leucine aminopeptidase and carboxypeptidase A*

●——● ^{3}H-labeled nuclear protein digested with leucine aminopeptidase

x___x ^{3}H-labeled nuclear protein digested with carboxypeptidose A

●----● ^{35}S-labeled E. coli protein digested with leucine aminopeptidase

x----x ^{35}S-labeled E. coli protein digested with carboxypeptidase A

* Reaction mixtures were prepared as described in "Methods" and incubated at 37°C. Aliquots were taken for the determination of radioactivity at the specified time intervals.

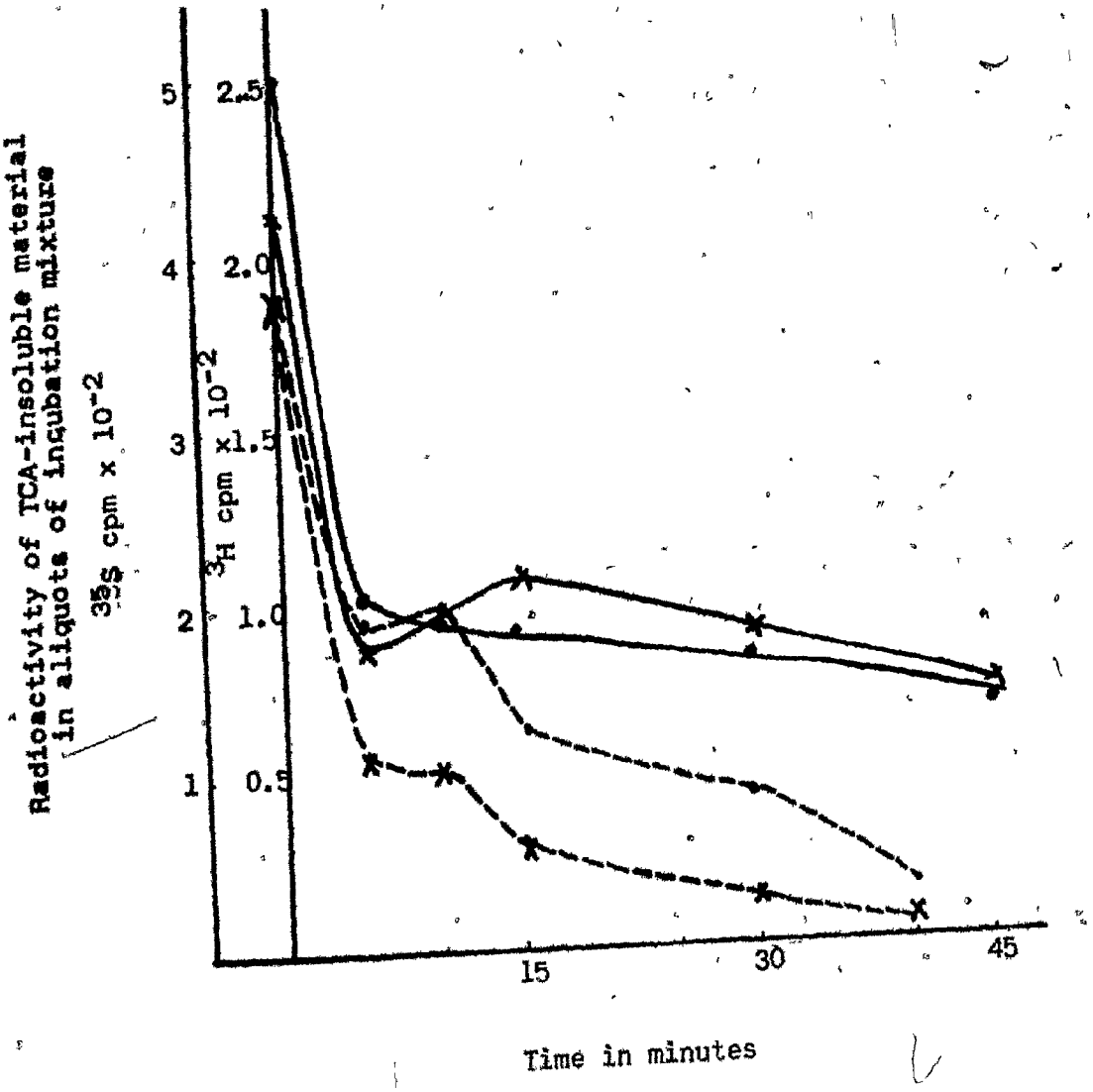

Radioactivity of TCA-insoluble material in aliquots of incubation mixture
^{35}S cpm x 10^{-2}
^{3}H cpm x 10^{-2}
5
4
3
2
1
2.5
2.0
1.5
1.0
0.5
15
30
45
Time in minutes

Figure 15 SDS Polyacrylamide gel electrophoresis of ^{3}H- labeled total nuclear proteins

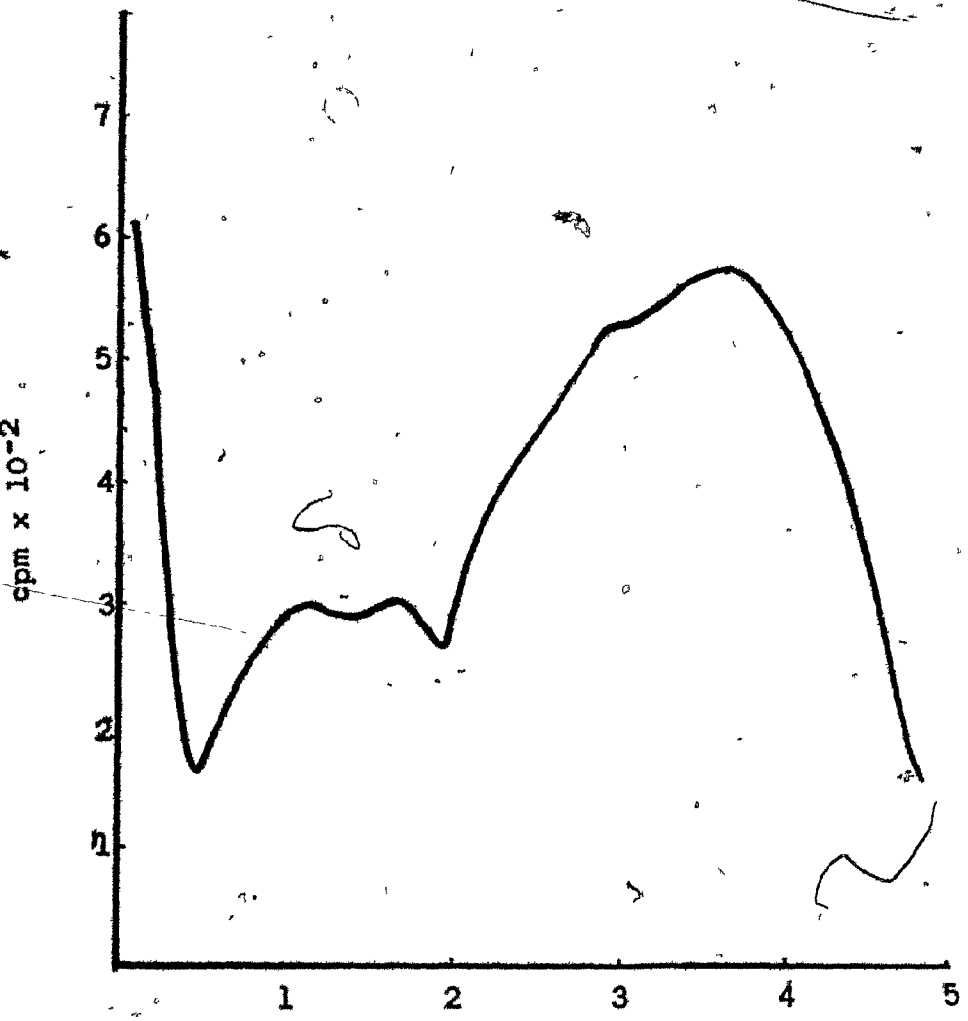

cpm x 10⁻²
7
6
5
4
3
2
1
1
2
3
4
5
Length of gel
cm from origin

Figure 16a Photograph of stained SDS polyacrylamide gel of ^{3}H-labeled chick embryo nuclear acidic proteins

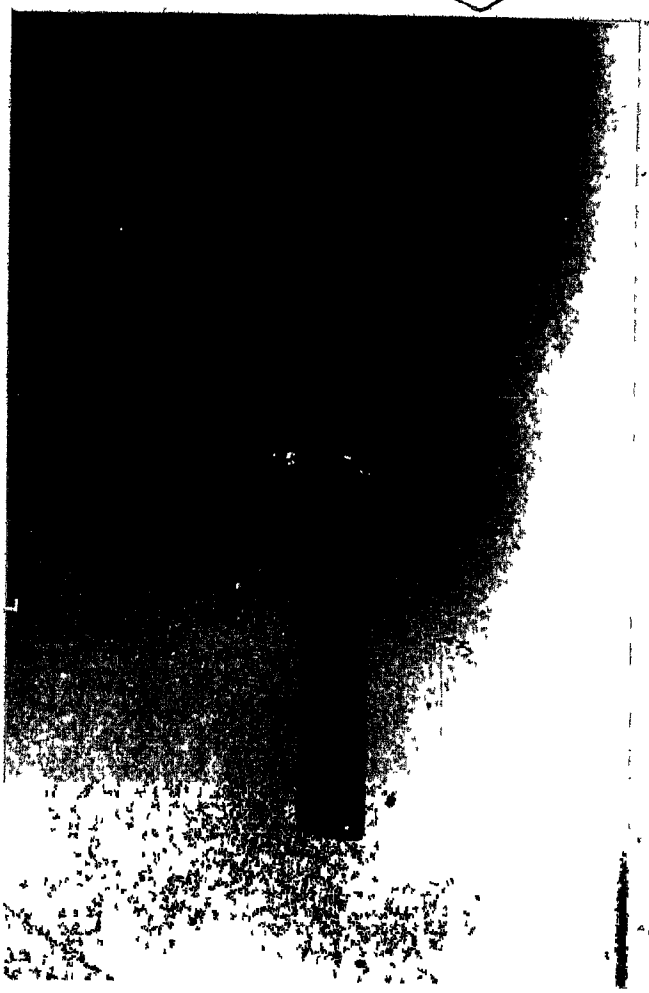
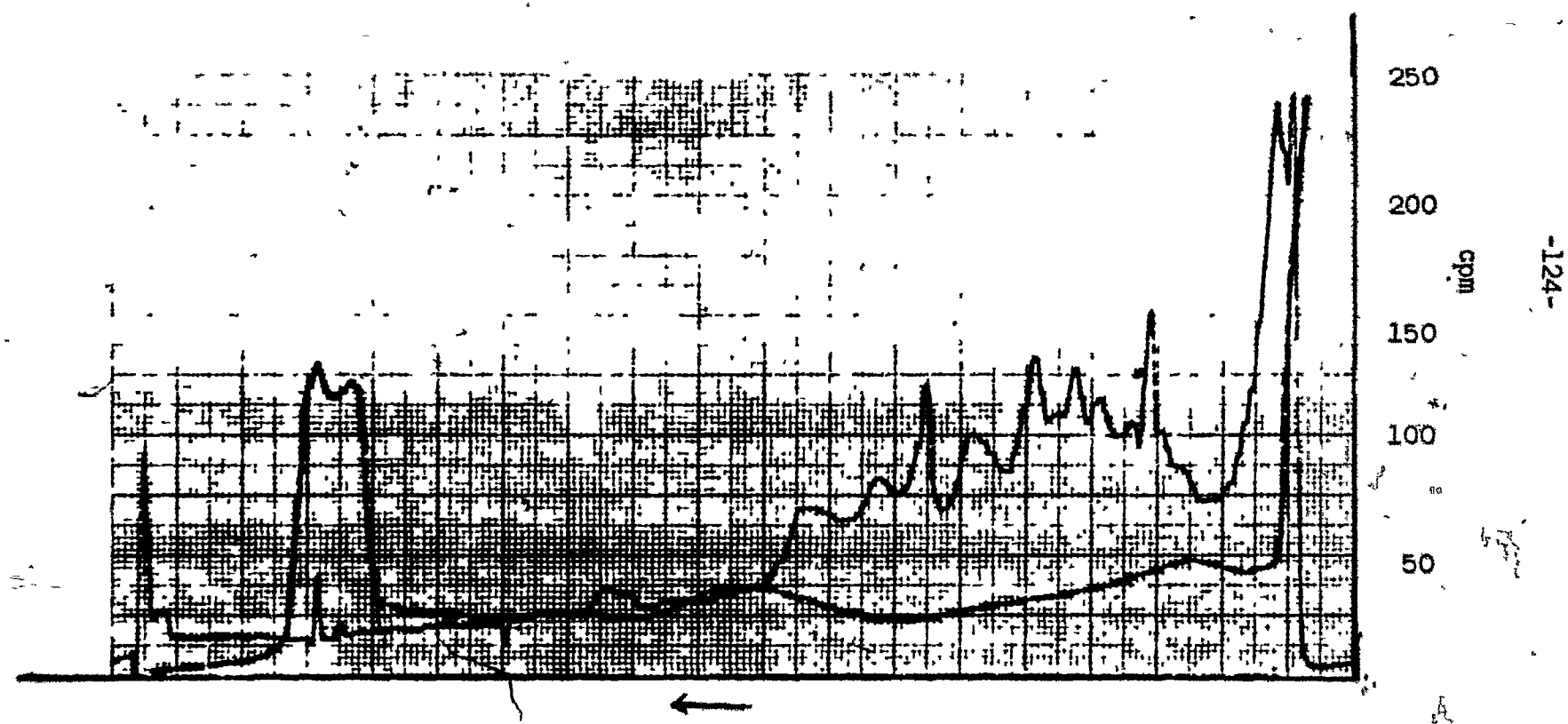


Figure 16b Scan of stained SDS polyacrylamide gel bearing ^{3}H-labeled chick embryo nuclear acidic proteins*

_____ Absorption of protein stain

°-------- Radioactivity of gel slices- cpm in ^{3}H

* Scale 1.5:1 cm


250
200
150
100
50
cpm
direction of electrophoresis

F. Amino acid incorporation by combined nuclear and mitochondrial fractions of chick embryo

Experiments were done in which a particulate fraction of the embryo homogenate, containing the nuclei and mitochondria, was incubated with ^{3}H-lysine. Enzyme assays for lactate dehydrogenase and glucose-6-phosphatase were done on the whole homogenate and this fraction, and the fraction was washed until the activity of either enzyme was no higher than 5-7% of that of the whole homogenate. Two washes were usually found to suffice.

The incorporation of ^{3}H-lysine by the supernatant fraction obtained in the initial isolation step was determined in two cases and found to range from 50-80% of the control nuclear value. No radioactive material could be recovered if this supernatant incubation mixture was treated by the procedures used for isolation of the two subcellular fractions after incubation. The incubation medium used is not optimal for amino acid incorporation by the microsomal fraction (139) or for the mitochondria (133). No attempt was made in these experiments to improve the level of amino acid incorporation by the mitochondria by substitution of the medium, because the incorporation of amino acid by the nuclear fraction was of primary interest.

Incorporation of ^{3}H-lysine by crude "nuclei plus mitochondria" fraction

The crude "nuclei plus mitochondria" fraction was isolated as described in "Methods" and incubated with 25 μCi ^{3}H-lysine and non-radioactive amino acids in AMO Medium. After incubation, the nuclear and mitochondrial fractions were isolated as described in "Methods", and the radioactivity, incorporated during joint incubation of the two fractions, was determined for each separately. Table 15 shows the results of two experiments in which amino acid incorporation by the components of this fraction was determined.

The effect of an exogenous energy-generating system on ^{3}H-lysine incorporation by mixed nuclear and mitochondrial fractions

Studies of the effect of an ATP-generating system were done on mixed nuclear and mitochondrial fractions for purposes of comparison of the response to isolated crude nuclear fraction (Table 6) and the nuclear portion of this mixture to an exogenous energy source. Nuclear and mitochondrial fractions were prepared from 4-day embryo, mixed in 1/1 proportions, and incubated with 10.3 μCi ^{3}H-lysine as described previously with the addition of 6 μmole phosphocreatine and 2.5×10^{-3} mg creatine kinase. The variation in radioactive lysine incorporation between duplicate samples was very high in these experiments, but there is an indication that an exogenous energy-generating

TABLE 15

The incorporation of ^{3}H-lysine by "nuclei plus mitochondria" fraction of 4-day chick embryo*

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot) ± S.D.	(net cpm)
Experiment I		
Blank.	2,122 ± 18	
Nuclear fraction	7,347 ± 722	5,225
Blank	393 ± 82	
Mitochondrial fraction	828 ± 124	435
Experiment II		
Blank	1,611 ± 22	
Nuclear fraction	5,423 ± 602	3,812
Blank	333 ± 64	
Mitochondrial fraction	703 ± 115	370

* Each reaction tube contained "nuclei plus mitochondria" fraction of 0.75 g wet weight whole 3-day embryo, 25 μCi ^{3}H-lysine, 19 non-radioactive amino acids and other components of AMO Medium as described in "Methods". After incubation, the tubes were chilled and the nuclear and mitochondrial fractions were isolated as described previously. The radioactivity of the isolated fractions was then determined.

TABLE 16

The effect of an exogenous energy-generating system on incorporation of ^{3}H-lysine by mixed nuclear and mitochondrial fractions of 4-day chick embryo*

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot) ± S.D.	(net cpm)	Incorporation as a percentage of control ± S.D.	(net cpm)
Experiment I				
Nuclear fraction				
Blank	246 ± 147		22 ± 12	
Control	1,143 ± 21	897	100	
Energy-generating system	2,402 ± 695	2,156	210 ± 60	240.3
Mitochondrial fraction				
Blank	307 ± 170		18 ± 10	
Control	989 ± 199	682	100	
Energy-generating system	1,677 ± 944	1,370	170 ± 74	200.8
Experiment II				
Nuclear fraction				
Blank	250 ± 143		19 ± 8	
Control	1,292 ± 328	1,042	100	
Energy-generating system	1,709 ± 685	1,459	132 ± 53	140.0
Mitochondrial fraction				
Blank	453 ± 289		51 ± 18	
Control	887 ± 30	434	100	
Energy-generating system	1,266 ± 208	813	142 ± 24	187.3

* The nuclear and mitochondrial fractions obtained from 0.83 g wet weight of 4-day embryo were mixed in 1/1 proportions and with 10.3 μCi ^{3}H-lysine as described in "Methods" with the addition of 6 μmole phosphocreatine and 2.5 x 10^{-3} mg creatine kinase. The fractions were then reisolated. Refer to Table 15 for methods. Each value represents the average of two determinations.

TABLE 17

The effect of cycloheximide on ^{3}H-lysine incorporation by "nuclei plus mitochondria" fraction of 4- to 5-day chick embryo*

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot (net cpm)		Incorporation as a percentage of control corrected for blank	
Experiment I				
Nuclear fraction				
Blank	64		3.3	
Control	1,907	1,843	100.0	
100 µg/ml Cycloheximide	316	252	13.5	13.6
Mitochondrial fraction				
Blank	38		16.8	
Control	226	188	100.0	
100 µg/ml Cycloheximide	192	154	85.0	81.9
Experiment II				
Nuclear fraction				
Blank	202		11.4	
Control	1,758	1,556	100.0	
100 µg/ml Cycloheximide	657	455	37.3	29.2
Mitochondrial fraction				
Blank	79		15.5	
Control	508	429	100.0	
100 µg/ml Cycloheximide	392	313	77.1	72.9
Experiment II				
Nuclear fraction				
Blank	449		1.8	
Control	25,397	24,948	100.0	
100 µg/ml Cycloheximide	9,883	9,434	38.8	37.8
Mitochondrial fraction				
Blank	140		11.3	
Control	1,230	1,090	100.0	
100 µg/ml Cycloheximide	890	750	72.3	68.8

* Samples of the "nuclei plus mitochondria" fraction of 5 g wet weight 4- to 5-day chick embryo were incubated with 10.3 µCi ^{3}H-lysine in AMO Medium as described in "Methods". Other procedures are as described in Table 15.

system stimulated radioactive lysine incorporation by both the nuclear and mitochondrial fractions. The results are given in Table 16.

The effects of cycloheximide and puromycin on ^{3}H-lysine incorporation by "nuclei plus mitochondria" fraction

Experiments were carried out in the same manner as described previously to determine the effects of cycloheximide and puromycin on amino acid incorporation by "nuclei plus mitochondria" fraction. Cycloheximide inhibited incorporation of radioactive lysine into both the nuclear and mitochondrial fractions of 4- to 5-day chick embryo as shown on Table 17. The results also show great variation in the amount of incorporation of radioactive lysine by a given quantity of material in separate experiments. Inhibition of the mitochondrial fraction by cycloheximide gives further evidence that the fraction was not adequately purified by the method used. It has been shown previously that cytoplasmic fraction reserved during preparation of purified nuclear fraction does not incorporate amino acid. This might have been due to inactivation of the fraction on standing due, for example, to degradation of RNA or ATP. As noted on page 125 the supernatant fraction obtained in these experiments was found to incorporate amino acid to some extent. If the abolition of incorporation into the cytoplasmic fraction is due to endogenous soluble ribonucleases,

and these ribonucleases are removed during purification of the nuclear and mitochondrial fraction more completely than are contaminating cytoribosomes, some increased level of incorporation due to these cytoribosomes might be observed.

The effects of puromycin were not studied extensively, and the results given in Table 18 are not felt to be conclusive. The large variation into the fraction may reflect differences in the degree of disruption of the nuclear fraction, or the age of the embryonic material.

The effect of chloramphenicol on amino acid incorporation by "nuclei plus mitochondria" fraction

The studies on the effect of inhibitors on amino acid incorporation by the "nuclei plus mitochondria" fraction were continued with experiments in which chloramphenicol was used. Incubation of the fraction was carried out in the usual manner in the presence and absence of 100 µg/ml chloramphenicol.

Chloramphenicol did not inhibit the incorporation of ^{3}H-lysine by the nuclear fraction of the "nuclei plus mitochondria" fraction, although the incorporation of lysine by the mitochondrial fraction was inhibited by 75% (Table 19). The observed lack of inhibition of the nuclear fraction by chloramphenicol was a surprising result in view of the findings of Trevitchick and Wainwright (144), who

found an almost total inhibitory effect of chloramphenicol on crude nuclear fraction (The data presented earlier on purified nuclear fraction had not yet been obtained.)

The effect of chloramphenicol on mixtures of purified nuclear and mitochondrial fractions

The results of the above experiments led to the hypothesis that the nuclear fraction is protected from inhibition by chloramphenicol in the presence of other components of the crude "nuclei plus mitochondria" fraction. The following experiments were done to test this hypothesis.

Purified nuclear and mitochondrial fractions were prepared, the nuclear fraction by the procedure in which 0.5% Triton is used, mixed in a 1/1 ratio, and incubated in the usual manner in the presence and absence of chloramphenicol. After incubation, the nuclear and mitochondrial fractions were again isolated and the radioactivity determined. The results were comparable to those of experiments in which "nuclei plus mitochondria" fraction was used. In both cases, the nuclear fraction was not inhibited by chloramphenicol. The results of three experiments are given in Table 20.

Determination of protein in recovered fractions

In one of the experiments on incorporation of amino acid by "nuclei plus mitochondria" fraction of 4-day chick

TABLE 18

The effect of puromycin on ^{3}H-lysine incorporation by "nuclei plus mitochondria" fraction of 3-day and 4-day chick embryos*

Puromycin concentration (M)	Incorporation of ^{3}H-lysine as percentage of control
Experiment I	
3-day chick embryo	
Nuclear fraction	
0	100.0
10^{-3} M	8.2
5×10^{-4} M	19.8
10^{-5} M	76.5
Mitochondrial fraction	
0	100.0
10^{-3} M	15.9
5×10^{-4} M	33.0
10^{-5} M	-
Experiment II	
4-day chick embryo	
Nuclear fraction	
0	100.0
5×10^{-4} M	37.6
10^{-5} M	94.0
Mitochondrial fraction	
0	100.0
5×10^{-4} M	12.3
10^{-5} M	12.5

* Each reaction mixture in Experiment I contained "nuclei plus mitochondria" fraction of 0.82 g wet weight 3-day chick embryo, and in Experiment II, 0.5 g of 4-day chick embryo. Other components included 25 µCi ^{3}H-lysine and AMO incubation medium as described in "Methods". Refer to Table 15 for standard methods.

TABLE 19

The effect of chloramphenicol on amino acid incorporation by "nuclei plus mitochondria" fraction of 4-day chick embryo*

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot) ± S.D.	Incorporation as a percentage of control corrected for blank
Experiment I		
Nuclear fraction		
Control	1,907 ± 53	100.0
100 µg/ml Chloramphenicol	1,801 ± 196	95.0 ± 10.0
Mitochondrial fraction		
Control	2,880 ± 428	100.0
100 µg/ml Chloramphenicol	605 ± 86	21.0 ± 3.0
Experiment II		
Nuclear fraction		
Control	2,270 ± 11	100.0
100 µg/ml Chloramphenicol	2,067 ± 315	91.0 ± 13.0
Mitochondrial fraction		
Control	2,956 ± 642	100.0
100 µg/ml Chloramphenicol	592 ± 102	20.0 ± 0.5
Experiment III		
Nuclear fraction		
Control	3,569 ± 152	100.0
100 µg/ml Chloramphenicol	4,992 ± 287	140.0 ± 8.0
Mitochondrial fraction		
Control	2,681 ± 79	100.0
100 µg/ml Chloramphenicol	928 ± 244	34.0 ± 6.0

* Each reaction tube contained "nuclei plus mitochondria" fraction of 0.55 g wet weight 4-day chick embryo, 25 µCi ^{3}H-lysine, 19 non-radioactive amino acids and other components of AMO Medium as described in "Methods". Isolation procedure as in Table 15. Each value is corrected for blanks, and represents the average of two determinations.

TABLE 20

The effect of chloramphenicol on mixtures of purified nuclear and mitochondrial fractions of 3- to 4-day chick embryo*

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot) ± S.D.	(net cpm)	Incorporation as a percentage of control, corrected for blank
Experiment I			
Nuclear fraction			
Blank	319 ± 103		
Control	1,510 ± 22	1,191	100.0
100 µg/ml Chloramphenicol	1,486 ± 79	1,167	98.0
Mitochondrial fraction			
Blank	327 ± 43		
Control	1,591 ± 100	1,264	100.0
100 µg/ml Chloramphenicol	673 ± 73	346	27.3
Experiment II			
Nuclear fraction			
Blank	213 ± 78		
Control	829 ± 39	616	100.0
100 µg/ml Chloramphenicol	860 ± 94	647	105.0
Mitochondrial fraction			
Blank	114 ± 11		
Control	606 ± 91	492	100.0
100 µg/ml Chloramphenicol	219 ± 58	105	21.5
Experiment III			
Nuclear fraction			
Blank	560 ± 237		
Control	2,572 ± 438	2,012	100.0
100 µg/ml Chloramphenicol	2,644 ± 315	2,084	102.8
Mitochondrial fraction			
Blank	499 ± 14		
Control	1,868 ± 204	1,369	100.0
100 µg/ml Chloramphenicol	825 ± 182	326	24.0

* Purified nuclear and mitochondrial fractions from 0.3 g wet weight chick embryo were prepared, mixed in 1/1 proportions, and incubated with 10.3 µCi ^{3}H-lysine as described in "Methods". Each value represents the average of 2 determinations. Refer to Table 15 for other methods.

embryo, determinations of protein by the Lowry method (202) were done in order to determine whether chloramphenicol caused observable degradation of fractions during the incubation period. No loss of material was observed in the chloramphenicol-treated samples relative to the others. The nuclear fractions recovered from two samples each of background, control, and chloramphenicol-treated samples contained an average of 35.6 ± 0.25 mg protein per sample, and the mitochondrial fractions, 18.3 ± 0.13 mg protein per sample.

The effect of chloramphenicol on amino acid incorporation by "nuclei plus mitochondria" fraction of primitive streak and 10-somite embryos

Further experiments were done on homogenates of freshly dissected primitive streak embryos. Embryos including the extraembryonic membranes were harvested at the primitive streak stage of development. Eight to ten embryos were used in each experiment. The "nuclei plus mitochondria" fraction was prepared as previously described. This fraction was not checked for cytoplasmic contamination, due to the scarcity of material, and it was assumed from earlier experiments that contamination was no greater than 10%. A preliminary experiment was done in which 50 μCi ^{3}H-lysine was used. In later work, the fraction was incubated with 103 μCi of ^{3}H-lysine and non-radioactive amino acids in AMO Medium in the presence and absence of chloramphenicol.

TABLE 21

The effect of chloramphenicol on the incorporation of ^{3}H-lysine by "nuclei plus mitochondria" fraction of chick embryos of the primitive streak stage

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot ± net cpm)		Incorporation as percentage of control corrected for blank
Experiment I			
Nuclear fraction			
Blank	70 ± 4		20.8	
Control	335 ± 58	265	100.0	
100 µg/ml Chloramphenicol	236 ± 12	166	70.5	62.6
Mitochondrial fraction			
Blank	378 ± 89		36.6	
Control	1,032 ± 108	654	100.0	
100 µg/ml Chloramphenicol	494 ± 151	116	47.8	17.7
Experiment II			
Nuclear fraction			
Blank	98 ± 21		3.4	
Control	2,834 ± 832	2,736	100.0	
100 µg/ml Chloramphenicol	421 ± 9	323	14.8	11.8
Mitochondrial fraction			
Blank	98 ± 1		9.8	
Control	1,004 ± 76	906	100.0	
100 µg/ml Chloramphenicol	705 ± 45	607	70.2	66.9

Experiment III #

Nuclear fraction				
Blank	28.4		13.7	
Control	206.6	178.2	100.0	
100 µg/ml Chloramphenicol	128.1	99.7	62.0	55.9
Mitochondrial fraction				
Blank	65.3		15.0	
Control	433.3	368.0	100.0	
10 µg/ml Chloramphenicol	277.5	212.2	64.00	57.6

* Each sample contained "nuclei plus mitochondria" fraction of 1.3-1.6 primitive stage embryos, 103 µCi ^{3}H-lysine, and other components as described in "Methods" and Table 15. Each value represents the average of two determinations.

The "nuclei plus mitochondria" fraction of 10 embryos was prepared and incubated with 50 µCi ^{3}H-lysine and other components as described in "Methods" and Table 15.

The pattern of results was very different from that obtained with older embryos. Chloramphenicol was found to inhibit incorporation of radioactive lysine by both the nuclear and mitochondrial fractions of the mixture. The results of these experiments are shown in Table 21.

Amino acid incorporation by the nuclear fraction of embryos at the 10-somite stage of development was also found to be inhibited by chloramphenicol in the presence of mitochondrial fraction. These results are shown in Table 22.

The results reported here differ from those of experiments on embryos at later stages of development, and suggested that at some time between the 10-somite stage and the 3- to 4-day stage, the nuclear fraction becomes resistant to chloramphenicol inhibition of amino acid incorporation in vitro in the presence of the mitochondrial fraction. The results of a single experiment with 2½-day embryo, in which the nuclear fraction of "nuclei plus mitochondria" fraction was inhibited by chloramphenicol, tentatively defined the period of the switch in sensitivity within a 12- to 18-hour span.

The effect of chloramphenicol on mixtures of purified nuclear and mitochondrial fractions of chick embryos at two stages of development

The final experiments were done on isolated nuclear and mitochondrial fractions from both 2- and 5-day embryo. Equal wet weights of embryonic material of both stages of development were homogenized and the nuclear and mitochondrial

fractions purified. These fractions were combined in four ways: 2-day nuclei with 2-day mitochondria, 2-day nuclei with 5-day mitochondria, 5-day nuclei with 2-day mitochondria, and 5-day nuclei with 5-day mitochondria. After incubation by the usual methods, the nuclear and mitochondrial fractions were again isolated and the radioactivity determined. The results indicated that some component of the mitochondrial fraction of 5-day chick embryo protects the amino acid-incorporating system of nuclear fraction of both stages from inhibition by chloramphenicol. The 2-day chick embryo mitochondrial fraction protects neither nuclear fraction. As previously stated, the mitochondrial fraction is highly contaminated. Incorporation of ^{3}H-lysine by the mitochondrial fractions of both stages is inhibited by chloramphenicol. These data present further evidence for differences in response to inhibitors of protein synthesis as a function of developmental stage, and are shown in Tables 23a, b.

TABLE 22

The effect of chloramphenicol on the incorporation of ^{3}H-lysine by "nuclei plus mitochondria" fraction of chick embryo at the 10-somite stage of development*

Sample	Incorporation of ^{3}H-lysine (cpm/aliquot ± S.D.)	(net cpm)	Incorporation as a percentage of control corrected for blank	
Experiment I				
Nuclear fraction				
Blank	147 ± 47		7.5	
Control	1,956 ± 108	1,809	100.0	
100 µg/ml Chloramphenicol	1,013 ± 58	966	51.8	47.8
Mitochondrial fraction				
Blank	155 ± 45		9.3	
Control	1,664 ± 91	1,509	100.0	
100 µg/ml Chloramphenicol	961 ± 9	806	57.7	53.4
Experiment II				
Nuclear fraction				
Blank	222 ± 57		8.5	
Control	2,592 ± 249	2,370	100.0	
100 µg/ml Chloramphenicol	607 ± 1	385	23.4	16.2
Mitochondrial fraction				
Blank	306 ± 92		29.2	
Control	1,046 ± 381	740	100.0	
100 µg/ml Chloramphenicol	552 ± 12	246	52.7	33.2

* Each sample contained "nuclei plus mitochondria" fraction of 1.3-1.6 10-somite embryos, 103 µCi ^{3}H-lysine, and other components as described in "Methods" and Table 15. Each value represents the average of two determinations.

TABLE 23a

The effect of chloramphenicol on the incorporation of ^{3}H-lysine by purified nuclear fractions of 2- and 5-day chick embryos in the presence of mitochondrial fraction of 2- or 5-day chick embryos*

Sample	Source of mitochondrial fraction of mixture	Incorporation of ^{3}H-lysine (cpm/ Experiment I	Expe
2-day chick embryo nuclear fraction	2-day chick embryo		
Blank		500.0	1-
Control		2,675.0	3
100 µg/ml Chloramphenicol		984.8	1,
	5-day chick embryo		
Blank		458.0	
Control		951.5	3,
100 µg/ml Chloramphenicol		956.2	3,
5-day chick embryo nuclear fraction	2-day chick embryo		
Blank		268.5	
Control		729.4	1,
100 µg/ml Chloramphenicol		498.6	
	5-day chick embryo		
Blank		255.6	
Control		1,390.3	2,
100 µg/ml Chloramphenicol		1,499.5	2,

* Purified nuclear and mitochondrial fractions of 2 g wet weight each of 2- and 5-day embryos were prepared, mixed in 1/1 proportions in all possible combinations, and incu with 25 µCi ^{3}H-lysine and other components as described in text. Refer to Table 15

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orporation of -lysine (cpm/aliquot) : I Experiment II	Incorporation as a percentage of control, corrected for blank Experiment I	Experiment II
1,001.6	100.0	100.0
3,646.8	22.2	13.2
1,351.7		
576.7	100.0	100.0
3,804.9	100.9	90.2
3,489.3		
391.8	100.0	100.0
1,453.6	49.9	26.9
678.4		
763.9	100.0	100.0
2,836.8	109.6	97.8
2,841.0		

2- and 5-day chick
ons, and incubated
to Table 15 for methods

TABLE 23b

The effect of chloramphenicol on the incorporation of ^{3}H-lysine by purified mitochondrial fractions of 2- and 5-day chick embryos in the presence of nuclear fraction of 2- or 5-day chick embryos*

Sample	Source of nuclear fraction of mixture	Incorporation of ^{3}H-lysine (cpm, Experiment I	Exper
2-day chick embryo mitochondrial fraction	2-day chick embryo		
Blank		125.6	
Control		792.0	1,
100 µg/ml Chloramphenicol		136.9	
	5-day chick embryo		
Blank		145.1	
Control		325.3	1,
100 µg/ml Chloramphenicol		188.8	
5-day chick embryo mitochondrial fraction	2-day chick embryo		
Blank		124.7	
Control		251.3	2,
100 µg/ml Chloramphenicol		172.5	
	5-day chick embryo		
Blank		252.2	
Control		848.7	1,
100 µg/ml Chloramphenicol		730.3	6

*Purified nuclear and mitochondrial fractions of 2 g wet weight each of 2- and 5-da embryos were prepared, mixed in 1/1 proportions in all possible combinations, and inc with 5 µCi ^{3}H-lysine and other components as described in text. Refer to Table 15 for

ified
nuclear

oration of sine (cpm/aliquot) Experiment II	Incorporation as a percentage of control, corrected for blank Experiment I	Experiment II
275.9		
1,478.9	100.0	100.0
425.1	1.6	12.4
397.1		
1,470.9	100.0	100.0
621.0	24.2	20.8
377.7		
2,172.2	100.0	100.0
910.1	37.7	29.6
535.5		
1,354.6	100.0	100.0
637.4	80.1	12.4

and 5-day chick
s, and incubated
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Discussion

Preliminary characterization of the nuclear fraction

The original aims of this study were to develop optimal incubation conditions for the incorporation of amino acids by chick embryo nuclear fraction and to study the effects of various inhibitors of protein synthesis on this process. As noted above, Trevithick and Wainwright made preliminary studies of amino acid incorporation by chick embryo "nuclear fraction" (144). They noted a great degree of variability among fractions in the level of amino acid incorporation, and attempts were made in this work to devise conditions of isolation and incubation of the nuclear fraction that would yield more consistent results.

The early experiments done in this work on the level of incorporation of amino acids by the crude nuclear fraction described in "Methods" showed very high variability. The substitution of the nexium described by Allfrey, Mirsky, and Osawa (AMO Medium) (141) for minimal medium used by Trevithick and Wainwright was shown to result in a much higher average level of incorporation of radioactive lysine by chick embryo nuclear fraction. The degree of variability among fractions was also reduced.

The level of incorporation of amino acid by calf thymus nuclei has been shown by Allfrey, et. al. to be

highly dependent on the relative proportions of sodium and potassium in the incubation mixture (141). This ratio differed greatly in the two media used in this work, and the change may have been of critical importance to the finding of increased amino acid incorporation by the nuclear fraction in AMO Medium.

The other components of the two incubation media were present in essentially the same amounts, with the exception of sucrose. Trevithick and Wainwright did not find any effect of added sucrose on the stability of the nuclear fraction or on the variability of the extent of amino acid incorporation. Determinations of the level of incorporation of amino acid by crude nuclear fraction in minimal medium plus sucrose were not repeated in this work.

The two media differed in pH by 0.4 units. No systematic study was made of the possible effects of this variation, or of the variation of the Na^+/K^+ ratio, as the emphasis of the work later shifted from this sort of study.

The procedure for isolation of the nuclear fraction differed from that of Trevithick and Wainwright (144) only with respect to the homogenization medium, which contained sucrose. Calcium chloride was present in both media. These compounds are generally considered to contribute to the stability of the nuclear fraction. Work by Incefy and Kappas (205) has indicated that divalent ions affect

the stability of nuclei isolated from chick embryo liver, and that magnesium has a greater stabilizing effect than does calcium. With the exception of the substitution of calcium by magnesium in the isolation procedure of these authors, the method is very similar to that described in this work. Some evidence is provided in the present study that an increased concentration of magnesium may enhance the extent of amino acid incorporation by the nuclear fraction.

The effects of sucrose and divalent ions on the stability of the isolated nuclear fraction and on the incorporation of amino acids by this fraction were not studied independently nor were they distinguished from the effect of variation of Na^+/K^+ ratio on amino acid incorporation.

The variability of the extent of amino acid incorporation by different preparations of nuclear fraction observed by Trevithick and Wainwright (144) was not entirely overcome in these studies (Table 11). There is good presumptive evidence that much of the variability observed in the course of this work was due to effects such as cold shock of the fertilized eggs during transport. Most of the embryo homogenates prepared during the winter months (October to April) of 1970-1971 showed negligible lactate dehydrogenase activity and amino acid incorporation, although morphological development was observed to be only

moderately retarded. Similar observations were made by Garside on the effects of cold shock of 1-day fish embryos (206).

The effect of an exogenous energy source on amino acid incorporation by isolated nuclei varies with the source of the nuclei, as has been noted in the "Literature Review". Most of the experiments described in this work indicate that addition of ATP or an energy-generating source enhances amino acid incorporation by both crude and purified nuclear fraction of chick embryo (Tables 6,14,16).

There is some precedent for the finding that ATP at 2 mM concentration inhibited amino acid incorporation by the whole homogenate (Table 6). Pronczuk, Baliga, and Munro (207) found that the synthesis of protein by a rat liver cell-free system could be inhibited by a high ATP concentration (2 mM and above), and that a twofold higher Mg^{++} level only partially overcame this inhibition. An increased concentration of ATP was found to inhibit GTP hydrolysis and to cause progressive disaggregation of polysomes. The concentrations of ATP and Mg^{++} used in the present work were within this order of magnitude. Similar results were reported by Allen and Schweet (208).

The "nuclei plus mitochondria" system

Wainwright found that the effects of inhibitors of protein synthesis on the onset of hemoglobin synthesis

in explanted chick blastodiscs indicated the participation of a non-cytoplasmic protein-synthesizing mechanism (169). The nuclei were of major interest to the author, but as the mitochondria also synthesize proteins, some investigation of this fraction was undertaken.

A low-speed particulate fraction containing both organelles, described earlier as "nuclei plus mitochondria" fraction, was therefore used in several experiments. After incubation with radioactive amino acid, the nuclei and mitochondria were isolated and the radioactivity determined.

The effects of cycloheximide and puromycin on incorporation of amino acid by the "nuclei plus mitochondria" fraction were studied (Tables 17,18). Inhibition of incorporation by the nuclear fraction was found in both cases to be higher than that observed by Trevithick and Wainwright (Table 18). These results may be due to possible effects of differences in the isolation procedure prior to incubation. Trevithick and Wainwright stated that impermeability of their nuclear fraction to puromycin might account for the low degree of inhibition they observed. The activity of the nuclear fraction isolated after incubation of the "nuclei plus mitochondria" was found to be inhibited by cycloheximide, though the degree of inhibition varied widely (Table 17). Later results of experiments on purified nuclear fraction seem to indicate that the observed effect of cycloheximide

in these experiments was on contaminating cytoplasmic components.

In the experiments on the mixed fraction, cycloheximide inhibited amino acid incorporation by the mitochondrial fraction by 15-30%, and puromycin, by 66% (Tables 17,18). These figures are comparable to those given by Beattie (132) and Kroon (137).

The effect of chloramphenicol on the mixed fraction was studied. It was expected, on the basis of preliminary results of Trevithick and Wainwright (144), that the incorporation of amino acid by the nuclear fraction would be found to be greatly reduced. The results shown in Table 19 were therefore rather surprising. No effect of chloramphenicol on the nuclear portion of the "nuclei plus mitochondria" was observed. This experiment was repeated several times and yielded the same result. In contrast, a preliminary experiment with nuclear fraction isolated with the use of 0.5% Triton-X-100 showed complete inhibition of incorporation in the presence of chloramphenical. Further study of this phenomenon was undertaken, with the working hypothesis that a component of the "nuclei plus mitochondria" fraction protects the nuclear fraction from inhibitory effects of chloramphenicol.

Further experiments were done to determine the effects of chloramphenicol on isolated fractions of both nuclei and mitochondria (Table 20). These fractions were assayed

for activity of marker enzymes and found to be essentially free of cytoplasmic contamination. Chloramphenicol inhibited amino acid incorporation into initial preparations of isolated nuclear fraction almost completely and into isolated mitochondrial fraction by 66%. Pre-purified fractions were mixed and incubated with amino acid. The nuclear fraction, when mixed with the mitochondrial fraction and incubated with radioactive lysine in the presence of chloramphenicol, incorporated amino acid to the same extent as did the control sample (Table 20, Expt. III).

Studies of the incorporation of radioactive lysine by the "nuclei plus mitochondria" fraction of earlier embryos in the presence and absence of chloramphenicol yielded very different results from those on 4- to 5-day embryos. The nuclear fraction of primitive streak and 10-somite embryos was found to be partially inhibited by chloramphenicol in the presence of the homologous mitochondrial fraction. It seems clear, from the results of the final experiments in which purified nuclear and mitochondrial fractions were prepared from embryos of the 2-day and 4- to 5-day stages and mixed in all possible combinations, that the "mitochondrial" fractions of the chick embryo develops, at about the 3- to 4-day stage, the ability to protect the amino acid-incorporating system of the chick embryo nuclear fraction from the inhibitory effects of chloramphenicol (Tables 23 a,b).

Electron microscopic examination of mitochondrial fraction prepared by the method described earlier revealed that the fraction was highly contaminated by other cytoplasmic components. Other standard methods of isolation were attempted and found to lead either to incomplete purification or destruction of the fraction, so the attempt was abandoned.

The drug-metabolizing activities of cells are, in general, resident in the microsomal fraction, and it may be that contaminating microsomes in the mitochondrial fraction inactivated the chloramphenicol in the mixed experiments, yielding the observed lack of inhibition of nuclear fraction. The finding that the ability of the fraction to effect this protection varies with developmental stage may be compared to studies of drug metabolism in humans. Infants have two chloramphenicol-metabolizing enzymatic activies, one of which is found in the neonate. The other develops at some time after birth (209). An analogous mechanism might be operative in the chick embryo.

It is not felt that the protective effect is due simply to the release of energy-yielding compounds from the mitochondria in response to chloramphenicol, perhaps as the result of mitochondrial breakdown. Later experiments on the combined effects of ATP and chloramphenicol on the incorporation of amino acid by purified nuclear fraction

gave results indicating that ATP enhances the inhibitory action of chloramphenicol, perhaps by stimulating entry of the drug into the fraction.

It would be of importance to clarify the relationship of the phenomena studied in these experiments to the onset of hemoglobin synthesis in the chick. As previously noted, Wainwright has shown the importance of a non-cytoplasmic protein-synthesizing system to the synthesis of hemoglobin. There is some evidence that a shift to relative insensitivity to chloramphenicol occurs around the 8- to 10-somite stages of development of the chick embryo (169).

Further attempts to obtain a highly purified preparation of mitochondria were not successful, however, and plans to repeat this work were abandoned in favor of a more comprehensive characterization of the activity of the purified nuclear fraction.

Characterization of the purified nuclear fraction

It was considered to be of great importance to demonstrate that the method of isolation of nuclear fraction yields a highly purified preparation. Nuclei prepared by the earlier method (i.e., with 0.5% Triton-X-100) were assessed by assay of marker enzymes as has been described. Later preparations of nuclei isolated with the use of 1.0 % Triton were examined by light and electron microscopy, enzymatic and chemical methods.

The activities of the non-nuclear enzymes glucose-6-phosphatase and cytochrome oxidase observed in samples of purified nuclear fraction were of the same order as those found by Anderson, et. al. (152) in preparations of purified rat prostate nuclei and lower than those determined by Incefy and Kappas (205) in preparations of chick embryo liver nuclei (see page 103). The latter authors did not remove the outer nuclear envelope with Triton-X-100 or another such agent, as was done in most of the present experiments.

The electron micrographs show no contamination of the nuclear fraction by cytoplasmic components with the exception of collagen fibrils, which were observed in one field. No perinuclear ribosomes are present (Figure 9).

The possibility of bacterial contamination which could contribute to the observed levels of amino acid incorporation by isolated nuclear fraction was explored in several experiments. Aliquots of the nuclear incubation mixture, when plated on blood agar, did not give rise to enough bacterial colonies to indicate any substantial contribution to the observed level of incorporation by bacterial contamination (152).

Experiments were done in which the nuclear fraction was artificially contaminated with large quantities of cytoplasmic fraction. Rather than contributing to the

observed level of amino acid incorporation, added cytoplasm inhibited incorporation into TCA-insoluble material. Some rationale for this finding has been discussed previously. It is felt that any low level of cytoplasmic contamination remaining in nuclear preparations could not have contributed substantially to the results. Cycloheximide was not found to inhibit amino acid incorporation into nuclear fraction prepared with 1.0 % Triton-X-100. This result contrasts with that obtained with the mixed fraction (Table 17) and was felt to give further evidence that cytoplasmic amino acid incorporation does not occur in the former preparation.

Anderson (152) has noted that relatively high concentrations of inhibitors are necessary to affect nuclear amino acid incorporation, presumably because purified nuclei are rather impermeable to them. This observation is supported by the present work, in which a high concentration of puromycin (10^{-1} M) was shown to be required to suppress amino acid incorporation by purified nuclear fraction. Disruption of the nuclear fraction by sonication rendered it more susceptible to inhibition of amino acid incorporation by puromycin (Figure 13).

Experiments on the effect of chloramphenicol on nuclear fractions purified with 1.0% Triton-X-100 did not show a complete inhibition of amino acid incorporation (Table 11) as did experiments mentioned previously.

This may reflect differences in stability of the preparations either due to the method of isolation, or variation in the strain of chicks used. It has been mentioned previously that several investigators in this department have found differences in several parameters from strain to strain, for example, enzymatic activity. At one point in the course of this work, shortly after a change in the flock at the suppliers, nuclear fractions were obtained which showed a consistent five-fold increase in the extent of incorporation, compared to most observations made, and which were unaffected by several different batches of chloramphenicol. Other parameters of metabolic activity were found to differ greatly among preparations of homogenates and subcellular fractions, including the activity of lactate dehydrogenase and the other marker enzymes.

The effects of RNase and of the respiratory inhibitors cyanide and dinitrophenol were studied only on the purified nuclear fraction. The concentrations used were comparable to those used by other workers who found inhibition from 30-75% (151,158,210,211). The respiratory inhibitors were not found to be effective as inhibitors of amino acid incorporation by purified nuclear fraction in this work, and, in fact, produced some stimulation. This might be further explored with higher concentrations of inhibitors and disrupted nuclear fraction.

Ribonuclease A was also found to stimulate amino acid incorporation by the nuclear fraction in some experiments, and to be ineffective as an inhibitor of amino acid incorporation in the majority of experiments. There are several precedents for this finding (141,151,158,159,210,211). The nuclear fraction may simply be impermeable to ribonuclease. It was hoped that the inhibitory effect of cytoplasmic fraction on incorporation of amino acid by the nuclear fraction might be attributed to the action of endogenous ribonucleases, but the results of the present experiments with ribonclease A do not allow such a simple interpretation.

It was possible to demonstrate that the incorporation of radioactive amino acid by nuclear fraction bears a linear relationship to the amount of fraction incubated. A linear uptake of amino acid as a function of time was also observed.

These results as a whole seemed to demonstrate that amino acid incorporation by nuclear fraction does indeed occur. It remained to be demonstrated that the amino acid is incorporated into internal positions of proteins by de novo protein synthesis. There are three lines of evidence to indicate that this is so.

The effect of puromycin on amino acid incorporation into nuclear fraction was studied in detail, as puromycin has been shown to be a general inhibitor of peptide chain elongation in all protein-synthesizing systems; prokaryotic,

eukaryotic, and that occurring in cell organelles. By the use of high concentrations or puromycin and of conically-disrupted preparations of nuclear fraction, it was possible to show a complete inhibition of amino acid incorporation by the fraction. This provided presumptive evidence that the amino acid taken up by the nuclear fraction was indeed being utilized by a protein-synthesizing system.

The pattern of digestion by exopeptidases of labeled material, recovered after incubation of the nuclear fraction by standard methods used to obtain crude protein extracts, provides a second indication that the label is incorporated into typical protein. This pattern is very similar to that observed for the digestion of labeled protein synthesized in vivo by E. coli. If the labeled amino acid were simply added to the termini of existent proteins, a rapid and complete loss of label from the TCA-insoluble fraction could have been expected very early in the course of the incubation with exopeptidase. A resistant core of labeled material was, however, found; both in the case of the E. coli protein and the nuclear labeled material.

Acidic nuclear proteins were extracted by standard procedures and examined by polyacrylamide gel electrophoresis. At least two labeled bands of material, which were stainable by Coomassie blue, were found to be radioactive. Other

non-labeled bands of protein were found on the gel. Zimmermann and co-workers (140) obtained similar results on electrophoresis of labeled material extracted from purified nuclei and nucleoli of HeLa cells after incubation with labeled amino acid. They concluded that specific protein synthesis occurred *in vivo*, both because two particular bands were also radioactive, and because other proteins were not labeled. This seemed to preclude non-specific addition of amino acid to pre-existent proteins. Similar reasoning may be applied in the case of the experiments described in this work.

It seems possible to conclude from the foregoing data that the nuclear fraction of chick embryo is able to incorporate amino acid *in vitro* and utilize it in protein synthesis.

Several speculations have been made about the possible role of nuclear protein synthesis in the living organism (159). These include suggestions that proteins synthesized in the nucleus might be involved in gene regulation in the course of differentiation, such as specific inducers or repressors of the synthesis of messenger RNAs. Amino acid incorporation into the nucleolar fraction might represent synthesis of proteins necessary for the processing of ribosomal RNA (163).

Enzymes endogenous to the nucleus, such as NAD^+ pyrophosphorylase, might be synthesized in the nucleoplasm (163).

Messenger RNA is associated with protein in the cytoplasm, which is lost on association of the messenger with ribosomal subunits (212). Such findings support the informosome concept proposed by Spirin (213), who postulates that mRNA is transported from the nucleus to the cytoplasm in association with proteins. Some or all of these proteins might be nuclear in origin.

In prokaryotes, ribosomes become associated with mRNA before the synthesis of the RNA is completed. An analogous residual coupling of transcription and translation has been proposed in eukaryotes which could account for the observed amino acid incorporation (214).

The theory that in situ synthesis of proteins concerned with RNA synthesis occurs in the nucleus is attractive to the author.

Work in this field has progressed to such an extent that one may expect prompt characterization of a protein synthesized by a well-purified nuclear fraction.

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