INVESTIGATIONS ON THE SYNTHESIS OF NUCLEIC ACID BY ISOLATED NUCLEI

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ABSTRACT

Nuclei extracted from wheat embryoes increase their nucleic acid content when incubated in a medium containing ATP, ribose, and a pyrimidine or pyrimidine precursor. The increase of nucleic acid in the nuclei consists of DNA and can be stimulated by the presence of orotic acid or by the replacement of ATP with ADP. While the DNA of the nuclei can more than double during incubation the RNA does not increase at all despite a possible three fold increase in the nuclear suspension. This is because the newly synthesised RNA is released into the medium. The chief stimulant for RNA synthesis is ascorbic acid which also causes the release of a considerable quantity of protein from the nucleus.

The energy source for RNA synthesis had to be ATP and it could not be effectively replaced by ADP or UTP. Experiments with nuclei extracted by a new non-aqueous technique showed that there is an active adenylic kinase present in wheat nuclei and work with aqueous and non-aqueous nuclei showed that ATP can be produced by glycolysis and that all the enzymes necessary to produce pyruvate from Fructose 1,6 Diphosphate were present. Pyruvate did accumulate and presumably could not be consumed by nuclei except by transaminase action.

Attempts were made to relate the RNA released by nuclei to the general pattern of RNA species known to occure in plant cells. Its high purine content sets it apart from any type previously examined from wheat but consideration of the rate of synthesis and molecular size as given by sedimentation analysis suggests that it may be a form of messenger RNA.

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INTRODUCTION

INTRODUCTION

considerable amount of evidence has been produced in the last few years showing that nuclei are able to synthesis nucleic acid. This evidence is almost entirely based on the incorporation of labelled nucleotides into nucleic acid (Rho 1961).

This thesis records work showing that nuclei isolated from wheat embryo are able to synthesise nucleic acids on a totally different scale during incubation. Both RNA and DNA can be increased to more than double the amount originally present and the rates of synthesis of each nucleic acid can be controlled to some extent by modifying the medium. The nuclei do not need to be supplied with a complete mixture of nucleotide triphosphates for synthesis to occur, but in view of the fact that they may already possess a pool of nucleotides it is not possible to say for certain that the pre-cursors that are in the medium are being incorporated into the nucleic acid.

While the synthesised DNA could all be sedimented with the nuclei after incubation, most of the RNA was released into the medium and could be examined separately. Protein could be released from the nuclei by treatment with ascorbic acid.

Following this initial stage of the investigation two other aspects were examined and recorded separately. Section B is concerned with the ability of isolated nuclei to produce ATP by glycolysis and by so doing establish a certain degree of independence of other organelles for their energy supply. Section C deals with the problem of characterising the RNA released from the nuclei during synthesis and discusses the regulation of cell metabolism by the nucleus.

Abreviations

RNA % C.	RNA expressed as a percentage of the RNA present in the zero time control.
DNA % C.	DNA expressed as a percentage of the DNA present in the zero time control.
NA % C.	Total Nucleic Acid expressed as a percentage of the total nucleic acid present in the zero time control.
mμME.	Millimicron Moles Equivalent (10^{-10} - Molar equivalents)
CTP.	Cytosine Triphosphate
UTP	Uridine Triphosphate

Materials

The wheat embryo was very kindly supplied by Dr. J. J. C. Hinton of the Research Association of British Flour-Millers. It was previously dry sieved to separate it from the bulk of the endosperm.

Chemicals were supplied mainly from BDH and were of "Analar" grade whenever possible. ATP and DPN were obtained from Sigma Chemical Co., Fructose 1, 6-Diphosphate from Boehringer, and Menadione (Vit. K_3) from nutritional biochemicals.

The Presentation of Results

Most of the results which demonstrate an increase or decrease of a compound are presented as a percentage of the ammount present at zero time. Any value less than 100% indicates a loss. To find the percentage increase 100% must be subtracted from the figures quoted. This method has the advantage of eliminating the need for negative signs to represent a decrease. Where the percentages are the mean value of duplicates or triplicates, the quantities of nucleic acid on which they are based are quoted in full in the appendix.

SECTION A

THE CONDITIONS FOR RNA SYNTHESIS

IN ISOLATED NUCLEI.

METHODS

The Preparation of Aqueous Nuclei.

The method is based on that of Johnson, Nasatir and Stern (1952) modified by the replacement of mill silk with nylon bolting silk 25μ mesh in the straining-procedure. All solutions were at 0-2°C. 10-20g of wheat embryo was ground in a coffee grinder or pestle and mortar and then stirred for ten minutes in 120 ml 2.0 M. sucrose + 1.0 mM. calcium chloride. The thick suspension was then strained successively through:-

2 layers of muslin
1 layer of nylon bolting silk
2 layers of flannelette.

Centrifugation of the strained suspension at 12,000 x G for 15 minutes gave a pellet of starch and nuclei. This pellet was suspended with a rubber 'policeman' in a supersaturated solution of sucrose (205 g sucrose + 100 g water) and centrifuged at 56,000 x G for 15 minutes in a Spinco-L centrifuge. The pellet contained starch and was discarded. The supernatant was diluted with a half volume of 0.5 M. sucrose + 3.0 mM. $CaCl_2$, giving a final concentration of 2 M. sucrose + 1.0 mM. $CaCl_2$. Centrifugation at

5,400 x G for 30 minutes gave a pellet of nuclei which was washed by layering over medium and centrifuging again at 5,400 x G for 30 minutes. Examination of the final pellet by light and electron microscopy has shown it to consist of intact and fragmented nuclei with only slight contamination of cell wall fragments and cytoplasmic debris (James and Richens 1963).

Precipitation of Nucleic Acid and Protein.

Trichloroacetic acid at 5% final concentration was routinely used for the precipitation of protein and nucleic acid. Occasionally other precipitating agents were used and the amount of RNA precipitated compared with that from the trichloroacetic acid method. 4% PCA and 80% ethanol, final concentrations were found to be satisfactory. Ethanol had the disadvantage of precipitating some of the sucrose from the incubation medium and perchloric acid is believed to degrade nucleic acid, even at 0°C (Hutchinson and Munro 1961). Slight acid degradation at this stage would make the DNA alkali-labile. No evidence for reduced DNA yield when using perchloric

acid for precipitation was ever observed. (Table I)

TABLE I

THE RNA, DNA, NA, YIELD AFTER PRECIPITATION WITH 5% PCA AND 5% TCA.

	5% PCA	5% TCA
RNA	61 mμ ME. Base	60 mμ ME. Base
DNA	37.5 mμ ME. Base	39.5 mμ ME. Base
Total NA	98.5 mμ ME. Base	99.5 mμ ME. Base

The solutions were always cooled immediately after precipitation, and after standing for 15 minutes at 0°C. they were centrifuged for a further 15 minutes also at 0°C. and at 3000 r.p.m. on an M.S.E. refrigerated centrifuge with swing-out head.

Washing of Precipitate

The final estimation of RNA, DNA and protein involved measurement of their nitrogen, phosphate, purines, pyrimidines, sugars and amino acids and so it was necessary to remove all other materials which would interfere. This was achieved by washing first in 5% TCA or 4% PCA to remove acid-soluble compounds such as nucleotides, phosphates and sugars. One wash in cold absolute alcohol removed any residual trichloroacetic acid and reduced the possibility of acid

hydrolysis during the subsequent removal of phospholipid and related substances which was achieved by washing twice with ethanol:chloroform (3:1) at room temperature and once in ethanol:ether (2:1). The final precipitate was dried with nitrogen. To facilitate the extraction procedure all operations, from incubation to the final precipitation, were carried out in stoppered centrifuge tubes of approximately 10 ml capacity.

Solubilisation of Nucleic Acid.

Initial attempts at solubilisation with 5% PCA at 90°C. for 15 minutes (Smillie and Krotkof 1960) were soon abandoned as the estimation of RNA and DNA, as distinct from total nucleic acid, was dependent on the diphenylamine reaction, and the quantities of precipitate available precluded the routine use of this reaction. The Schmidt-Thannhauser procedure of alkaline digestion was used and found satisfactory for RNA but digestion in 0.3N. KOH at 37°C. was carried out for only 90 minutes instead of 18 hours. The 60 minutes recommended by Hutchinson and Munro (1961) in their review was found to be rather near the lower time limit required for complete solubilisation of RNA. Incubation

of extracted nucleic acid for 18 hours proved that there was no further increase in U.V. absorbence or orcinol colour after the first 60 minutes. . . .
At no stage during the alkaline hydrolysis was sufficient deoxyribose released into the supernatant to give a reaction with indole.

Alkaline hydrolysis took place in a total volume of 1 ml and a precipitate of DNA and protein was obtained by addition of 4.0 ml 5% PCA followed by centrifugation at 0°C. The pellet was washed in 5% PCA.

The precipitate was then taken up in 5% PCA and incubated for 15 minutes at 90°C. The protein and potassium perchlorate were spun down and the supernatant used for DNA estimations. The use of 72% PCA at 100°C. for 60 minutes as recommended for base analysis (Markham 1955) was found to destroy the deoxyribose completely and to give rather low ultraviolet absorption values. By solubilisation of DNA with 5% PCA it was found possible to obtain values by U.V. absorption, phosphate estimation and deoxyribose estimation in close agreement. (Table II)

TABLE II.

THE RELATION OF DEOXYRIBOSE PHOSPHATE AND U.V. ABSORPTION DETERMINATIONS OF DNA.

Deoxyribose	U.V. abosrption	Phosphate
	80 mμME.	80 mμME.
534 mμME.	529 mμME..	

The Quantitative Estimation of Nucleic Acid

Base Estimation.

The purine and pyrimidine bases of RNA and DNA absorb strongly at about 260 mμ and this property forms one of the easiest and most accurate methods for estimating nucleic acid. The solutions of solubilised RNA and DNA could be read directly on a Unicam SP 500 Spectrophotometer using a 1cm. cuvette. The solution was always compared with pure solvent as control which had been treated in exactly the same way as the experimental solutions. With RNA this was 1.0 ml 0.3N potassium hydroxide + 4.0 ml 5% PCA and with DNA 5.0 ml 5% PCA. After absorption measurements aliquots of the solutions were taken for sugar or phosphate determinations, and solvent from the control cuvettes used for reagent blanks.

For routine estimations the absorption was

read at 260, 280 and 310 mμ and occasionally at 255 and 265 mμ. The peak usually lay at 260 mμ and it is on the absorption at this wavelength that most calculations are based, but when RNA synthesis was active the peak moved towards 257 mμ.

The absorption at 280 mμ gave an indication of any interference by protein derivatives. Absorption at 310 mμ was usually very low, as is to be expected with solutions of nucleic acids. Occasionally an entire spectrum was scanned to obtain information regarding the base composition of the nucleic acids present (see section III). The absorption coefficient used was based on that of Markham (1955) and was:

$$\frac{\text{Absorption value} \times 1000 \times \text{volume}}{12} = \text{m}\mu\text{ME. Base.}$$

Usually, however, the results were written as a percentage of the control at zero time. The U.V. absorption values obtained were usually more consistent than phosphate or sugar estimates and so formed the basis of calculations with either of the latter two values as confirmation.

Ribose estimation

Ribose was estimated by the orcinol method of

Markham (1955) without any modification. At least 100 mμME of RNA sugar was needed to give a reliable absorption value, and for this reason the method was only infrequently used. In addition it was found that alkaline hydrolysis liberated some material which exaggerated the apparent RNA content. Ribose estimations could be used to give a measure of RNA liberated by the nucleus or extracted from nuclei. (Table III)

TABLE III

THE RNA OF NUCLEI AND MEDIUM ESTIMATED BY THE ORCINOL AND U.V. ABSORPTION METHODS.

	U.V. absorption	Ribose estimation
RNA without nuclei	149 μg	134 μg
RNA with nuclei	290 μg	1120 μg

Deoxyribose Estimation.

Two methods were used for determining the sugar content of DNA. The diphenylamine reaction of Dishe (1930) was used at first but proved too insensitive for the quantities available and so the more sensitive though more laborious indole method of Ceriotti (1952) was used.

To 2.0 ml of DNA solution was added 1.0 ml of 0.04% indole in water and 1.0 ml of concentrated hydrochloric acid in a stoppered test tube. The solutions were well shaken before incubating at 100 °C. for 10 minutes. The pale yellow solution was extracted three times with chloroform and then the absorption of the washed solution was read at 490 mμ on a Unicam SP 500. This method requires 10-30 μg DNA. Occasionally it was necessary to centrifuge the tubes gently to obtain a completely clear aqueous phase.

Phosphate Estimation.

The method of Chen, Toribara and Warner (1956) was used almost exclusively after comparison with the method of Allen(1940) which was found to be too insensitive, and Bartlett (1959) which was less convenient and had no advantages. The ashing technique was a modification of that of the Allen method in that the phosphate was liberated by perchloric acid alone. 1 or 2 ml of RNA or DNA solution in 5% PCA was pipetted into a test tube and heated at 100°C. until the volume was about 0.5 ml. The tubes were then placed in an oven at 175°C. and left overnight, during which time they dried out completely. 4.0 ml water was added to the dry tube

and incubated with occasional shaking for 30 minutes at 37°C. to ensure complete solution of the phosphate, and then 4.0 ml reagent was added and the incubation at 37°C. continued for another 90 minutes. The reagent consists of:-

2 volumes distilled water

1 volume 6 N. sulphuric acid

1 volume 2.5% ammonium molybdate

1 volume 10% ascorbic acid

The blanks and standards were treated in exactly the same way so that any inaccuracy caused by the perchlorate solutions would occur in all tubes. The resulting colour was read on a Unicam SP 500 at 820 mμ. This method as described is suitable for 1-8 μg phosphorous.

Isolation of Nucleic Acid

In order to test the ability of DNA to withstand alkaline hydrolysis it was necessary to extract from wheat germ a quantity of undergraded DNA. The extraction method used was based on that of Colter, Brown and Ellem (1962). 20 g wheat embryo were ground in a coffee grinder, then stirred in 90 ml of 1 M. sodium chloride with phosphate buffer pH 7.3. 90 ml of water saturated phenol was added and then mixed in a

Servall omnimixer for 30 minutes at 90 volt setting. The strained liquid was centrifuged for 60 minutes at 2680 G. All centrifugations were at 5°C. The supernatant was centrifuged for 15 minutes at 26,000 x G in a Spinco L centrifuge. The aqueous phase was removed and extracted twice with an equal volume of 90% phenol. The extraction consisted of vigorous shaking for 5 minutes followed by centrifugation for 30 minutes at 2680 G. The aqueous layer was extracted three times with ether. Nitrogen was bubbled through for 10 minutes to remove any trace of ether and the solution was left overnight in a refrigerator. Centrifugation resulted in a precipitate, which was discarded, and the supernatant was poured into an equal volume of ethanol and the fibrous precipitate collected on a glass rod. This precipitate consisted of approximately 30% DNA, 70% RNA.

In order to obtain cleaner DNA preparations for base analysis the method was modified by extracting the nucleic acid into the medium before adding the phenol. In order to reduce loss by deoxyribonuclease action 1.0 mM sodium citrate was added. Citrate would remove divalent cations from active sites where they would stimulate deoxyribonuclease and inhibit ribonuclease. The sodium ion stimulates ribonuclease. After three extractions with

phenol the aqueous solution was poured onto an equal volume of ethanol and the precipitate collected by spooling. The spooled precipitate was dried, suspended in medium and precipitated again. At this stage there was still almost as much RNA present as DNA and so the final precipitate was incubated in a 1 mg/ml solution of ribonuclease for 90 minutes at 37°C. A single phenol extraction and then two precipitations removed the enzyme protein and RNA breakdown products.

Estimation of Protein

Total nitrogen was determined by the method of Sunderland, Heyes and Brown (1957). The acid and solvent washed precipitate was dissolved in 1.0 ml 0.3N KOH and triplicates of 0.01 ml were transferred to a digest tube and the hydrolysis and titration procedure followed without modification.

The most rapid and convenient method for obtaining an approximate value for protein content is that of Lowry et al (1951). After the removal of DNA the protein residue was taken into solution in 0.4 ml 1.0N sodium hydroxide and the method followed without modification. Comparison was made with solutions of soluble casein which had been precipitated, solvent

washed and treated with KOH and 5% PCA in exactly the same way as the unknown solutions.

A more significant but rather more laborious method of estimation is to hydrolyse the protein and estimate the amino acid content. Hydrolysis was performed with 6N. hydrochloric acid in special thick walled pyrex tubes at 120°C. for 12 hours. The tubes were flushed with nitrogen before being sealed and incubated.

After hydrolysis the tubes were opened and dried under vacuum. The dry precipitate was then ready for amino acid analysis.

Amino Acid Determination

1.0 ml of nutralised protein hydrolysate or acid soluble fraction from nuclei was treated according to the method of Yem and Cocking (1955). There were no modifications made to the method and the standard curve was obtained with L-Glutamic acid.

RESULTS

The impulse for this research came from results obtained by James and Richens (1963) which indicated that isolated nuclei from Wheat embryo were able to synthesise protein. Attempts were made to obtain nucleic acid synthesis under the incubation conditions required for the synthesis of protein - 10mM. Glutamate 10mM ATP and 20mM Phosphate buffer pH 7.0. None of these early attempts showed any synthesis of nucleic acid. Eventually a medium was found which was capable of giving a net increase of nucleic acid. Later when reinvestigating the minimal incubation requirements with a different batch of wheat embryo it was found that this time even in a medium containing no pyrimidine precursors there was a small but consistent increase in nucleic acid. Nuclei from a third batch of embryo were quite incapable of increasing their nucleic acid content in a wide range of media. These extreme examples illustrate the wide variability of the plant material, a variability which was not so apparent when investigating protein synthesis.

<u>Condition for DNA Synthesis.</u> Preliminary attempts to obtain DNA synthesis with a simple ATP-Glutamate system were not successful, but synthesis was achieved in a medium containing the pyrimidine pre-cursor, orotic acid. (Table IV)

TABLE IV

DNA SYNTHESIS IN THE PRESENCE OF OROTIC ACID

Conditions of incubation	DNA % C.
Medium	100
Medium + 1.0mM. Orotic Acid	120

Medium: 10mM. Glutamate, 10mM. ATP, 10mM. Ethyl Carbamate, 10mM. D-Ribose, 10mM. L-Ascorbate, 1.0mM. $MgSO_4$, 1.0M. Sucrose, 0.5mM. $CaCl_2$, 20mM. Phosphate buffer pH 7.0.

Total Vol. 2.0ml. Incubate 60 min. at 30°C.

This synthesis of DNA could be inhibited by adding Glucose to the medium (Table V).

TABLE V.

THE INHIBITION OF DNA SYNTHESIS BY GLUCOSE

Conditions of Incubation	DNA % C.
Medium	109
Medium + 4mM. Orotate	173
Medium + 10mM. Glucose	99
Medium + Orotate + Glucose	111

Medium: 10mM. Glutamate, 10mM. ATP, 10mM. Ribose, 10mM. Ethyl carbamate, 10mM. Ascorbate, 1mM. $MgSO_4$, 1.0M. Sucrose, 0.5mM. $CaCl_2$, 20mM. Phosphate buffer pH 7.0.

Total Vol. 2.0ml. Incubate 60 min. at 30°C.

The omission of ATP also resulted in DNA synthesis and a system containing only glutamate ethyl carbamate and D-ribose, all in 10mM. concentration gave a 7% increase. Replacement of ATP by ADP even in the presence of glucose gave a much more considerable increase (Table VI).

TABLE VI

THE EFFECT OF ADP AND ASCORBATE ON DNA SYNTHESIS

Conditions of Incubation.	DNA % C.
Medium + ATP	91
Medium + ADP	117
Medium + ATP + Ascorbate	95
Medium + ADP + Ascorbate	116

Medium contained: 10mM. Glucose, 10mM. Glutamate 10mM. Ethyl Carbamate, 10mM. D-Ribose 10mM. L-Ascorbate, 1mM. $MgSO_4$, 10mM. Bases from RNA hydrolysis. 50mM. Phosphate buffer pH 7.0, 1.0M. Sucrose, 0.5mM. $CaCl_2$.

Total Vol. 4.0ml. Incubated 120 min. at 30°C.

The replacement of ATP with UTP in the presence of glucose made no significant difference.

The mechanism for removing the oxygen atom from ribose nucleotides to convert them to deoxyribose nucleotides is not known but it is conceivable that such a process might be facilitated by compounds capable of accepting or giving electrons. Three such compounds were used, cystein, DPN, and Vit. K_3. Cystein is a possible Hydrogen

donar, DPN will accept hydrogen ions to become an electron donor. Vitamin K_3 is a good electron acceptor capable of carrying electrons from one compound to another (Szent-Gyorgyi 1960). The addition of these compounds to an ascorbate containing medium could be expected to result in a change in the electron status of some compounds and possibly stimulate the normal processes of reduction of nucleotides. (Table VII).

TABLE VII.

THE EFFECT OF COFACTORS ON DNA SYNTHESIS

Conditions of Incubation	DNA % C.
ADP Medium	127
" " + 8.9mM. Cystein	148
" " 0.89mM. DPN + 0.89mM. Menadione	162
" " " " + 8.9mM. Cystein	122
ATP Medium	118
" " 8.9mM. Cystein	128
" " 0.89mM. DPN + 0.89mM. Menadione	146
" " " " + 8.9mM. Cystein	155

Medium: 8.9mM. Glutamate, 8.9mM. Glucose, 8.9mM. Ethyl carbamate, 8.9mM. D-Ribose, 8.9mM. Ascorbate, 10mM. nucleotides from RNA hydrolysate, 0.89mM. $MgCl_2$, 8.9mM. ATP or ADP. 0.89M. Sucrose, 0.4mM. $CaCl_2$, 27mM. Phosphate buffer pH 7.0.
Total vol. 4.0ml. Incubation for 120 min. at 30°C.

The results listed in Table VII show that there is a large increase in DNA in a medium containing DPN and Vit. K_3 (Menadione). In every case ATP was not so effective as ADP as energy source. The increase caused by cystein is more suprising as this amino acid is not usually regarded as being an important co-factor. The reduction of DNA when all three compounds were present in an ADP system indicates some antagonism but the resistance of the ATP system to any such reduction suggests that the adenosine nucleotide is actually involved in part of the action of these co-factors. No duplicates were run with this experiment and no attempt was made to repeat it so care must be used in interpreting the results. It can be concluded however that compounds capable of giving or receiving electrons can influence the synthesis of DNA.

Ascorbate alone has only a very limited effect on DNA synthesis. Sometimes it produces a slight inhibition (Table VIII) but more often there is no effect at all (Table VI)

DNA is much less influenced by the addition of purine and pyrimidine bases to the medium than is RNA. The addition of a complete mixture of nucleic acid bases and sugars at 3.0mM. concentration together with 10mM. ATP and Glutamate resulted in a 15% loss of DNA during the incubation. Deoxy-D-ribose was found to be less effective as sugar source than D-ribose (Table IX).

TABLE VIII.

EFFECT OF ASCORBATE ON DNA SYNTHESIS

Conditions of incubation	DNA % C.
Medium + 43mM. Ascorbate	96%
Medium + 10mM. Ascorbate	106%
Medium only	116%

Medium: 10mM. ATP, 10mM. Glutamate, 10mM. Ethyl Carbamate, 10mM. Ribose, 10mM. Orotic Acid. 1.0M. Sucrose, 0.5mM. $CaCl_2$, 20mM. Phosphate buffer pH 7.0.
Total Vol. 2.0ml. Incubated 60 min. at 30°C.

TABLE IX.

DEOXY-D-RIBOSE AND D-RIBOSE EFFECT ON DNA SYNTHESIS.

Conditions of Incubation.	DNA % C.
Medium + 10mM. Ribose	119
Medium + 10mM. Deoxy-D-Ribose	105

Medium: 10mM. ATP, 10mM. Glutamate, 10mM. Orotic Acid, 1.0mM. Folic Acid, 1.0mM. Sodium Methotrexate 20mM. Phosphate buffer pH 7.0. 1.0mM. $MgCl_2$, 1.0M. Sucrose, 0.5mM. $CaCl_2$.

Total Vol. 3.0ml. Incubated 60 min. at 30°C.

One batch of wheat embryo was consistently different from any of the others used in that it could produce large quantities of DNA in a medium containing only ATP and ribose or ethyl carbamate (Table X).

TABLE X.

INCUBATION IN MINIMAL MEDIUM

Incubation Conditions	DNA % C.
Medium + 13mM. Ribose + 13mM Carbamate	194%
Medium + 13mM. Ribose	268%
Medium + 13mM. Carbamate	183%

Medium: 13mM. ATP, 26mM Phosphate Buffer
pH 7.0, Trace $MgCl_2$. 1.0M. Sucrose
0.5mM. $CaCl_2$.
Total Vol. 1.5ml. Incubated 60 min. at 30°C.

The addition of glutamate and omission of Mg^{++} had little effect (Table XI)

TABLE XI

MINIMAL MEDIUM BUT WITHOUT Mg^{++}

Condition of Incubation	DNA % C.
Medium + 10mM. Carbamate + 10mM. Ribose	188
Medium + 10mM. Carbamate + 10mM. Glutamate	176
Medium + 10mM. Ribose + Carbamate 10mM. Glutamate	205

Medium: 10mM. ATP, 20mM. Phosphate Buffer
pH 7.0, 1.0mM. Sucrose, 0.5mM. $CaCl_2$.
Total Vol. 2.0ml. Incubation 60 min. at 30°C.

The batch of embryo producing such active nuclei is here referred to as 'active' embryo and the nuclei derived from it 'active' nuclei. The observation that there is a difference in the acid soluble material of 'active' nuclei compared with others is described further on.

A point of interest is the apparant independance of RNA and DNA synthesis. In the conditions of Table X there was considerable RNA synthesis - equal to that of DNA, but in the conditions of Table XI there was no RNA synthesis at all.

Conditions for RNA Synthesis

The first successful synthesis of RNA was obtained with a complex medium containing the bases and sugars known to occur in nucleic acid (Table XII).

TABLE XII

RNA SYNTHESIS IN A MEDIUM CONTAINING PURINES, PYRIMIDINES AND PENTOSES.

Conditions of Incubation	RNA%C.	NA%C.
Nuclei + 10mM.ATP + 10mM.Glutamate	70.5	63.5
Nuclei + Complete medium	278	135

Medium : 0.3mM.Adenine, 0.3mM.Guanine
0.3mM.Cytosine, 0.3mM.Uracil, 0.3mM.Thymine
0.3mM.D-Ribose, 0.3mM.Deoxy, D-Ribose
10mM.ATP, 10mM.Glutamate, 50mM.Phosphate buffer
2M. Sucrose, 0.5mM.$CaCl_2$ pH 7.0
Total Volume 5.0ml. Incubated 60 mins. at 30°C.

The large increase in RNA is characteristic of nuclei extracted from wheat embryo and on twelve occasions the total RNA of the nuclear suspension was found to have more than doubled.

Although Deoxy-, D-ribose was not an active source of the deoxyribose of DNA (p. 29) it did permit RNA synthesis but was not so efficient as D-ribose (Table XIII). It was subsequently omitted from the incubation media. In this experiment the initial 'complex medium' was simplified by replacing the pyrimidine bases with orotic acid (Table XIII).

TABLE XIII

EFFECT OF RIBOSE AND DEOXYRIBOSE ON RNA SYNTHESIS.

Conditions of Incubation	RNA%C.	NA%C.
Medium + 10mM. D-Ribose	225%	121%
Medium + 10mM. 2 Deoxy, D-Ribose	119%	100%

Medium : 10mM, ATP, 10mM. Glutamate,

1.0mM Folic Acid, 1.0mM Orotic Acid,

1.0mM $MgCl_2$, 1M. Sucrose, 0.5mM $CaCl_2$.

Total Volume of 3.0ml. Incubated 60 mins. at 30°C.

According to the review of Reichard (1959), carbamyl phosphate is the precursor of orotic acid. The only readily available source of carbamate was ethyl carbamate supplied by BDH. It was found that 10mM. ethyl carbamate provided an adequate source of pyrimidine

for nucleic acid synthesis (Table XIV). It was previously shown (Table XIII) that orotic acid could act as pyrimidine source but when orotic acid was added to the carbamate medium, partial inhibition resulted (Table XIV). This was the first indication of base inhibition.

TABLE XIV

OROTIC ACID INHIBITION OF CARBAMATE INDUCED RNA SYNTHESIS

Conditions of incubation	RNA%C.	NA%C.
Medium only	197%	224%
Medium + 0.5mM Orotic acid	164%	217%

Medium : 10mM. ATP, 10mM. Glutamate,
10mM. Ethyl carbamate, 10mM. Ribose
1.0mM. Magnesium chloride, 50mM. Phosphate buffer
1M. Sucrose, 0.5mM. $CaCl_2$ pH 7.0
Total Volume 3.5ml. Incubation for 60 mins. at 30°C.

The reduction of RNA synthesis by Orotic Acid in the presence of carbamate introduced the possibility that other bases may also reduce the basic rate obtained in the carbamate medium. The results of an investigation into this possibility were not

conclusive. The pyrimidine bases seemed unable to give any additional formation of RNA, and orotic acid again gave an appreciable inhibition (Table XV).

TABLE XV

THE EFFECT OF SINGLE BASES ON RNA SYNTHESIS

Conditions of Incubation	RNA mμME. BASE		RNA % of C.
Zero Time Control	102		
" " "	114	106	100
" " "	105		
" " "	106		
Medium without bases	455		429
"	544		513
" + 1.0mM Guanine	-(lost)		
" "	675		637
" + 1.0mM Thymine	397		375
" "	482		455
" + 1mM Uracil	530		500
" "	549		518
" + 1.mM Cytosine	-(lost)		
" "	430		405
" + 1.0mM Cytidine	465		438
" "	273		258
" + 0.5mM Orotic Acid	301		284
" " "	322		304

Table XV (cont'd)

Medium : 10mM. Glutamate, 10mM. Glucose,

10mM Ribose, 10mM Ascorbate, 1mM $MgSo_4$

20mM. Ethyle Carbamate, 50mM. Phosphate Buffer pH 7.0.

Total Volume 4.0ml. Incubation for 120 mins. at 30°C.

(Full details of this experiment are given because there was such wide differences between duplicates.)

Guanine was the only base to give any increase at all. In order to remove the very high initial increase of the carbamate medium, an experiment was designed to identify more closely any base with an inhibiting effect. In this case the carbamate was replaced with a mixture of bases and any inhibiting effect sought by omitting one or more bases (Table XVI).

TABLE XVI

THE EFFECT OF OMITTING BASES FROM THE MEDIUM

Conditions of Incubation	RNA as% of C.
Total medium	174%
Medium without Guanine	150%
" " Uracil	181%
" " Cytosine	180%
" " Thymine	214%
" " Uracil and Cytosine	212%
" " Uracil and Thymine	254%
" " Cytosine and Thymine	167%
" " Any bases	189%

Total Medium : 10mM. Glutamate, 10mM. Glucose,
10mM. Ascorbate, 10mM. Ribose, 5.0mM. ATP, 60mM.
Phosphate buffer pH 7.0, 0.1mM. Guanine, 0.25mM.
Cytosine, 0.25mM. Thymine, 0.25mM. Uracil.
1M. Sucrose, 0.5mM. $CaCl_2$.
Total Volume 2.0ml. Incubation for 120 mins. at 30°C.

The only conclusion that could be drawn from this experiment was that omission of any one or two pyrimidine bases resulted either in no change or in a slight increase in RNA. Only when guanine was omitted was there any significant drop in synthesis, so confirming the results in Table XV. The surprising fact emerging from this experiment was the apparent synthesis of RNA in a medium devoid of any direct pyrinidine precursor. The fact that carbamyl phosphate can be synthesised from carbox dioxide, ammonia and ATP could suggest the possibility that De Novo synthesis is taking place, possibly with the utlisation of amino groups from glutamate. The presence or absence of such an enzyme system, or of a pool of pyridine precursors could explain the wide differences in activity of different batches of embryo. The results of Table XV are the highest ever obtained.

The final stage of the investigation of base inhibition would be the addition of single bases to a medium devoid of any carbamate. This was unfortunately not possible as the supply of embryo was exhausted. The new batch was found to have completely differing characteristics.

To continue the investigation into the effect of bases and related compounds a solution of 2 and 3 phosphate nucleotides was obtained by the alkaline hydrolysis of commercial RNA. The composition of this sample was found by the method described in Section C and was:

Adenylic Acid 2.6mM/10mM Total Base
Guanylic Acid 2.0mM/10mM Total Base
Cytidylic Acid 2.4mM/10mM Total Base
Uridylic Acid 3.0mM/10mM Total Base

Although the 2,3 phosphate nucleotides are not regarded as the direct precursors of RNA the hydrolysate did give an increase over the rate with carbamate alone (Table XVII).

TABLE XVII

THE EFFECT OF RNA HYDROLYSATE ON RNA SYNTHESIS

Conditions of Incubation	RNA %C.
Medium	255%
Medium + 10mM Hydrolysate	315%

Hydrolysate : 10mM.with respect to phosphate.

Medium : 6mM. ATP, 10mM.Ethyl
Carbamate, 10mM. Ribose, 10mM.Glucose. 1mM. $MgSO_4$,
50mM. Phosphate Buffer pH 7.0, 1 M. Sucrose
0.5mM. $CaCl_2$.

Total Volume 4.0ml. Incubation for 120 mins. at 30^oC.

There is little doubt that nuclei supplied with an energy source and mixture of nucleic acid components can synthesise RNA. It would appear that the nuclei are able also to use low grade precursors for pyrinidine synthesis but it was not possible to establish the possible synthesis of purines. ATP had always to be added for synthesis as the source of energy as well as adenine.

An attempt to replace ATP was made and a single experiment was performed in which UTP was used (Table XVIII). The results are quite unambiguous,

UTP could not replace ATP and actually had an inhibiting effect on RNA synthesis in an ATP containing medium.

TABLE XVIII

THE EFFECT OF UTP ON RNA SYNTHESIS

Conditions of Incubation	RNA%C	NA.%C.
Medium alone	91%	93%
Medium + 4.4mM UTP	107%	98%
Medium + 4.4mM UTP + 6.25mM ATP	113%	100%
Medium + 6.25mM. ATP	204%	127%

Medium : 4.7mM. Glutamate, 0.97mM. Folic Acid, 4.7mM. Ethyl carbamate, 0.47mM. $MgCl_2$ 4.7mM Ribose, 4.7mM. Glucose, 125mM Phosphate 1 M. Sucrose, 0.5mM $CaCl_2$. Buffer pH 7.0.
Total Volume 4.0ml. Incubation for 60 mins. at 30°C.

ADP was usually unable to replace ATP as the energy source for RNA (Table XIX), although it was able to support DNA synthesis (Table VI). Ascorbic acid when added to an ADP medium did enable some RNA synthesis to proceed but only at a fraction of the rate for ATP + Ascorbate (Table XIX).

TABLE XIX

THE ASCORBATE STIMULATION OF RNA SYNTHESIS IN AN ATP AND ADP SYSTEM

Conditions of Incubation	RNA %C	NA %C
Medium + 6.0mM.ATP	268%	159%
Medium + 6.0mM.ADP	84%	112%
Medium + ATP + 10mM.Ascorbate	630%	248%
Medium + ADP + 10mM.Ascorbate	172%	133%

Medium : 10mM, Glucose, 10mM.Ethyle carbamate, 10mM.Ribose, 10mM.RNA Hydrolysate, 1mM. $MgSO_4$, 50mM.Phosphate buffer pH 7.0, 1M Sucrose, 0.5mM. $CaCl_2$.

Total Volume 4.0ml. Incubation for 120 mins. at 30°C.

The ascorbate stimulation of RNA synthesis is one of the outstanding factors of this investigation. Ascorbic acid at 10mM concentration had two main effects on nuclei, it doubled the yield of RNA and it released a large proportion of the nuclear protein. In the inactive nucleus a certain amount of RNA is released into the medium by both ascorbate and dehydroascorbate but it has so far not been possible to test this latter compound for its ability to stimulate RNA synthesis.

It was shown in Table VII that menadione, DPN and cystein could effect DNA synthesis. Their influence on RNA synthesis was much less marked and only menadione and DPN in an ATP system could give any real increase (Table XX).

TABLE XX

THE EFFECT OF COFACTORS ON RNA SYNTHESIS

Conditions of Incubation	RNA % C.
ADP MEDIUM	149
" " + 8.9mM.Cystein	166
" " + 0.89mM.DPN + 0.89mM. Menadione	158
" " + DNP, Menadione + Cystein	152
ATP Medium	286
" " + 8.9mM.Cystein	203
" " + 0.89mM.DPN + 0.89mM. Menadione	338
" " + DPN, Menadione, Cystein	270

(There were no duplicates in this experiment).

Medium : 8.9mM.Glutamate, 8.9mM.Glucose,
8.9mM.Ethyl carbanate, 8.9mM.D-Ribose,

Table XX (Cont'd)

8.9mM. Ascorbate, 0.89mM $MgCl_2$, 10mM RNA nucleotides from hydrolysate, 8.9mM ATP or ADP. 0.89M Sucrose, 0.5mM. $CaCl_2$.

Total Volume 4.0ml. Incubation for 120 mins. at 30°C.

The glucose effect is typical of the problems involved in making a precise analysis of factors influencing RNA synthesis. Because it could inhibit DNA synthesis (Table V) it was used when trying to establish the conditions for RNA synthesis, in order to prevent differences caused by removal of substrate for DNA. Glucose appeared to have no effect on RNA and many of the highest RNA increases were obtained with media containing glucose. (Tables XV, XIX). Later, with a different batch of embryo, glucose was found to cause a considerable inhibition of RNA synthesis (Table XXI).

TABLE XXI

THE GLUCOSE INHIBITION OF RNA FORMATION

Conditions of Incubation	RNA % C.
Medium	208%
Medium + 10mM Glucose	112%

Table XXI (Cont'd)

Medium : 10mM. Glutamate, 10mM Ethyl
Carbamate, 10mM Ribose, 10mM Ascorbate,
1mM $MgSO_4$, 50mM Phosphate buffer pH 7.0,
1M Sucrose, 0.5mM $CaCl_2$.

Total Volume 2.0ml. Incubation for 60 mins. at 30°C.

The variation in the effect of glucose on RNA synthesis shown by nuclei from different batches of embryo was merely one facet of an overall variation in synthetic capacity. An extreme example occurred with a final batch of embryo which produced nuclei capable on one occasion of large increases in RNA in a medium lacking either carbamate or ribose. This was the only occasion, out of five extractions, that RNA synthesis occurred at all with this particular batch (Table XXII).

TABLE XXII

CARBAMATE AND RIBOSE REQUIREMENT FOR RNA SYNTHESIS

Conditions of Incubation	RNA % C
Medium + 13mM Carbamate + 13mM Ribose	360
Medium + 13mM Carbamate	335
Medium + 13mM Ribose	308

Medium : 13mM. ATP, 26mM. Phosphate buffer pH 7.0
Trace of $MgCl_2$. 1M Sucrose, 0.5mM $CaCl_2$.

Total Volume 1.5ml. Incubated for 120mins. at 30°C.

Another aspect of the heterogenity of nuclei is reported later and it may well be that these metabolic differences reflect a genuine morphological difference of the nuclear extractions. The basis of the variation is partly accounted for by different types of starting material but other factors must be involved, and it seems likely that there could be either some selection of different types of nuclei from the same embryo, or possibly a rapid change in the composition of the nucleus immediately after extraction during which it loses, or gains, its synthetic ability. Whatever the cause, an examination of the composition of the nuclei should give some information of the most important environment of all - the site of synthesis.

Conditions for Total Nucleic Acid and Protein Synthesis

Usually the maximum increases of nucleic acid occurred with maximum increases of RNA (Table XII, XIV) because the relative increases in RNA were so much greater than those of DNA. In an active DNA synthesising system the reverse is true. The chief value of a total NA figure is to demonstrate a net increase in precipitable nucleotide, so removing any doubts that the increases are simply an apparent increase of one nucleic acid at the expense of another. This could be due either to a genuine RNA - DNA movement, or a change in alkaline resistance or lability of the nucleic acids, resulting from an actual physical change or a deficiency of the method of analysis. In all the figures quoted to indicate synthesis there was a net increase in total nucleic acid. The conditions for total nucleic acid synthesis cannot be regarded as so significant as those for the individual RNA and DNA synthesis. In many experiments the confusion caused by possible competition between RNA and DNA synthesising systems was removed by adding glucose to the incubation medium and so inhibiting DNA synthesis. Competition with other possible enzyme systems could not be eliminated and it is likely that protein syntheis in particular would be

competing for amino groups and ATP. Adenylic deaminase for example is known to be active in pea nuclei (Turner 1961) and preliminary attempts showed that ethyl carbamate could provide the amino groups for alanine synthesis from pyruvate (Section B).

Generally no attention was paid to protein synthesis measured as increases in total nitrogen, but in the case of chloramphenicol stimulation a comparison was made between RNA and protein increases (Table XXIII).

Table XXIII

CHLORAMPHENICOL EFFECT ON RNA AND TOTAL PRECIPITABLE NITROGEN.

Conditions of Incubation	RNA % C	N_2 % C
Basic Medium	70.5	144%
Basic Medium + 2mM. Chloramphenicol	85.5	99.9%
Enriched Medium	278	92%
Enriched Medium + 2mM. Chloramphenicol	450	74%

(no duplicates, but experiment repeated)

Basic Medium: 10mM. ATP, 10mM. Glutamate,

Enriched Medium also contained: 0.32mM. Adenine, Guanine, Cytosine, Uracil, Thymine, Ribose and Deoxyribose, 50mM. Phosphate buffer pH 7.0. 2M. Sucrose, 0.5mM. $CaCl_2$.

Total Volume: 5.0ml. Incubated 60 min. at 30°C.

Total Nitrogen was investigated on one other occasion, with very active embryo material. The DNA values are given in Table XI. RNA increases were less than 20% but total nitrogen increased proportionately with DNA giving a maximum of 209% of control. In this incubation there was a doubling of DNA and Protein and a slight increase of RNA but the final proportions of each of the three components was still within the limits of variation for nuclei immediately after extraction (see page 49).

The chemical composition of extracted nuclei

Nucleic acid accounts for approximately 10% of the total acid insoluble nitrogen of the nucleus. Of this 10% the RNA proportion varies from 35 - 60%. The cause of this variation is unknown but is probably due either to a loss of RNA from the nucleus or to the presence of cytoplasmic "tags". The absence of any visual evidence of adhering cytoplasm makes this latter possibility less likely. One observation that was made but not supported biochemically was that nuclear preparations occasionally differed in the number and size of their nucleoli suggesting that the chemical composition may reflect a significant difference in the nuclei themselves. It is most unlikely that there would be any loss of DNA from nuclei during extraction and there is no possibility of gaining any by cytoplasmic contamination, so it may be more significant to relate the RNA as a percentage of DNA instead of RNA at zero time. This basis of estimation has removed anomalies between differing extractions with similar incubation conditions (Table XXIV), but has the disadvantage of relating

the quantity of one compound to that of another. If it could be demonstrated that all the DNA, or a fixed percentage of it, participated in RNA synthesis then it would be justified but in view of the known heterogeneity of DNA and the presence of nuclear fragments probably consisting of enzyme free deoxyribonucleoprotein, it is more reliable to refer the increase in RNA to the initial quantity of RNA.

TABLE XXIV

THE INCREASE IN RNA RELATED TO INITIAL RNA (R) AND INITIAL DNA (D).

Conditions of incubation	RNA % R		RNA % D	
	V	VI	V	VI
ADP + Ascorbate + Medium	70%	49%	30%	53%
ATP + Ascorbate + Medium	498%	186%	210%	203%

Medium as given for Tables V and VI.

As DNA synthesis proceeds so the composition of the nucleus changes. It is possible for a "40% Nucleus" to become a "60% Nucleus" after an hour's incubation. Increases in RNA do not change the composition of the actual nucleus because a corresponding amount of RNA is released into the medium. While the rate of release of DNA into the medium can be explained in terms

of passive breakdown the release of RNA must be a form of extrusion as it only occurs under conditions of active synthesis (Table XXV).

TABLE XXV

THE RELEASE OF RNA INTO THE MEDIUM

Conditions of Incubation	RNA % C	
Control supernatant	7	] 100
Control precipitate (nuclei)	93	
Experimental supernatant	69	] 152
Experimental precipitate (nuclei)	83	

Experimental medium : 0.67mM.Glutamate, 0.67mM.Glucose
0.67mM.$MgSO_4$, 6.7mM.Ascorbate, 67mM.Ribose,
67mM.ATP, 6.7mM.ethyl carbamate, 33mM.
Phosphate buffer pH 6.8
1M Sucrose, 0.25mM.$CaCl_2$.

Total Volume 2.0ml. Incubated 24 hours at 30°C.

The nuclei were separated from the medium by centrifugation.

TABLE XXVI

PROTEIN LOSS DURING ASCORBATE INCUBATION

Conditions of Incubation	Protein % C
Control supernatant	-
Control precipitate (nuclei)	100
Experimental supernatant	45
Experimental precipitate (nuclei)	60

Medium : 6.7mM.Glutamate, 6.7mM.ATP, 6.7mM.Ribose
6.7mM.Ascorbate, 6.70mM.ethyl Carbamate,
0.67mM.Uracil, 0.67mM.Guanine, 1M Sucrose,
0.5mM.$CaCl_2$.

Total Volume 5.0 ml. Incubated 60 mins at 30°C.

TABLE XXVII

THE RELEASE OF RNA BY NUCLEI PRETREATED WITH ASCORBATE

Conditions of Incubation	mμME.RNA BASE	
I	14.2	] 77.6
II	63.4	
III	145.2	

I 10mM Ascorbate only. 60 mins at 30°C.

II Full medium. 60 mins at 20°C. after ascorbate treatment.

III Full medium + 10mM ascorbate. 60 mins at 30°C

Table XXVII (Cont'd)

Medium : 10mM Glutamate, 10mM ATP, 10mM ethyl carbamate, 1.0mM Ribose, 1.0mM Guanine, 25mM phosphate buffer pH 7.0. Trace $MgCl_2$. 1.0M Sucrose 0.5mM $CaCl_2$,

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A quite considerable change in the composition of the nuclei occurred when they were incubated in the presence of ascorbic acid. Under such conditions a large proportion of the nuclear protein was released into the medium despite the absence of any appreciable synthesis (Table XXVI). The depleted nuclei retained their integrity and enzyme activity and could be removed from suspension by centrifuging 5 mins. at 12000 x g, and then resuspended and incubated for RNA synthesis. Under normal conditions of protein synthesis there is no release of protein into the medium (James and Richens, 1963).

Although RNA synthesis will proceed after ascorbate treatment it does not do so as rapidly as when incubated with medium + ascorbate (Table XXVII). Under conditions of no synthesis ascorbate still caused the release of some RNA and DNA. Dehydroascorbic acid also released protein and nucleic acid

from the nucleus but so far its effects have not been fully investigated (Table XXVIII). A preliminary analysis of the ascorbic acid using the method of Schaffert and Kingsley (1955) suggests that under the conditions of incubation most of the ascorbic acid is oxidised to dehydroascorbate. This factor has not been examined further.

TABLE XXVIII

THE RELEASE OF RNA AND DNA FROM NUCLEI BY ASCORBIC AND DEHYDROASCORBIC ACID.

Conditions of Incubation	RNA%C	DNA%C	DNA% Total	
Ascorbate supernatant	56.5	51.6	24.2	]100
" precipitate	51.8	161	75.8	
Dehydroascorbate supernatant	44.7	61.3	25	]100
" precipitate	51.8	194	75	

DNA % Total = DNA as a percentage of the Total DNA of that sample of nuclei. It represents the proportion distributed in precipitate or supernatant.

Medium : 25mM.ATP, 50mM.Phosphate buffer pH 7.0, 10mM.ribose, 10mM.ethyl carbamate, 0.5mM. orotic acid, 0.5mM.guanine, 1.0M.sucrose, 0.5mM.$CaCl_2$, 20mM.ascorbate or dehydroascorbate.

Total volume 4.0ml. Incubated 60 mins. at 30°C.

The quantities of DNA released from the nuclei by ascorbate varied with the source of embryo. The earlier extractions released only about 2-5% but the latest "active" batch released 25% (Table XXVIII).

The displacement of protein from nuclei is not a simple salt effect as on many occasions. there has been no protein detected in the medium after ascorbate treatment. This was especially the case when the nuclei had been previously incubated for RNA synthesis. No detailed analysis was made of the protein released from the nuclei. A large proportion could be precipitated at pH 10.6, a characteristic of arginine rich histone (Allfrey 1961). Acid hydrolysis and chromatography revealed at least six distinct ammino acids but no attempt was made to identify them.

Nuclei extracted in 2M. Sucrose 1mM. $CaCl_2$ retain a pool of acid soluble material which absorbs ultraviolet light. This fact is of importance when assessing the utilisation of substrate for RNA synthesis as it may be possible that all the necessary nucleotides are in fact

already in the nuclei. The gross absorbtion of the soluble fraction after precipitation of nucleoprotein in 5% PCA was usually between 14-30% of the nucleic acid absorbtion. The latest 'active' batch of embryo was exceptional in that the soluble material was of far greater importance, being on one occasion 133% of the total Nucleic acid.

The presence of ATP in the incubation media meant that there was no problem with regard to purine source. The bulk of the work with base pre-cursors was concerned with pyrimidines and it is possible that if all the U.V. absorbing material were pyrimidine then the nuclei could almost double the yield of RNA without using any added pre-cursors. The very large increases in RNA described in Tables XV and XIX could not possibly be explained in this way. It is more likely that the significance of this acid soluble material lies in the possible role of co-factor. It was found that the absorbtion peak varied from extraction to extraction and was usually either at 260mμ or 280mμ.

(Fig. 1) This change in absorbtion peak reflects a balance of at least two compounds. The

relative balance of these components is changing during the actual extraction, and incubation of nuclei in sucrose resulted in a loss of the U.V. material from 29.7% of total nucleic acid to 17.7% after 60 minutes. An attempt was made to separate the residual and released absorbing material by centrifuging the nuclei from the medium and examining the spectrum of the medium and the acid soluble fraction of the precipitate. (Fig. 2). 73% of the total absorbance was contained by material released into the medium and had a maximum absorbtion at 278mμ. The remaining 27% released from the nuclei by acid treatment had a broad peak at approximately 257mμ.

The relation of the soluble material of active and inactive nuclei has not been closely investigated but it was observed that nuclei from one batch of inactive embryos absorbed only at 260mμ and the latest 'active' embryos produced nuclei which on one occasion absorbed at 280mμ when active and 260 mμ when inactive. Nuclei from the same embryo absorbed at 265 and 270 on two occasions when DNA synthesis only occurred.

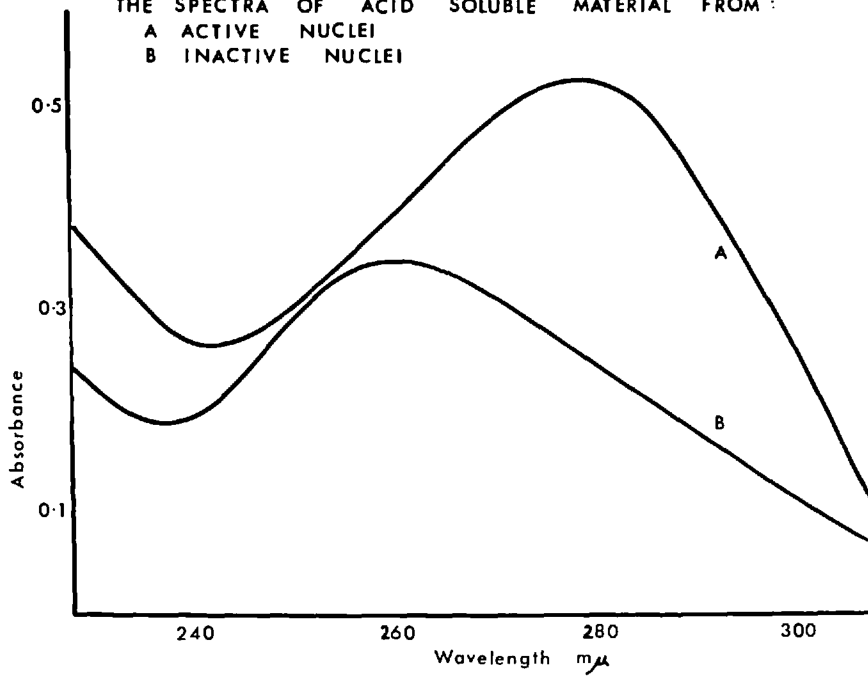


Fig. 1
THE SPECTRA OF ACID SOLUBLE MATERIAL FROM :
A ACTIVE NUCLEI
B INACTIVE NUCLEI
0·5
0·3
0·1
Absorbance
A
B
240
260
280
300
Wavelength mμ

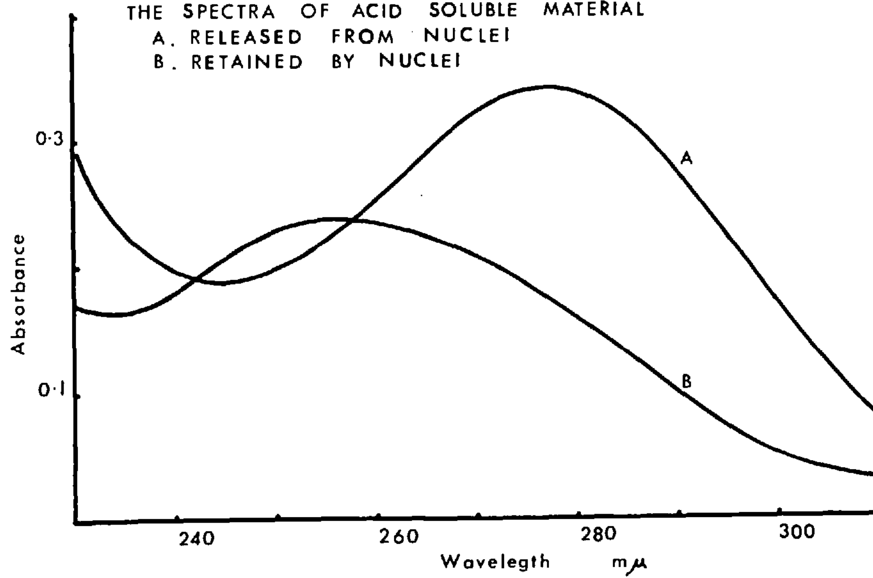


Fig. 2
THE SPECTRA OF ACID SOLUBLE MATERIAL
A. RELEASED FROM NUCLEI
B. RETAINED BY NUCLEI
0·3
0·1
Absorbance
A
B
240
260
280
300
Wavelegth mμ

On one occasion the ethanol:chloroform supernatants were examined and it was found that there was an increase in solvent soluble material absorbing in the UV during incubation in sucrose.

An examination of the acid soluble free amino groups of nuclei showed that only 1.5-2.5% of the total nitrogen of the nuclei occurred in this form. With nuclei from the very active embryo the figure was as high as 10% and so occurred in approximately equimolar concentration with total nucleic acid nitrogen. The exact status of these amino groups is not known as not only amino acids, but some purine and pyrimidine bases and nucleotides give a reaction with the method used.

DISCUSSION

The outstanding result of the experiments recorded in this section was the repeated synthesis of both RNA and DNA by nuclei isolated from wheat embryo. The amount of nucleic acid synthesised by these nuclei is far greater than has been previously observed for any others. Why should they be so suitable for nucleic acid synthesis? There are probably two main reasons, the method of extraction, and the type of plant material. The use of 2M. Sucrose 1mM. $CaCl_2$ as extracting medium keeps the nuclei intact but without membranes. (James 1963). Allfrey (1957) compared nuclei extracted in organic solutions with those extracted in 0.25M. Sucrose 3mM. $CaCl_2$ and found very little difference in the composition of the soluble constituents. 1.0M. or 2.0M. Sucrose would reduce diffusion even more than 0.25M. Evidence was found for the restriction of diffusion by Sucrose while working on another problem. 0.8 ml. 1.0M. Sucrose was layered over 0.8 ml. 1.3M. Sucrose containing gentian violet. After 3 days there was still no gentian violet in the 1.0M. layer.

The plant material from which the nuclei were obtained is meristematic and up to the time of extraction the cells are dormant. Nuclei from meristematic cells of peas are dehydrated (Lyndon 1963) and wheat embryo cells are so dry that they can be extracted with petroleum spirit directly without damaging the enzyme complement (Section B). A compact dehydrated nucleus would be much more resistant to mechanical breakage during extraction. Microscopic examination of the tissue shows that the cells are dominated by the large nuclei and it is likely that in the early stages of development the nucleus also retains biochemical dominance over the cell.

The possibility that bacterial contamination was producing the nucleic acid was investigated by plating a nuclear suspension in medium before and after incubation, onto agar enriched with sterile medium. After incubation for two weeks at 30°C. it was found that the "before incubation" plate had 73 colonies and the "after incubation" plate, 55 colonies. Each had two fungal colonies. 5% of the incubation

suspension was plated in both cases. The attempts to examine the requirement for pre-cursors was the most disappointing aspect of the work. It soon became apparent that there was often more variation from one extraction to another than from one type of medium to another. The main points that emerged were that carbamate was an effective pre-cursor for both RNA and DNA and that in its presence orotic acid increased DNA but reduced RNA synthesis. It was also shown that under the experimental conditions deoxy-ribose was not a suitable source of sugar for DNA, though ribose apparently was.

The partial inhibition of RNA synthesis by orotate was unexpected as carbamate was an adequate source of pyrimidine pre-cursor and the free bases, end point of pyrimidine synthesis, had only the very slightest inhibitory effect. The possible reason for this anomaly may be that orotic acid is exceptional in possessing a carbo-xyl group attached to a pyrimidine ring.

Ribose was the only sugar found to be actively utilised for nucleic acid synthesis.

Deoxy-ribose was only of limited value as sugar source for RNA and no use for DNA. A similar observation was made by Coddington and Sorensen (1963) who found that deoxyATP inhibited DNA synthesis. Deoxyribose is not known to occur at any stage of nucleic acid synthesis, reduction of the sugar residue taking place at the nucleotide stage (Reichard 1960).

UTP also had a strong inhibitory effect despite the fact that it is a known precursor of CTP. Both of these nucleotide triphosphates can be incorporated into RNA by isolated pea nuclei (Rho 1961).

The inhibition of DNA synthesis by glucose was rather suprising as it has been suggested that glucose is bound to hydroxymethyl cytosine and involved in DNA synthesis (Kornberg, 1959). However, use was made of the specific inhibition of DNA synthesis by glucose to establish more closely the factors effecting RNA synthesis, until it was found that with nuclei from one batch of embryo, glucose had an inhibiting effect on RNA synthesis as well as DNA synthesis (Table XXI). An examination was made of the specific

effect of potassium and sodium ions on nucleic acid synthesis but no requirement or inhibition was observed with either. Magnesium was usually present in the medium but its omission made no appreciable difference. It is likely that the nuclei retain sufficient salts during extraction to maintain normal metabolic conditions.

The other outstanding result of this section was the increase in RNA synthesis which resulted when ascorbate was present in the medium. Bagatytreva and Znamenskaya (1962) found that ascorbate denatured DNA apparently by separating the two strands of the twin helix, but with nuclei, ascorbate had another effect as well, it released a large proportion of the nuclear protein. It has been demonstrated by Allfrey et al (1963) that histones restrict the ability of DNA to act as the template for RNA synthesis. It is likely that ascorbate stimulates RNA synthesis by releasing the histone from the chromosomes and so leaving them free for RNA synthesis. There is some evidence that ascorbate may be the natural histone releasing agent, as it has been reported by

Spagg (1959) that ascorbate and dehydroascorbate occur in the developing meristem, and recent work by Andrews (1961) has shown that ascorbate accumulates in the wheat embryo during vernalisation. Under the conditions of incubation much of the ascorbate was oxidised to dehydroascorbate even before it was added to the nuclei and so it is possible that it is the oxidised form that is most active. It was demonstrated that dehydroascorbate could release protein and RNA from nuclei but no evidence has been obtained that it can cause the increase in RNA synthesis.

Ascorbate did not influence DNA synthesis and it is apparent that the stimulus required to obtain RNA or DNA synthesis is quite different. The ATP inhibition of DNA was of especial interest as it introduces the possibility that phosphorylation of ADP may be involved in the mechanism of DNA synthesis. It has been demonstrated by Allfrey (1958) that DNA is needed for nuclear phosphorylation and more recently Mc Ewen (1963) has shown that histone will inhibit the phosphorylation. One possible connection between

this data is that electron transport along the DNA molecule is needed to induce, or maintain, DNA synthesis. It has been established (Bradbury-1961) that DNA can act as a semiconductor and transmit electrons along its length from donor to acceptor. This means that energy can be transmitted with the electron and could be drawn off at any position on the DNA molecule. It has been shown by Kornberg (1959) that the deoxyribonucleotides required for DNA synthesis are required to be phosphorylated to the triphosphate level so it can only be assumed that as the nucleotide triphosphates are polymerised in close contact with existing DNA so an electron passes from the nucleotide to the template DNA or nascent DNA as a free electron, and passes along the molecule to an electron acceptor such as DPN. The reduced acceptor would need to be continuously reoxidised, a process which might be dependent on ADP. The addition of Vitamen K and DPN, two good electron acceptors (Szent-Gyorgyi 1960), resulted in an increase in DNA synthesis, even in the absence of an excess of ADP. No further work was attempted in support for this hypothesis

and as nothing is known of the ability of nuclei to oxidise or reduce an ascorbate-Cystein - DRN, Vit. K system containing ATP or ADP little reliance can be put on the actual mechanism other than to conclude that co-factors involved in phosphorylation do influence DNA synthesis and that two at least of these co-factors, DPN and ADP, occur in nuclei in comparatively large quantities. (Osawa 1957).

If a system of electron transfer along DNA does occur then there is a ready explanation regarding the peculiar effects of bases, as all the nucleic acid bases are fairly good electron donors, and could interfere with the sub-molecular structure of DNA simply by creating an adverse electronic environment. This in turn could prevent the normal functioning of DNA in the replication process.

The significance of DNA synthesis is difficult to assess in the absence of a close analysis of the physical state of the nuclei. Anything up to 100% increase could be explained simply as replication of the DNA which would

normally preceed nuclear division. Before considering the implication of an increase of more than 100% it must be established that there is in fact no increase in the number of nuclei during incubation. The fact that the bulk of the DNA can be sedimented with the nuclei indicates that it is remaining a part of the nuclear structure but in the "active" nuclei which showed the greatest increase in DNA there was a much larger percentage released into the medium and in fact the increase remaining in the nuclei was less than 100%. The most likely explanation seems to be that DNA will increase until there is complete replication and will then stop unless there is some means of releasing the DNA from the nucleus, in which case replication can continue.

The RNA situation is less complicated because nuclei from meristematic cells can be expected to produce relatively large quantities of RNA to maintain cell development. The status of this RNA is discussed later.

The release of histone from the nucleus

is of especial interest as this is very likely to be one of the mechanisms by which RNA synthesis is restricted. It has been shown by Allfrey et al (1963.) that it is necessary to remove histone from DNA before it will function as template for RNA synthesis. If ascorbate or dehydroascorbate are the protein releasing compound then one of the basic mechanisms of nuclear control of cell metabolism can be related to naturally occurring substances which exist in chemical equilibrium in the cell and which are known to vary in their concentration during the development of the cell. It is of interest that indole compounds such as indole acetic acid have a similar ability to donate or accept electrons as ascorbate, a fact which may be of importance in their function as growth factors.

It must not be assumed that ascorbate gives complete control over nuclear metabolism. It is obvious from the very erratic results (less than 50% of incubations gave any nucleic acid increase at all) that ultimate control lies in the nucleus and that other factors, possibly related to the content of the acid soluble material, are involved and exercise ultimate control.

SECTION B

GLYCOLYTIC PHOSPHORYLATION BY

ISOLATED NUCLEI.

METHODS

Isolation of non-aqueous Nuclei.

20g wheat embryo was stirred in petroleum spirit (30 - 40°C B.P.) for 5 mins., decanted and the procedure repeated. After drying on filter paper at room temperature the now partially defatted embryo was ground in a coffee grinder and stirred in 70% Carbontetrachloride/Cyclohexane for five mins. The suspension was strained through two layers of muslin, homogenised briefly in a polypropylene tube and then strained through a single layer of nylon bolting silk, 25µ mesh. A crude Nuclear-Starch pellet was obtained by centrifuging the solution 5 mins. at 3000 r.p.m. and purification of the nuclei achieved by suspending the pellet in 70% Carbon tetrachloride and layering onto a density gradient. This gradient was made by injecting 25 ml. 80% Carbon tetrachloride under 20 ml. 70% Carbon tetrachloride. Centrifuging the loaded gradient for 15 mins. at 3000 r.p.m. gave a pellet of starch and a narrow layer of nuclei at the centre of the gradient. The Nuclear layer was pipetted off and centrifuged through 70% Carbon tetrachloride

5 mins. at 3000 r.p.m. The resulting precipitate consisted mainly of nuclei and nuclear fragments.

Isolation of "Light Fraction" Particles

The 70% supernatant from the first Nuclear centrifugation was diluted to 65% with cyclohexane, centrifuged, and the pellet suspended in 75% Carbon-tetrachloride 25% cyclohexane. After another centrifugation the supernatant was diluted again to 60% and centrifuged. This precipitate consisted of mitochondria, small plastids and about 10% nuclear material with attached cytoplasm.

Glycolytic Phosphorylation

Stern & Mirsky have shown that wheat germ nuclei contain all the enzymes necessary to produce Pyruvic acid from fructose 1, 6 - diphosphate, but although ATP was needed to obtain Pyruvic Kinase activity no attempt was made to demonstrate the accumulation of ATP.

Incubation medium

10mM D-3-Phosphoglyceric Acid

10mM Adenosine Monophosphate

20mM Potassium hydrogen Phosphate buffer pH 7.3

Trace Magnesium-Sulphate

The D-3-Phosphoglyceric Acid was purchased as the Calcium salt and converted to the potassium by dissolving in 0.1N Hydrochloric Acid and passing through a 3 cm. column of Decalso 'F' in the potassium form.

The incubation medium was added direct to the dry nuclear pellet and homogenised. 1.0 ml. samples were pipetted into 1.0 ml. Cold absolute ethanol and the resulting precipitate removed by centrifugation. 1.0 ml. of the supernatant was used for the estimation of pyruvic acid and 0.1 ml. was chromatogramed.

Estimation of Pyruvic Acid

1.0 ml. of the incubation supernatant containing 0.5 ml transformed medium was added to 1.0 ml of 2:4 Dinitrophenylhydrazine, saturated solution in 2N. HCl.

After standing 2.0 hrs. at room temperature 2.5 ml water was added followed by 5.0 ml ethyl acetate. The hydrazine and hydrazone derivatives were extracted into the ethyl acetate by shaking. The aqueous phase was removed, washed once with ethyl acetate and discarded. The ethyl

acetate extracts were pooled, washed once with water, and the hydrazone derivatives extracted into 1% sodium bicarbonate. After washing once with bicarbonate the ethyl acetate solution of hydrazines was discarded and the pooled bicarbonate was washed three times with fresh ethyl acetate.

The bicarbonate solution was measured on a Unicam SP 500 spectrophotometer at 375 mμ or on an EEL colorimeter using filter OBIO. Pyruvate standards containing up to 5mμMole Equivalents per ml were similarly treated.

Chromatography

1.Chromatography of Pyruvic acid.

The bicarbonate solution was acidifed with HCl and the colour extracted into 2.0 ml ethyl acetate. This was dried on a rotary film evaporator, dissolved in 0.1 ml ethyl acetate and 10μl chromatogramed. The chromatogram was developed in Butanol, Ethanol, Ammonium (7:1:2) for 4 hrs. The 2:4 Dinitrophenylhydrazone derivitive of pyruvic acid formed two spots owing to the formation of cis-trans isomers.

2. Chromatography of Adenosine nucleotides.

100μl of the ethanol supernatant was applied to

Whatman No. 1 paper and developed in isobutyric acid, 1.0N, ammonia, 0.1N.Diamino-ethane-tetra-acetic acid (Versene). (100:60:1.6 v/w).

Good separation was obtained and the spots could be cut out, eluted in 0.1N HCl and the solution read directly on the spectrophotometer.

Occasionally use was made of the solvents, isobutyric acid, water, concentrated ammonia (132:66-:2 v/v) and ammonium sulphate, sodium hydrogen phosphate pH 6.8, n- propanol (60:100:2 w/v/v).

RESULTS

Figure 3 shows the relation of pyruvate accumulation and phosphate binding with time. The ten minute lag is characteristic of this system without added ATP. The addition of ATP at 1 mM concentration or the replacement of AMP with ADP removed this lag phase but gave no stimulation during active pyruvate formation (Table XXIX).

Fig.3 PYRUVATE ACCUMULATION AND PHOSPHATE BOUND TO NUCLEOTIDE IN AN AMP MEDIUM.

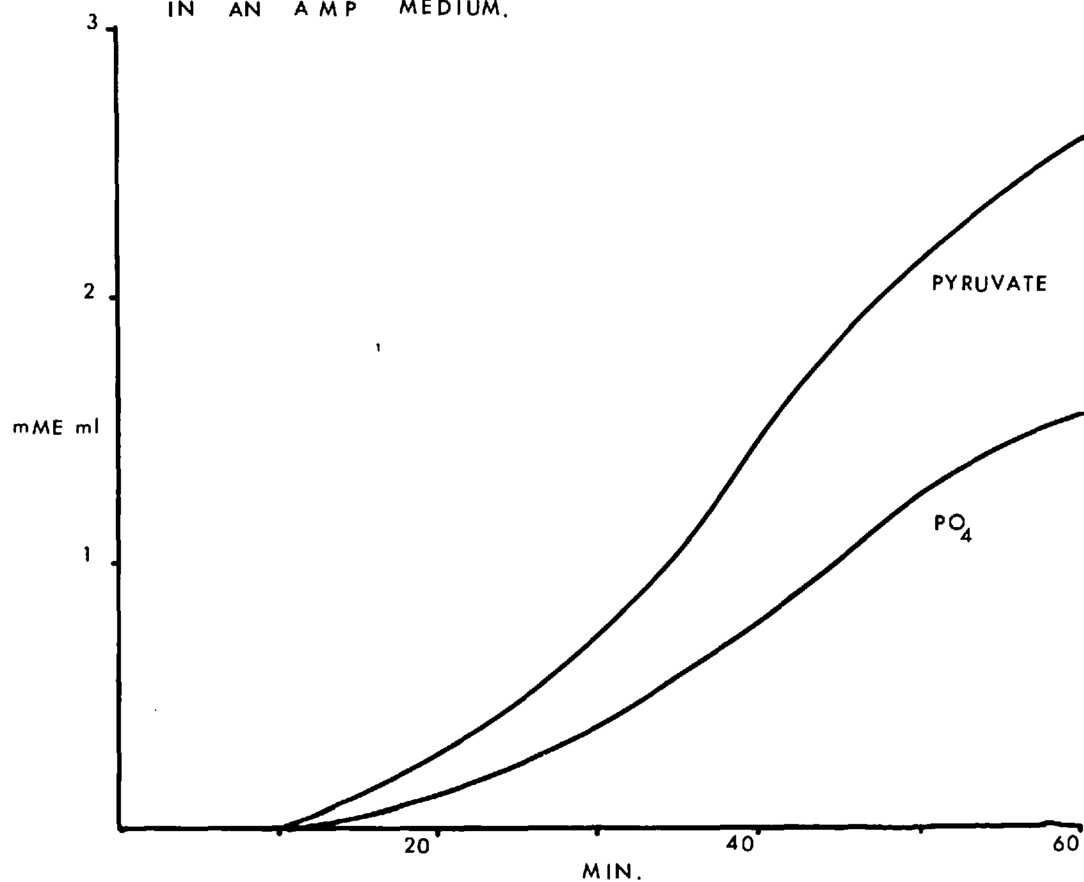


TABLE XXIX

THE ABSORPTION OF PYRUVATE DERIVATES FORMED EITHER WITH AMP OR ADP AS ADENOSINE SOURCE.

O.D.

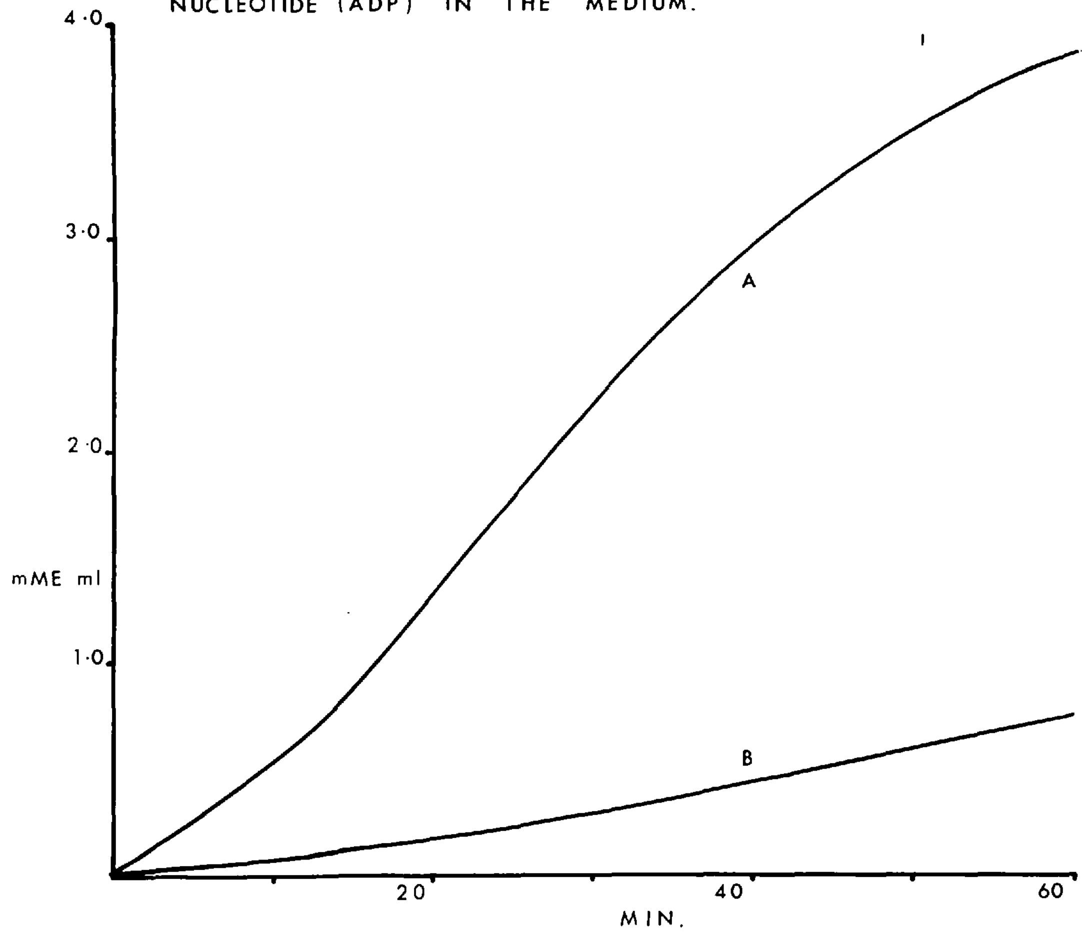
Minutes	Absorption of Pyruvate Derivatives	
	AMP	ADP
0 - 5	-	0.12
5 - 10	0.02	0.24
10 - 20	0.65	0.54
20 - 40	1.29	1.08
0 - 40	1.96	1.98

The production of pyruvate was accompanied by an equivalent binding of phosphate.

$$\frac{\text{PHOSPHATE BOUND TO ATP AND ADP}}{\text{PYRUVATE FORMATION}} = 50\text{-}85\%$$

Because of the small quantity of nuclei available it was not possible to extract enough endogenous adenosine nucleotides to chromatogram but that these nuclei do contain at least trace ammounts of ADP is shown by the fact that they were able to produce small ammounts of pyruvate in the absence of any nucleotide substrate. (Fig. 4)

Fig.4 PYRUVATE ACCUMULATION WITH (A) AND WITHOUT (B) ADENOSINE NUCLEOTIDE (ADP) IN THE MEDIUM.



To confirm that all the glycolytic enzymes were present in these nuclei, Fructose-1, 6 Diphosphate with ATP was used instead of 3 Phosphoglyceric acid. A uniform increase in pyruvate was observed over a period of 60 minutes (Fig. 5).

The conditions of isolation of the nuclei make it impossible to preclude the possibility that small numbers of other organelles may be included in the nuclear fraction and so could be responsible for much of the observed activity.

The possible interference of mitochondria and other lighter particles was examined by incubating nuclei and "light fraction" in a medium which should stimulate mitochondrial activity.

Medium:

D3-Phosphoglyceric Acid	10mM final conc.
AMP	10mM
K_2HPO_4 pH 7.2	10mM
Succinate	1 mM
DPN	1 mM
$MgSO_4$	Trace

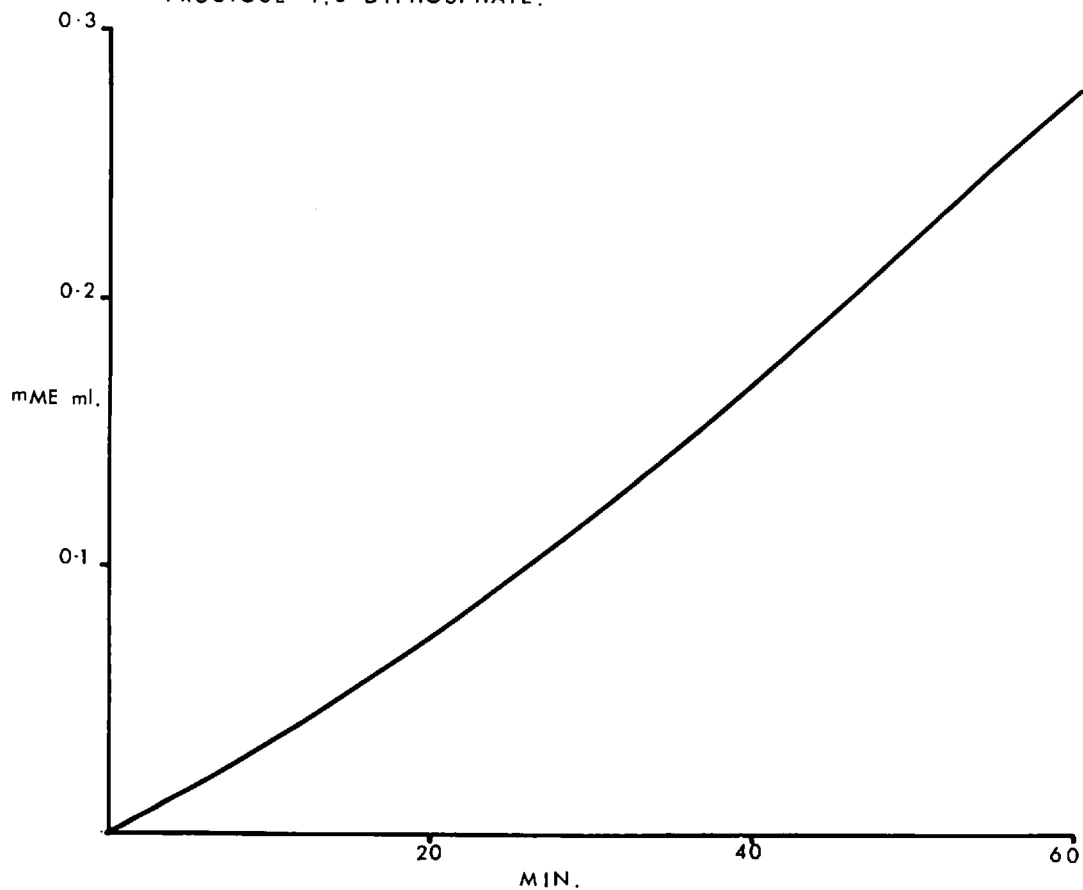


Fig.5 PYRUVATE ACCUMULATION IN A MEDIUM CONTANING FRUCTOSE 1,6 DIPHOSPHATE.

The three conditions of incubation were:

100% light fraction

50% light fraction + 50% nuclei

100% nuclei

The result is given in Table XXX .

TABLE XXX

THE PYRUVATE FORMATION BY NUCLEI AND "LIGHT FRACTION" PARTICLES.

Min. Incubation	mμM pyruvate/mg Dry Wt.		
	Light fraction	L.F. and Nuclei	Nuclei
0 - 30	13.0	18.5	19.7
30 - 60	- 13.0	1.3	24.3
0 - 60	Nil	19.8	44.0

It is obvious that the light fraction effect is quite different from the nuclear fraction, as the initial synthesis, which may be due to the 10% nuclear contamination, is followed by catabolism.

The effect of mitochondria was also shown by adding aqueous mitochondria, extracted by the method of James and Richens, to the basic

system after 50 mins. The ATP synthesis was stimulated but ADP began to decrease. Table XXXI.

TABLE XXXI

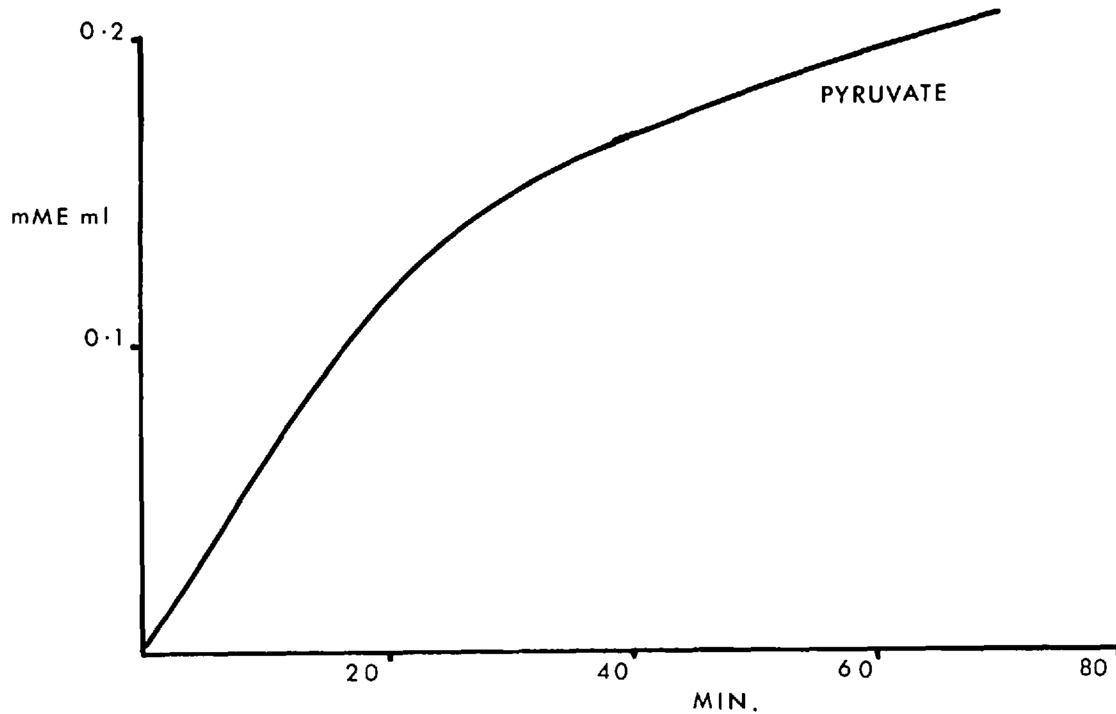
THE ADP AND ATP ACCUMULATION BY NUCLEI AND THE EFFECT OF ADDING AQUEOUS MITOCHONDRIA

	Basic System		Mitochondria Added
	30 min.	50 min.	80 min.
ADP	1.8	2.7	2.6 μM/ml
ATP	1.3	2.6	4.5 μM/ml
$\frac{ATP}{ADP+ATP}$	42%	49%	64%

The other possible source of error is the precipitation of soluble enzymes onto the nuclear surface during extraction in the non-aqueous solutions. To test for this, nuclei extracted in aqueous media were used (Johnson, Nasitir and Stern, 1952). The incubation medium was the same as for non-aqueous nuclei and the result is shown in Fig. 6 .

The fact that AMP, with or without ATP could act as substrate for phosphorylation suggests

Fig. 6 PYRUVATE ACCUMULATION BY AQUEOUS NUCLEI



the presence of an enzyme capable of performing the reaction: AMP $\longrightarrow$ ADP. The most likely enzyme for this is adenylic kinase which catalyses the reaction: AMP + ATP $\rightleftharpoons$ 2 ADP. This enzyme is important also as a possible source of ATP in a medium supplied only with ADP as energy source.

Adenylic Kinase.

This enzyme has been assumed to be present in nuclei for some time

Reaction mixture:	ADP	10mM.
	Phosphate buffer pH 7.2	200 mM.

Incubation was at 30°C. 0.5 ml. aliquots were removed at intervals and injected into 0.5 ml. cold ethanol. After centrifugation 100µl of the clear supernatant was chromatogramed, using the solvent isobutyric acid, ammonia, water (66:1:33). The chromatogram was developed for 18 hrs., dried and then all spots visible under Ultraviolet light were removed, cut into 1 mm. strips and soaked in 5.0 ml. 0.5N. HCl at room temperature for 18 hrs. Solutions were read on a spectrophotometer and the concentration of base calculated (Table XXXII).

TABLE XXXII

THE FORMATION OF AMP AND ATP FROM ADP.

	mμ Mole Equivalent of Base/Spot		% Total Base
	0 min.	120 mins.	120 mins.
ADP	479	190.5	40
AMP	-	162.0	34
ATP	-	123.7	26
Total	479	476.2	100%

$$\frac{\text{ADP}}{\text{ATP + AMP}} = 66.7\% \qquad \frac{\text{ATP}}{\text{AMP + ADP}} = 43.3\%$$

There was no loss or gain in the total amount of adenosine, merely a redistribution of the bound phosphate groups. Phosphate equivalents were worked from the base values (ATP x 3)(ADP x 2). (TableXXXIII).

TABLE XXXIII.

THE REDISTRIBUTION OF PHOSPHATE DURING KINASE ACTION.

	mμ Mole Equivalent of Phosphate/Spot		% Total Base
	0 min.	120 mins.	120 mins.
ADP	958	381	41.7
AMP	-	162	17.7
ATP	-	371	40.6
Total	958	914	100

$$\frac{ADP}{AMP + ATP} = 71.5\%$$

It can be concluded that the nucleus is able to produce ATP from ADP by Kinase action with the by-production of AMP.

Discussion

ATP has a double role in nucleic acid synthesis, as energy source and as substrate. The fact that each of the five nucleotides involved must be raised at least to the diphosphate and possibly to the triphosphate state indicates a high energy requirement. (Kornberg 1959) (Hurwitz 1958). James et al (1960) were unable to trace any cytochrome activity in these nuclei and in their later paper (1963) demonstrated the utilization of ATP produced by mitochondria. The presence of a full complement of glycotytic enzymes was demonstrated by Stern and Mirsky (1952) but they made no attempt to show that ATP or pyruvate accumulated. The incubation conditions did not promote protein or nucleic acid synthesis and so reduced the loss of ATP from the system. On the basis of one molar equivalent of phosphate being bound to the nucleotide for each molar equivalent of pyruvate formed, up to 85% of the expected yield was identified as ADP or ATP. Accumulation of pyruvate does not occur in normal cells and its accumulation by these isolated

nuclei may be due to a number of factors, nuclear disorganisation, loss of any additional substrate or enzymes required for its further metabolism, or a general inability of these nuclei to use this compound. Incubation of non-aqueous nuclei with pyruvate and glutamate did result in measurable transamination with the formation of alanine. Transamination also proceeded when the glutamate was replaced with ethyl carbamate. The presence of a very active transaminase in nuclei is also suggested by the results of James and Richens (1963) who found that glutamate alone was an adequate source of amino acid for the synthesis of protein containing a wide range of amino acids. Transaminase does not give a reduction of total organic acid and it is likely that there is either some other use for these acids, such as acetylation of amino acids or they are lost to the surrounding cytoplasm to be consumed in the oxidative processes of mitochondria. The ability of mitochondria to consume pyruvate is shown in Table XXX and illustrates the interdependance of organelles for substrates. The nuclei may obtain ATP direct by glycolysis but they could also rely

on mitochondria to produce additional quantities of this energy source by oxidising the glycolytic end product, pyruvic acid.

The ability of ADP to produce a net increase in nucleic acid (Table VII) was probably the result of kinase action rather than glycolysis but kinase could not give a net increase in phosphate bound to nucleotide and could not produce any ADP or ATP from AMP alone. Undoubtedly kinase would produce the conditions for glycolysis when AMP was added as substrate, as only ADP can accept the phosphate bond in glycolysis (Stern and Mirsky 1952) but it is necessary to assume that trace ammounts of ADP or ATP were already contained in the nuclei. This assumption was verified experimentally by inducing the production of pyruvate in the absence of any added nucleotide. (Fig. 4) The lag at the beginning of many experiments with AMP as substrate emphasizes the relation of the two end products of glycolysis, pyruvate and bound phosphate, and confirms the assumption that the phosphate bound results from glycolysis. The utilisation of Fructose 1, 6-Diphosphate showed that a complete range of glycolytic enzymes was available.

Despite their reputed solubility the glycolytic enzymes were not completely lost from the nuclei extracted in 2M Sucrose and an increase in pyruvate could be established when 3-phosphoglyceric acid was used as substrate.

The nuclei used in this work are meristematic and, up to the time of use, are in a resting state. Such nuclei occupy a position in the centre of a cell surrounded by dense, mitochondria filled cytoplasm. The mitochondria would be the chief sources of energy at a time when the cell is at the maximum rate of growth and development. It is conceivable that the drain of oxygen by the mitochondria would maintain the nucleus in a state of near anaerobiosis. Under these conditions glycolytic ATP production would provide a useful source of energy and the by-production of pyruvate would provide substrate for the surrounding Mitochondria.

There was always a requirement for AMP or ADP during nucleic acid synthesis and it is clear that a part of this requirement could be satisfied by the nucleus itself at least during the meristematic stage of cell development when RNA synthesis is at a maximum (Lyndon 1963).

SECTION C

THE CHARACTERISATION OF RNA RELEASED FROM ISOLATED NUCLEI DURING INCUBATION.

METHODS

The Isolation of Microsomes.

All procedures for isolating microsomes and related subcellular structures relied on the initial preparation of a crude microsomal pellet. 20 g wheat embryo was ground very finely and stirred in 30 ml. of medium.

Medium: 0.4M. Sucrose
0.05M. Phosphate buffer pH 7.2
0.004M. Magnesium chloride
0.035M. Potassium chloride

The thick suspension was strained through Muslin and then centrifuged 10 min. at 2400 x g to remove cell debris before centrifuging 20 min. at 10,000 x g to obtain a crude mitochondrial preparation. The final supernatant was centrifuged 60 min. at 105,000 x g to obtain the crude microsome pellet. Samples of one old batch of embryo would not produce a pellet at 105,000 x g and had to be centrifuged 30 min. at 176,000 x g. Further centrifugation for 16 hrs. at 105,000 x g gave an additional pellet. The final batch of embryos, which produced very active nuclei, gave a good compact pellet after 30 min. at 105,000 x g. Because of the high

inherent synthetic ability of the microsomal fraction from these active embryoes (Table XXX) they were cleaned by washing in extraction medium, or in medium containing 0.3% sodium deoxycholate. The washing procedures affected not only the snythetic ability but also the composition of the microsomal fraction. A comparison of the nitrogen values taken immediately after precipitation, and then after washing three times in Ethanol:Chloroform (3:1) showed that in the initial pellet there was a fall in total precipitable nitrogen of about 70%. After washing either in medium or deoxycholate there was only a slight loss after ethanol:chloroform extraction. Deoxycholate had an additional effect in that it reduced the pellet produced by centrifuging 30 min. at 40,000 x g to less than 10% of its former value. A further small pellet could be obtained by subsequent centrifugation for 60 min. at 173,000 x g but to get a really good pellet it was necessary to centrifuge 14:5hrs at 105,000 x g. (Table XXIV).

Table XXIV

THE LOSS OF ACID INSOLUBLE NITROGEN AFTER WASHING MICROSOME PREPARATIONS IN CHLOROFORM:ETHANOL.

Microsomal fraction	Before washing	After washing
I	323μg.N_2	97μg.N_2
II	326μg.N_2	345μg.N_2
III	* 17.6μg.N_2	*27.1μg.N_2
IV	-	292μg.N_2

*Method not accurate for such small quantities.

Microsome I Basic crude microsome preparation sedemented 30 min. at 105,000 x g.

Microsome II. Crude preparation washed in medium. Final sedementation 30 min. at 105,000 x g.

Microsome III. Crude preparation washed in medium containing 0.3% sodium deoxycholate. Final sedementation 30 min. at 105,000 x g.

Microsome IV. The supernatant from III centrifuged 105,000 x g 14.5Hrs.

All increases or decreases of protein were measured as total nitrogen by the micro-keldahl method described in section A. Preliminary treatment consisted of precipitation and washing in 5% TCA followed by three additional washings in chloroform:ethanol (1:3).

Sephadex Chromatography.

5.0 g of Sephadex G25 was suspended in 20mM. Phosphate buffer pH 7.0. The very fine particles were poured away with the supernatant. A 10 cm. long column was made by pouring the sephadex slowly into a tapered glass tube partially blocked with glass wool at the lower end. Even distribution of the particles is very important and this was attained by having the tube filled with water through which the sephedex grains passed. The water was dripping from the column at approximately 1 drop in 20-30 seconds, the rate of flow used during the experiments, and providing a degree of compaction stable under the experimental conditions.

To maintain the original degree of dispersion of particles, 20mM. Phosphate buffer pH 7.0 was used for every stage of the sephadex proceedure. From data supplied with the G25 it

was calculated that water regain would equal 12 g. The total volume of the column was 17 ml. This meant that there was an external volume of 5 ml. This calculation was first tested with Indian ink and it was found that 5.0 ml. could be loaded into the column and eluted completely with 10.0 ml.

The RNA released from the nuclei was applied directly to the column. The effluent was discarded and the RNA collected in the first 10.0 ml. eluate.

This RNA could be used without further adjustment for microsomal activation experiments. First traces of the soluble medium were eluted after 15-20 ml. had passed through the column.

Ribonuclease Digestion of RNA was performed on the solvent washed precipitate in 1.0 ml., of a 1 mg./ml. solution of RNase at pH 7.0. Incubation continued for two hours at 37°C. before 3.0 ml. Ethanol was added, the precipitate removed, and the absorbtion measured at 260 mμ. This procedure was applied to acid insoluble material released in media with or without ascorbic acid, as well as to

the residual nuclei.

Spectroscopy The spectra of RNA and DNA were obtained from a Perkin Elmar Infacord Spectrophorometer. Later, a Unicam SP 800 was also used. In both cases the reference cell contained true solvent and the scan was made at fast speed - 2 min. for the range 200 to 350 mμ.

The Determination of Base Composition. To determine the base composition of the released RNA the washed and dried precipitate was hydrolysed in 0.3N KOH at 37°C. for 18 hrs. before being brought to pH 8.0 with perchloric acid and then chromatogramed. The chromatogram was run in a Smith 2 way tank using either No. 1 or 20 Whatman paper. The first solvent was: -

Ammonia	2 ml.
Water	66 ml.
Isobutyric Acid	132 ml. (Pabst 1956)

After 14-16 hrs. the chromatogram frame was taken out, dried and then run at 90° to the first direction in:

Ammonium sulphate	60 g.
Sodium Phosphate buffer pH 6.8	100 ml.
n-Propanol	2 ml.

(Pabst 1956)

These two solvents give a very good separation of the 2 and 3 nucleotides released from RNA by alkaline hydrolysis.

The chromatograms of RNA hydrolysis products were examined under u.v. light and the absorbing spots cut out and eluted in 5.0ml. 0.1N HCl at overnight room temperature. The absorbtion of the solutions was measured at their maximum and at 260mμ. The base concentration was calculated using the methods of Markham (1955) and Siekevitz and Palade (1959). The former method was the maximum absorbtion and the latter the absorbtion at 260mμ.

Ultracentrifugation. A single attempt, involving three solutions, was made to obtain the sedimentation data on the nuclear incubation products. The centrifugation was made at Guinness Research Laboratories with a Spinco model E ultra-centrifuge. The supernatant solutions were dialysed 24Hrs. and concentrated by blowing cold air over the dialysis bag. Owing to the method of concentration the final salt content was not known and so it was not possible to correct the 'S' values for salt effect but in view of the nature of the salt this effect should be slight. The dialysis medium contained 200mM. Phosphate pH7.0, 10mM. $MgCl_2$, and centrifugation was at 20.8°C.

RESULTS.

The RNA released from the incubating nuclei was of especial interest because it could be easily examined free from the mixture of RNA and DNA of the intact nuclei. It was also important because it represented a possible link between the nucleus and the medium or external cytoplasm and might well be the means by which the nucleus controls the general cell metabolism.

Ribonuclease Digestion

Confirmation that the excreted material was in fact RNA was obtained by solubilising it with RNase. After the digestion with RNase and precipitation of protein and other non-hydrolysed material with ethanol, the absorption of the 80% ethanol supernatant was measured at 260mμ against a blank consisting of RNase solution similarly precipitated with ethanol (Table XXV). The quantity of RNA of the nucleus is comparatively unchanged after incubation, there being a fall of only 14%. The supernatant of the experimental nuclear incubation now contains a quantity of RNA released from the nuclei during the incubation. This gives a total increase of 64% over the zero time control. A RNase test was made on

the nuclear supernatant of an incubation mixture lacking ascorbate. RNA was found to be present but there was no value obtained for either total zero time control or for the nuclear precipitate.

TABLE XXV

RIBONUCLEASE DIGESTION OF NUCLEAR AND SUPERNATANT RNA.

Fraction Examined	Absorbtion at 260mμ	RNA % C.
Zero Time Control RNA	0.42	100
Supernatant RNA	0.36	85.7
Nuclear RNA	0.33	78.5
RNA . Total from Incubated Nuclei	0.69	164.2

Medium : 25mM. ATP, 10mM Ribose, 10mM. ethyl carbamate, 0.5mM. orotic acid, 0.5mM. guanine, 20mM. ascorbate, 1M sucrose, 0.5mM. $CaCl_2$. 50mM. Phosphate Buffer pH 7.0

Total incubation volume 4ml. Incubated 120 mins. at 32°C.

Base Composition

Even before it was discovered that RNA was released from the nuclei it was apparent that after incubation the absorbtion peak of the total RNA had moved towards 257mμ. This was repeated in extractions from three different batches of embryo. This consistent change must be due either to the nuclei themselves or to some factor in the medium. The latter possibility could be safely discounted when it was found that media containing either guanine or cytosine produced RNA with identical absorbtion spectra (Fig. 7).

When the nuclei were separated from the supernatant it was found that the nuclear RNA absorbed at 265mμ, much the same as the zero time control but the supernatant RNA absorbed at 257mμ (Fig.). The different absorbtion peaks must reflect a different base composition. Of the nucleotides released into solution by alkaline hydrolysis CMP is exceptional in absorbing at 280mμ. Cytosine can consequently constitute only a small proportion of the bases of the released RNA. GMP and AMP absorbing at 255mμ could bring the RNA absorbtion peak closer to the observed 257mμ. Several attempts were

Fig. 7. SPECTRA OF RNA FROM NUCLEI INCUBATED IN A MEDIUM CONTAINING CYTOSINE (C) OR GUANINE (G)

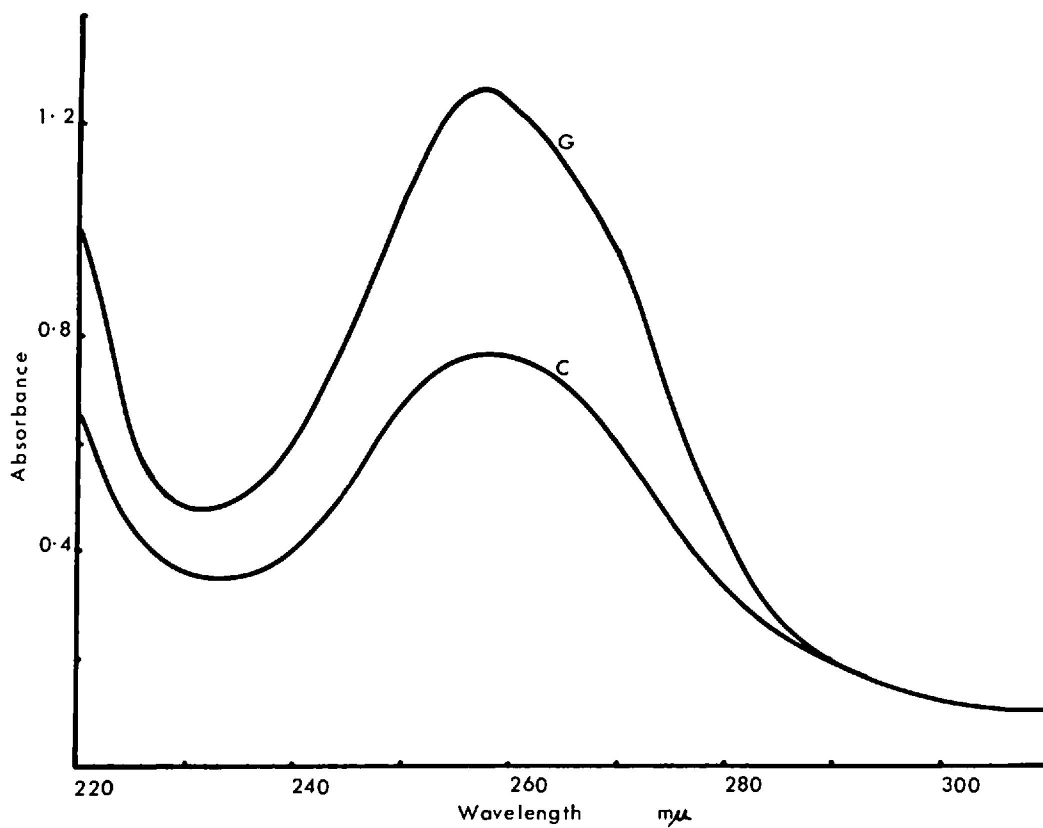
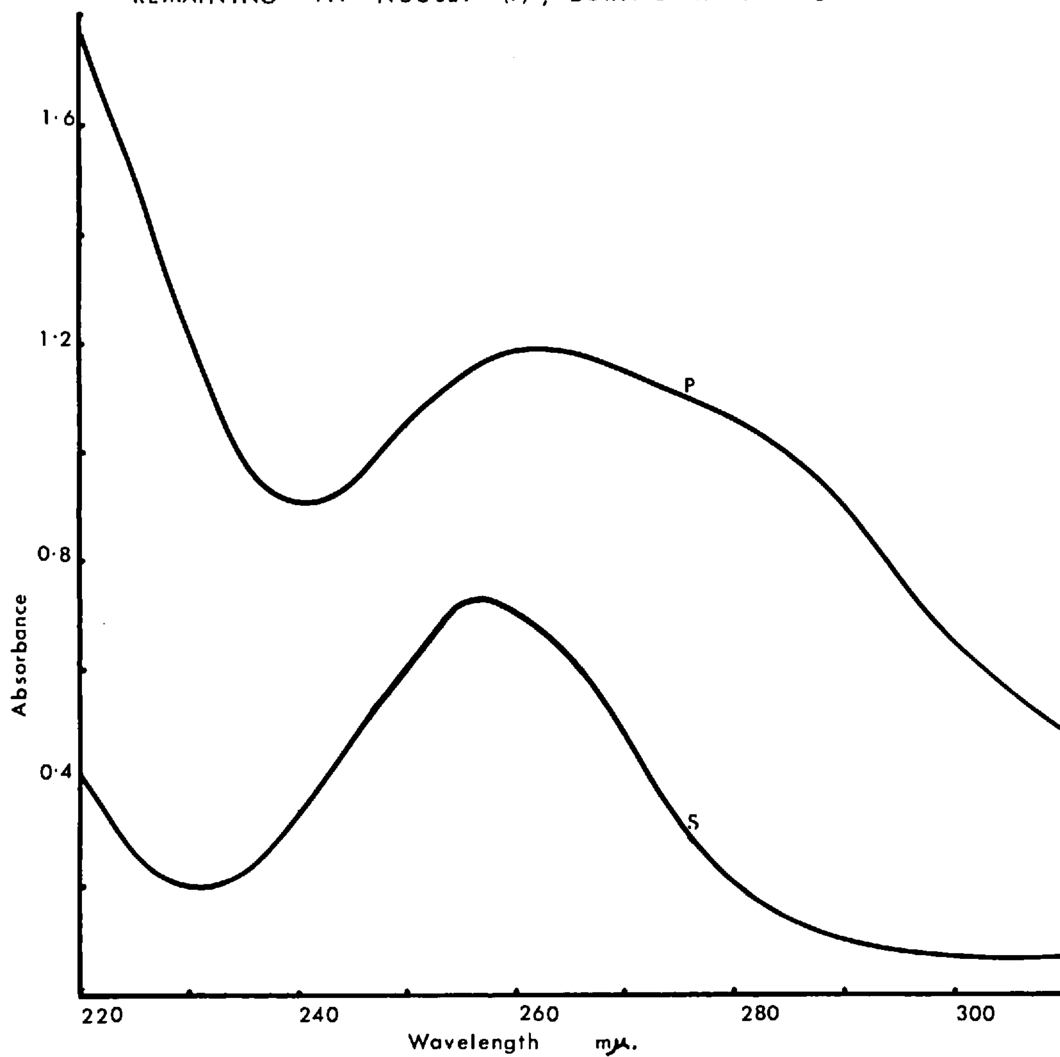


Fig.8. THE SPECTRA OF RNA, RELEASED FROM NUCLEI (S) AND REMAINING IN NUCLEI (P), DURING INCUBATION



made to hydrolyse the RNA with 0.3N. KOH to the nucleotide stage, and then chromatogram and estimate the rleative proportions of each nucleotide. Preliminary efforts yielded only faint spots of AMP and GMP but finally enough RNA was obtained to show all four nucleotides. The spots were eluted and the extinction values of Siekevitze and Markham were used to relate the absorbance to molar concentration (Table XXVI). These results confirmed the previous conclusion that the released RNA was poor in cytidine and rich in purines.

TABLE XXVI

THE BASE COMOSITION OF RNA RELEASED FROM NUCLEI AND PUBLISHED DATA ON WHEAT DNA AND RNA

Base	% Molar Concentration				
	RNA_1	RNA_2	RNA_3	RNA_3	DNA
Adenine	32.3	31.6	21.8	21.5	27.2
Guanine	37.7	36.8	29.7	31.7	22.6
Cytosine	13.0	14.1	23.6	23.3	22.8
Uracil	17.0	17.6	21.8	23.5	-
Thymine	-	-	-	-	27.4
$\frac{C + G}{A + U}$	1.04	1.03	1.22	1.22	0.83
$\frac{G + U}{A + C}$	1.21	1.19	1.13	1.23	1.00
$\frac{A + G}{U + C}$	2.34	2.16	1.13	1.14	0.99

RNA_1 RNA released from nuclei and base composition calculated by the method of Siekevitze (1959).

RNA_2 RNA released from nuclei and the base composition calculated by the method of Markham (1955).

RNA_3 Composition of soluble RNA of wheat, published by Glitz (1963).

RNA_4 Composition of ribosomal RNA of wheat, published by Glitz (1963).

DNA Composition of DNA of wheat, published by Ergle (1961).

The composition of this RNA gives no information regarding its function. If it had been identical with that of the parent DNA then there might have been reason for comparing it at once with messenger RNA (Loening 1962). This unusual base composition cannot be related to any known nucleic acid fraction from wheat (Table XXVI). The size of the molecule is of importance as transfer RNA is smaller than either messenger or ribosomal RNA.

Sedimentation characteristics.

The sedimentation analyses of RNA released during synthesis following ascorbate treatment, and RNA released during synthesis in the presence of ascorbate are given in Table XXVII and the schlieron picture in Fig. 9. Both the s values obtained are far too high to be given by transfer RNA, especially in view of the fact that in such a high phosphate concentration the RNA would be expected to coil more tightly (Hall 1958), and so sediment like a smaller particle. Another interesting point is that dialysis for 24 hrs. against 20mM phosphate buffer pH 7.0, 10mM $MgCl_2$ caused much of the RNA to precipitate. Transfer RNA was originally identified because of its solubility in water and very dilute salt solutions and methods for isolating it depend on this fact (Brubaker, 1963).

TABLE XXVII

SEDIMENTATION OF RNA RELEASED FROM NUCLEI :

I IN MEDIUM AFTER ASCORBATE PRE-INCUBATION

II IN MEDIUM WITH ASCORBATE

Supernatant	S at 20.8°C.
RNA I	8.1
RNA II	7.4

NOTE: Protein released from the nuclei during the ascorbate pre-incubation gave no schlieron figure at all.

Ascorbate Medium: 30mM ascorbate pH 7.0, 1.0M sucrose 0.5mM $CaCl_2$.

Incubation Medium: 10mM glutamate, 10mM ATP, 10mM ethyl carbamate, 1.0mM ribose, 1.0mM guanine, 1M sucrose, 0.5mM $CaCl_2$, trace $MgCl_2$, 25mM phosphate buffer.

Fig 9
THE SCHLIERON PATTERN OF RNA

Upper ; Medium without Ascorbate but with Ascorbate pre-incubation.

Lower : Ascorbate in the medium.

Only on one occasion was any protein synthesis observed to occur with RNA synthesis. On the few occasions protein was measured there was only one or the other and this fact does make it rather unlikely that the RNA formed was in fact ribosomal RNA. However it was occasionally found that even in the absence of ascorbate treatment there was some release of protein with the RNA but no ribosome nucleoprotein was observed in the ultracentrifuge

The s value of 7.4 - 8.1 is similar both to that found by Hall and Doty (1958) for ribosomal RNA and for the value for messenger RNA by Gros *et al* (1961).

The effect of released RNA on microsomes

The three types of RNA are characterised not only by their chemical composition and structure but also by their function. If the released RNA was messenger RNA then it is possible that it would aid ribosomes to produce protein. A crude microsome pellet could contain all the RNA and enzymes necessary for protein synthesis except possibly messenger RNA which is believed to have a short life (Loening 1962).

Microsomes are a cytoplasmic fraction isolated by centrifugation (Claude 1937) and later shown

to consist of endoplasmic reticulum and ribosomes (Palade 1958) when the crude "inactive" microsomes were incubated with medium there was no synthesis. If the supernatant from a nuclear incubation was added there was an increase in total precipitable nitrogen. If the RNA was precipitated from the nuclear incubation medium with alcohol then there was no increase (Table XXVIII). A very slight increase occurred if the RNA was cleaned of medium by passing through a column of sephadex (Table XXIX). The use of "active" microsomes at this time introduced the problem that the increase which occurred in the absence of RNA primer was so great that any extra-increase caused by RNA was obscured (Table XXIX).

Attempts were made to obtain a fraction of microsomes from the "active" embryo which were not so active. This was finally attained by washing either in Medium or in medium containing 0.3% sodium deoxycholate. These fractions contained very little lipid (Table XXIV) and presumably consist of ribosomes without reticulum. It has not yet been possible to add RNA to these washed microsomes.

The initial RNA content of ribosomes is often 10-100 times greater than that which is added so induced synthesis would be extremely difficult to measure without using isotopes

TABLE XXVIII

THE EFFECT OF RNA ON PROTEIN SYNTHESIS BY MICROSOMES.

Conditions of Incubation	µg Nitrogen	N2%C.
Zero control	1125.6	100%
Microsomes in medium	1100.4	97%
Microsomes + medium + RNA	1244.4	110.5%
Microsomes + medium + precipitated RNA	1156.4	102%

Medium for RNA synthesis: 10mM ATP, 10mM ribose, 10mM ethyl carbamate, 20mM phosphate buffer, 1.0mM guanine, trace $MgCl_2$, 1.0M sucrose, 0.5mM $CaCl_2$.

Medium for protein synthesis (Used in all ribosome incubations). 10mM ATP, 10mM glutamate, 1.0mM alanine, 1.0mM arginine, 1.0mM deoxyphenylalanine, 1.0mM glycine, 1.0mM histidine, 1.0mM leucine, 1.0mM lycine, 1.0mM methionine, 1.0mM phenylalanine, 1.0mM proline, 1.0mM tryptophan, 1.0mM Valine, 0.1mM tyrosine.

TABLE XXIX

THE EFFECT OF SEPHADEX CLEANED RNA ON

PROTEIN SYNTHESIS BY MICROSOMES.

Conditions of Incubation	μg nitrogen	N_2%C.
Zero control	902.07	100
Microsomes + medium	1521.80	169
" " + RNA	1578.73	175(174)
RNA only	10.69	-

Incubation media the same as Table XXVIII.

The value in brackets represents the increase of nitrogen after subtraction of the RNA added.

DISCUSSION

It has long been known that the control of cellular metabolism depends on the regulation of enzyme activity. While the enzymes can be controlled at the site of action by substrate availability, product inhibition and general metabolic inhibitors it is also possible to exercise control by limiting the supply of enzyme. The general pattern of the genetic control of enzyme synthesis is summarised by Brenner et al (1962.i,ii.). The information for the structure of the enzyme is contained in the DNA chromosome and is conveyed to the site of synthesis by means of an RNA with the exact base sequence of the DNA for that particular enzyme (uracil replacing thymine). This information-carrying RNA is called messenger RNA and it travels from the nucleus to the ribosome. The ribosomes contain an RNA which is probably only of structural significance, and cannot synthesise protein until they are primed with mRNA. Activated amino acids are transported from the free cytoplasm to the ribosome by transfer RNA (tRNA). Each amino acid has a

specific tRNA and each tRNA attaches itself to a specific combination of three bases on the mRNA. The amino acids are then joined together by enzymes on the ribosome and when complete a new protein is released and then folds into its' active form to be an enzyme. The actual control lies in the production of mRNA. This form of RNA is believed to have only a very short life and needs to be continuously released from the DNA template to re-code the ribosomes.

The RNA released from the nuclei during incubation differs appreciably from the RNA remaining in the nucleus. The first hint of this difference came when spectra of the total precipitate were found to shift the peak of absorbtion after incubation from 260-265 to approximately 257. This shift in absorbtion was confirmed by examining the spectra of the nuclear and supernatant-RNA separately. According to Cohn (1955), the four nucleotide-3-phosphates released by alkaline hydrolysis absorb at 257 mμ (Adenine and Guanine nucleotides) 262mμ (Uracil nucleotide) and 278 (Cytosine nucleotide). It is

clear that the RNA in the medium must contain either Guanine or Adenine in large quantities and very little cytosine, as absorbtion at 280 is relatively low. There is one factor involved in this analysis which may be misleading, the nuclear RNA hydrolysate may contain protein degradation products which would absorb at about 280mμ. From examination of the nuclear spectrum of Figure 8 this seems very likely, and so a part of the difference may be explained as contamination. It is clear then that if this is so, the protein released from the nucleus with RNA is different from that remaining, in that it has negligible absorbtion under these conditions.

The composition of the RNA was clearly not influenced by the bases of the incubation medium (Fig. 7) as cytosine and guanine absorb at opposite ends of the RNA spectrum but produced no changes in the RNA absorbtion peak.

It was obviously necessary to obtain the base composition by hydrolysis and chromatography. Although the method for accomplishing this had been tried and found satisfactory with commercial RNA there was the problem of obtaining enough

released RNA to chromatogram. On three occasions when chromatograms were run only one or two spots could be seen. These were probably guanine and adenine, the former compound being identified by its blue fluorescence as well as by its r.f. Eventually a preparation was obtained which gave enough material for all four base derivatives to be identified and estimated (Table XXVI). The high purine content is most unusual for RNA but does confirm the spectroscopic result obtained previously. It also perhaps explains the requirement for guanine discussed in Section A.

The base composition of the released RNA is different from that of wheat ribosomal or transfer RNA. It is likely that both ribosomal and transfer RNA are heterogeneous groups and may possess types of RNA with a base composition similar to that of the released RNA, quite different from the average values obtained for the groups as a whole.

There is additional evidence that it is not tRNA that is released from the nuclei. The wheat tRNA has a sedimentation value of 4.1

(Glitz 1963) a value characteristic of tRNA from many tissues (Luborsky 1962) and quite different from that of the released RNA. In addition tRNA is usually isolated from other cell macromolecules by dissolving in water or dilute salt solutions but most of the released RNA was insoluble in dilute salt solutions. Although the released RNA had none of the physical characteristics of tRNA the ultimate test-the ability to bind amino acids and transfer them to sites of protein synthesis- was never tried.

Ribosomal RNA usually occurs associated with protein, forming stable nucleoprotein units with relatively high sedimentation values. Glitz (1963) gave a value of 18 and 24s for the ribosomal RNA but it is possible that there was still protein attached. No partical of 18s or over was observed and there was no apparent association of protein with the RNA dispite the high magnesium concentration. The conditions for RNA synthesis were not suitable for protein synthesis possibly because of competition for substrate and ATP and so any ribosomal

RNA formed would either react with pre-existing protein or remain in a free state until such protein was synthesised.

The most likely role of the released RNA is that of transmitting the genetic information from the DNA chromosome to the site of protein synthesis. This would require that the mRNA had a base composition identical to the parent DNA (Perutz 1962) but in a highly differentiated tissue it is possible that only 20% of the DNA is passing information to the sites of synthesis (Frenster 1963). It is not unexpected that the mRNA could have a base composition totally different from that of the total DNA.

The sedimentation value for mRNA from E. coli was found by Gros et al (1961) to be 8s. This agrees closely with the value for the released RNA. Another characteristic is the very rapid rate of synthesis of mRNA not only in lower organisms but in plants (Loening 1962).

Confirmatory evidence that it was mRNA that was released from the nuclei would be obtained if it was shown able to induce protein

synthesis in ribosomes. The microsomal fraction obtained from the inert embryos showed some signs of being useful for this purpose. Very slight increases of protein were obtained when it was incubated with RNA excreted from the nuclei but nothing at all when it was incubated alone. The microsomes from the 'active' embryos were rather more difficult because they showed a high endogenous rate of protein synthesis before RNA was added. From the data on the composition and sedementation characteristics of these ribosomes (Table XXIV) it is likely that the inert material was not fully maturated and produced ribosomes lacking endoplasmic reticulum and mRNA. The 'active' embryos produced ribosomes attached to endoplasmic reticulum and already coded for protein synthesis. Washing with medium removed much of the lipid material of the reticulum but deoxycholate was needed to finally clean the ribosomes of reticulum and mRNA, leaving them dispersed and difficult to sediment.

It seems likely that the most rewarding avenue for continuing the attempt to obtain RNA stimulated protein synthesis is with the deoxycholate

washed microsomes which should contain only ribosomes (Palade 1958) and possibly associated enzymes and tRNA.

It is perhaps suprising that the RNA released from nuclei extracted from several different batches of embryo and incubated in different media had the same absorbtion peak and presumably the same peculiar base composition. This uniformity is in marked contrast to the very variable synthetic capabilities both of extracted nuclei and microsomes. It is likely that the resting nucleus is pre-conditioned so that when suitable conditions occur it can commence the synthesis of RNA for a specific purpose. It is clear that ultimate control lies in the nucleus and none of the results presented indicate how this control could be removed but it is apparent that the addition of a suitable substrate can encourage the synthesis of RNA which is then exuded by the nucleus presumably to code the pre-existing ribosomes for enzyme snythesis. At the early stages of germination of the wheat seed the bulk of enzyme synthesis may be limited to a very narrow range of enzymes

possibly involved in the utilisation of storage materials in the cell. Wheat germ is a well known source of acid phosphatase and lipase. There is also the possibility that cell structures are being synthesised, the most likely being the cellulose wall and lipoprotein endoplasmic reticulum.

It has been proposed (Brenner 1961.i) that nuclei control cell development by synthesising mRNA to code pre-existing ribosomes for a specific protein. This would require that the RNA be synthesised rapidly at the onset of rapid cell development, and that the RNA so formed, be exuded from the nuclei into the ribosome filled cytoplasm. Both these aspects are satisfied by the RNA released from wheat embryo nuclei and described in this section.

APPENDIX

APPENDIX I.

Details of Table V.

THE INHIBITION OF DNA SYNTHESIS BY GLUCOSE

Conditions of Incubation	DNA		DNA % C.
	mμME.Base	Mean	
Zero Time Control	84 79 79	81	100
Medium	(69) 86 90	88	109
Medium + Orotate	142 (66) 138	140	173
Medium + Glucose	86 75 79	80	99
Medium + Orotate + Glucose	100 - 79	90	111

Figures in brackets not used in calculation of mean.

APPENDIX II.

Details of Table VI

DNA SYNTHESIS IN THE PRESENCE OF OROTIC ACID

Conditions of Incubation	DNA mµME.Base	DNA Mean	DNA % C.
Zero Time Control	- 58	58	100
Medium + ATP	57 49	53	91
Medium + ADP	72 64	68	117
Medium + ATP + Ascorbate	55 56	56	95
Medium + ADP + Ascorbate	67 -	67	116

Figures in brackets not used in calculation of mean.

APPENDIX III

Details of Table VIII

THE EFFECT OF ASCORBATE ON DNA SYNTHESIS

Conditions of Incubation	DNA		DNA %C.
	mμME.Base	Mean	
Zero Time Control	47 51 -	49	100
Medium + 43mM. Ascorbate	44 51 46	47	96
Medium + 10mM. Ascorbate	57 50 50	52	106
Medium only	62 54 54	57	116

Figures in brackets not used in calculation of mean.

APPENDIX IV

Details of Table X

INCUBATION IN MINIMAL MEDIUM

Conditions of Incubation	DNA mμME.Base	DNA Mean	DNA %C.
Zero Time Control	35 35 -	35	100
Medium + Ribose + Carbamate	62 73 68	68	194
Medium + Ribose	90 97 (60)	94	268
Medium + Carbamate	61 70 60	64	183

Figures in brackets not used in calculation of mean.

APPENDIX V

Details of Table XI

MINIMAL MEDIUM BUT WITHOUT Mg^{++}

Conditions of Incubation	DNA mμME.Base	DNA Mean	DNA% C.
Zero Time Control	40 44 38	41	100
Medium + Carbamate + Ribose	75 78 (92)	77	188
Medium + Carbamate + Glutamate	75 71 71	22	176
Medium + Ribose + Carbamate + Glutamate	(106) 79 89	84	205

Figures in brackets not used in calculation of mean.

APPENDIX VI

Details of Table XIII

THE EFFECT OF RIBOSE AND DEOXYRIBOSE ON RNA SYNTHESIS.

Conditions of Incubation	RNA		
	mμME.Base	Mean	RNA % C.
Zero Time Control	84 81	83	100
Medium + Ribose	185 188	187	225
Medium + Deoxyribose	99 99	99	119
Conditions of Incubation	Total Nucleic Acid		
Zero Time Control	356 335	346	100
Medium + Ribose	430 410	420	121
Medium + Deoxyribose	356 336	346	100

APPENDIX VII

Details of Table XIV

OROTIC ACID INHIBITION OF CARBAMATE INDUCED RNA SYNTHESIS.

Conditions of Incubation	RNA mμME.Base	RNA Mean	RNA % C.
Zero Time Control	73 65	69	100
Medium	131 140	136	197
Medium + Orotate	129 103	113	164

Conditions of Incubation	Total Nucleic Acid mμME.Base	Total Nucleic Acid Mean	RNA % C.
Zero Time Control	172 172	172	100
Medium	370 399	385	224
Medium + Orotate	387 360	374	217

APPENDIX VIII.

Details of Table XVI

THE EFFECT OF OMMITTING BASES FROM THE MEDIUM.

Conditions of Incubation	R.N.A. mμME.Base	R.N.A. Mean	RNA % C.
Zero Time Control	- 201	201	100
Medium	333 367	350	174
Medium without Guanine	301 300	301	150
Medium without Uracil	380 348	364	181
Medium without Cytosine	382 341	362	180
Medium without Thymine	417 444	431	214
Medium without Uracil, Cytosine	426	426	212
Medium without Uracil, Thymine	509	509	254
Medium without Cytosine, Thymine	335	335	167
Medium without any bases	381 378	380	189

APPENDIX IX

Details of Table XVIII

THE INHIBITING EFFECT OF UTP ON RNA SYNTHESIS.

Conditions of incubation	R.N.A.		
	mµME.Base	Mean	RNA % C.
Zero Time Control	85 90 94 87	89	100
Medium	75 86	81	91
Medium + UTP	106 84	95	107
Medium + UTP + ATP	103 99	101	113
Medium + ATP	199 163	181	204
Conditions of Incubation	Total Nucleic Acid		
	mµME.Base	Mean	NA % C.
Zero Time Control	265 252 299 275	273	100
Medium	247 261	254	93
Medium + UTP	275 260	268	98
Medium + UTP + ATP	270 277	274	100
Medium + ATP	364 328	346	127

APPENDIX I[illegible]

Details of Table XIX

THE ASCORBATE STIMULATION OF LNA SYNTHESIS
IN AN ATP AND ADP SYSTEM

Conditions of Incubation	R.N.A.		
	mμME.Base	Mean	RNA % C.
Zero Time Control	.. 25	25	100
Medium + ATP	58 75	67	268
Medium + ADP	21 -	21	84
Medium + ATP + Ascorbate	157 142	150	600
Medium + ADP + Ascorbate	- 43	43	172

Conditions of Incubation	Total Nucleic Acid		
	mμME.Base	Mean	RNA % C.
Zero Time Control	83	83	100
Medium + ATP	115 149	132	159
Medium + ADP	93 (64)	93	112
Medium + ATP + Ascorbate	215 197	206	248
Medium + ADP + Ascorbate	110	110	133

Figures in brackets not included in mean.

APPENDIX XI

Details of Table XXI

THE GLUCOSE INHIBITION OF RNA FORMATION

Conditions of Incubation	R N A		RNA % C.
	mµME.Base	Mean	
Zero Time Control	75 69 77 72	73	100
Medium	(108) 163 141	152	208
Medium + Glucose	83 99 74	82	112

APPENDIX XII

Details of Table XXII

CARBAMATE AND RIBOSE REQUIREMENT FOR RNA SYNTHESIS

Conditions of Incubation	R N A mμME.Base	Mean	RNA % C.
Zero Time Control	38 35 38 35	37	100
Medium + Carbamate + Ribose	168 126 107	133	360
Medium + Carbamate	140 120 113	124	335
Medium + Ribose	152 100 89	114	308

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