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THE NUCLEIC ACIDS OF GERMINATING CEREALS

IN RELATION TO VERNALISATION

ABSTRACT

The main purpose of this investigation was to provide experimental evidence for the involvement of the nucleic acids in vernalisation. The effect of vernalisation on cereal growth is discussed and related to current ideas concerning biochemical aspects of differentiation. Various ways in which the nucleic acids may be estimated in plants are discussed in detail.

The RNA concentration was similar in the embryos of the spring and winter races of Petkus rye both before and after a cold treatment (3° to 5^oC) and in embryos grown at 25^oC to a stage comparable in morphological development to those in the cold. There was considerable synthesis of RNA in cold treated embryos. In the embryos of Cappelle winter wheat germinated at 25^oC, nucleic acid concentration reached a maximum after about 48 hr. at 25^oC and then decreased both in embryos kept longer at this temperature and in those transferred to the cold after 48 hr. at 25^oC. The amount of nucleic acid per embryo steadily increased in the warm over the 4 day and in the cold over the 40 day periods studied and there was little difference in content in cold and warm grown embryos of comparable dry weight. The cold treatment, however, reduced to a marked extent the rate at which nucleic acid content increased. No difference was found in the base compositions of DNA, soluble RNA and microsomal RNA of the embryos of cold grown, warm grown and ungerminated rye grain. Studies of the incorporation of C^{14} into the nucleic acids of cold and warm grown rye embryos also failed to show differences that could be attributed to initiation of the flowering processes by vernalisation. Any change related to these processes must therefore be slight.

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INTRODUCTION

The effect of the environment on the genetic material of plants has long been of interest to plant physiologists. It was first suggested by Klebs nearly fifty years ago that there was such an effect (see Salisbury, 1963) and in Russia biological thinking was dominated for many years by the claim of T. D. Lysenko that he could convert one species of wheat to another by a cold treatment. Attention was drawn more specifically to the effect of vernalisation on the nucleic acids by claims that the processes taking place during cold treatments are autocatalytic (Lang, 1952; Finch & Carr, 1956). For instance it seems that the cells produced long after the termination of the treatment are modified, indicating that the cold effect is self-perpetuating. Also the kinetics of the vernalisation response are said to be those of an autocatalytic reaction (Purvis, 1948). Since the nucleic acids are known to provide a pattern for their own synthesis, it seems possible that they are intimately involved in this process. Advances in the last decade in the theory of the action and in the biochemistry of the nucleic acids have resulted in a greatly increased volume of

research being carried out on these substances and
several studies have been made of changes in their
content in plants during a cold treatment. Results
of these investigations were inconclusive and there-
fore further investigation appeared justified.
An additional justification is that there is abundant
evidence that the nucleic acids are involved in the
differentiation of tissues and therefore it is prob-
able that they are in some way implicated in changes
occurring during a cold treatment.

In this introduction the important details
of the response of cereals to cold are summarised
and an attempt made to relate them to current ideas
concerning biochemical aspects of differentiation.
Although vernalisation has been defined as 'the
acquisition or acceleration of the ability to flower
by a chilling treatment' (Chouard, 1960), for reasons
that will be elucidated in the following paragraphs,
the term is used here only to denote the cold treat-
ment.

Numerous investigations have been carried
out of the effects of various heat and light treat-
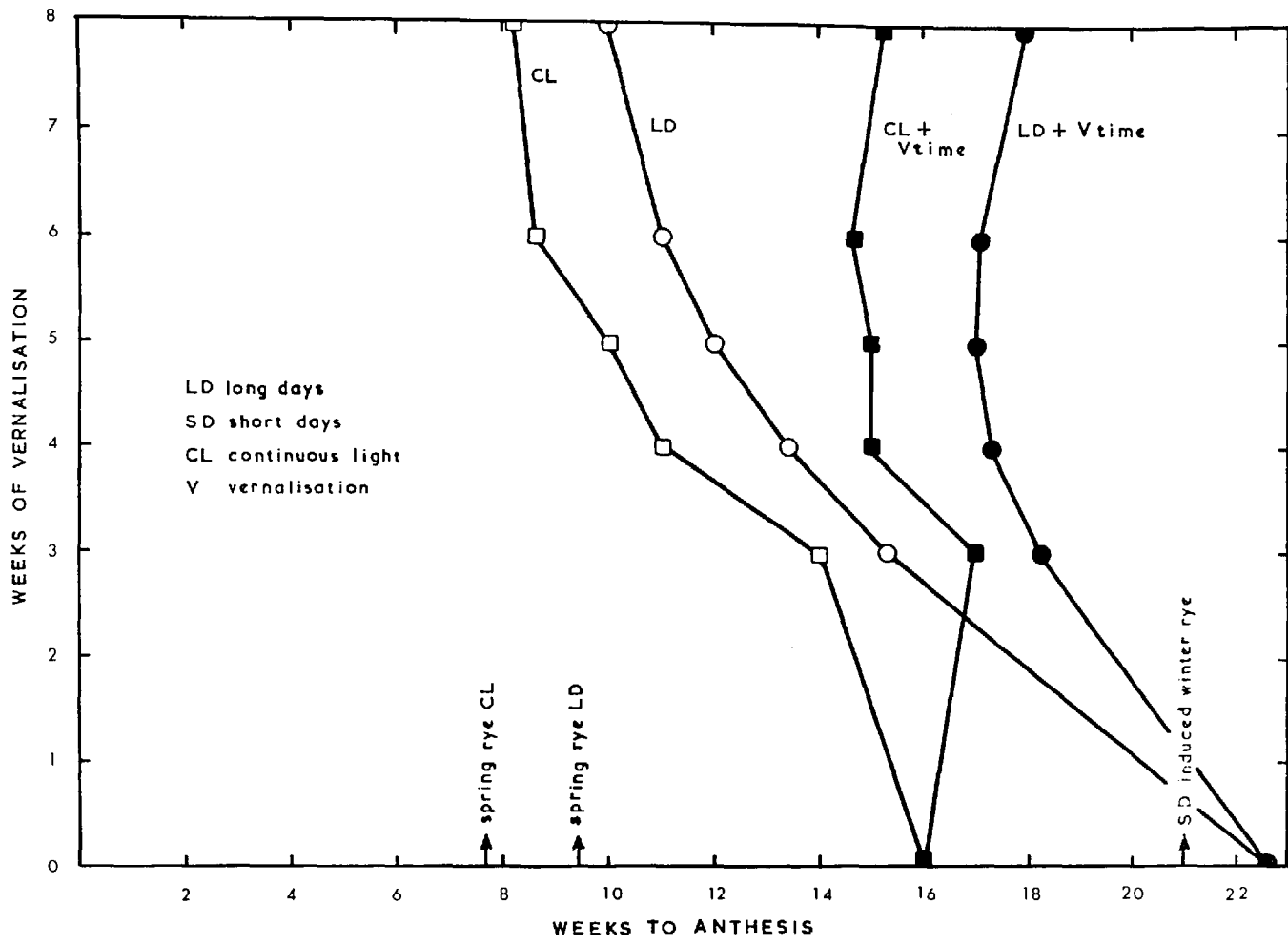ments on the flowering of the spring and winter

FIGURE I   RELATIVE TIMES TO ANTHESIS OF PETKUS RYE GRAIN AFTER VARIOUS LIGHT AND VERNALISATION TREATMENTS. DATA DERIVED FROM THOSE OF GOTT, GREGORY & PURVIS (1955)

races of Petkus rye. Since the photoinduction of flowering in these plants is directly involved with the responses to cold, the consequences of different photoperiodic treatments will also be considered.

Vernalisation is not an absolute requirement for flowering in winter rye since anthesis occurs in the absence of a cold treatment. Without vernalisation earliest flowering is attained in Petkus rye in continuous light (Gott, Gregory & Purvis, 1955). In winter rye this occurs after about 16 weeks and in the spring race in a little less than 8 weeks (Figure 1). Time to flowering steadily increases in winter rye as the daylength is decreased and in long days of 17 hours flowering occurs after about 23 weeks (Jewiss, Gregory & Purvis, 1959) and in spring rye after 9 weeks. Neither spring nor winter rye flower in days of less than 11 to 12 hr. (Purvis, 1934) but ears are differentiated within the leaf sheaths and die without emerging. The short days, therefore, do not inhibit the inception of flowering but prevent further development. In fact the initiation of flowers in unvernalised winter rye is earlier in short days

than in natural long days, though later than in
continuous light (Gott, Gregory & Purvis, 1955).
Transference to long days or continuous light after
a succession of short days (optimal duration 6 to
8 weeks) results in continued development and early
ear formation ensues.   Clearly in the winter rye
there are differences in the photoperiodic require-
ments for the various stages of flower formation.

The effect of vernalisation may be assessed
either by measurement of the time to flowering from
the end of the cold treatment or alternatively by
recording the time to anthesis from the commence-
ment of germination.   If only the time to anthesis
is considered there is, within limits, earlier
flowering the longer the duration of the low temper-
ature (Figure 1).   Maximum effect is achieved
after about 8 weeks cold treatment by which time
the grain responds in the same way as the spring
rye to subsequent photoperiodic treatments (Purvis,
1948).   In this sytem of measurement the effects
of various treatments are compared in plants grown
from embryos that have reached similar stages in
morphological development.   No account is taken
of the cold period during which reactions leading

to flowering take place and therefore it is impossible to compare the rates of such reactions occurring in cold grown plants with those grown at higher non-vernalising temperatures. In order to make this comparison total time to anthesis must be considered.

On this basis (Figure 1) vernalisation of winter rye apparently has little or no effect in reducing the time to flowering when vernalised plants are compared with plants grown throughout in optimal lighting conditions (continuous light). From this it would seem that continuous light and vernalisation promote flowering to a similar extent. The cold treatment nevertheless considerably reduces the time to anthesis when the plants are grown subsequently in 17 hr. days. Under these conditions maximum effect is achieved after 5 to 6 weeks of vernalisation when the period to anthesis is reduced from 23 to 17 weeks, while longer cold treatments delay flowering. Since the acceleration of flowering by the cold treatment is apparently not unique, the definition of vernalisation stated earlier in this introduction is not applicable and

hence the term is used only to denote the cold treat-
ment.

A pronounced result of vernalisation is
the decrease in number of leaves produced before
flowering occurs in plants grown subsequently in
either long days or continuous light.   Without
vernalisation 19 to 23 leaves are produced in a
17 hr. day and as day length is increased this
number steadily decreases to between 14 and 17
in continuous light (Jewiss, Gregory & Purvis, 1959).
With increase in duration of vernalisation there
is a decrease in leaf number, plants grown after-
wards in continuous light having about 1 leaf less
than those in long days.   In plants vernalised for
8 weeks, the leaf numbers are approximately 7 and
8 respectively which corresponds with those of spring
rye grown in comparable lighting conditions.   A
cold treatment of up to 13 weeks may further reduce
the leaf number in winter rye to 5 in some instances
when the plants are grown afterwards in continuous
light.

There is a close relationship between time
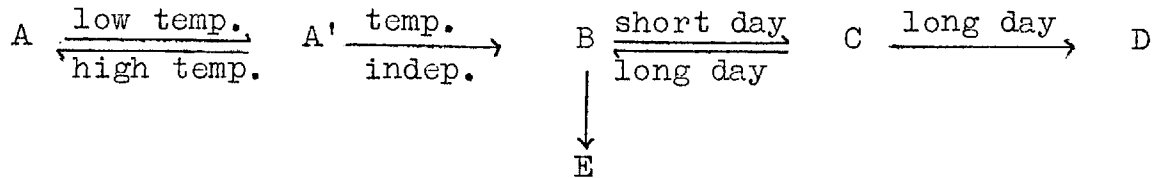to flowering and the number of leaves produced.   At

non-vernalising temperatures leaf production occurs approximately at the rate of 1 leaf per week (Gott, Gregory & Purvis, 1955) and it appears that any treatment which increases or diminishes the period of growth to flowering proportionately affects the leaf number until the maximum (25) or minimum (5) is reached. During vernalisation leaf production is suppressed and consequently final leaf number reduced.

Apparently therefore at the low temperatures progress to flowering is not affected (and may be stimulated) but vegetative growth is greatly diminished. This progress may, in fact, be entirely independent of growth since vernalisation can still be effective at temperatures of -2°C which stop growth processes (Grif, 1958). Temperatures as low as -4.5°C still have some effect while progress to flowering occurs at almost the same rate between 1° and 9°C and temperatures as high as 12°C still have a stimulatory effect in plants compared to those grown in natural long days without chilling. An additional factor supporting the contention that initiation of the processes which lead to flowering is not dependent on growth is the fact that the processes

occur in grain vernalised under conditions of restricted moisture which diminish growth. The reactions leading to flowering may occur as soon as the moisture content is increased to about 40 per cent of the dry weight. They proceed more rapidly at 45 per cent moisture but at higher levels further increase in rate is small (Lojkin, 1936). The experiments of Purvis (1940) in which she grew embryo fragments on nutrient agar, indicate that these reactions occur in the shoot apices.

A clue as to the nature of the substances formed in the cold and involved in flowering of winter rye is derived from the reversal or complete annulment of the chilling effect by temperatures above $15^{o}C$ after the cold treatment. Flowering is delayed and there is increased vegetative growth. Less and less reversion occurs as the length of time in the cold is increased and after 8 weeks vernalisation the plants are virtually unaffected by a subsequent high temperature treatment. This indicates that the product of the initial processes leading to flowering is thermolabile and that from it a stable substance is formed. To accommodate these and some of the other findings that have been

discussed, a schema has been proposed for the re-
actions leading to flowering in winter rye (for
details see Gott, Gregory & Purvis, 1955; Jewiss,
1958).

$$A \xrightarrow[\text{high temp.}]{\text{low temp.}} A' \xrightarrow[\text{indep.}]{\text{temp.}} B \xrightarrow[\text{long day}]{\text{short day}} C \xrightarrow{\text{long day}} D$$

$$B \downarrow$$

$$E$$

A is a hypothetical substance contained in
dormant grain and in the cold it is converted to thermo-
labile A' from which in turn heat stable B is formed.
It is assumed that in embryos of spring rye B is al-
ready present.   B is converted into flower initiating
precursor C from which in turn the substance res-
ponsible for spikelet development (D) is formed.   A
leaf promoting reaction is represented in the schema
as B ———➔ E.   This is to account for the increased
number of leaves produced during periods of growth
in short days.

Several criticisms of this schema may be
made.   Firstly, no account is taken of the fact
that flowering may occur in winter rye in the absence
of any cold treatment.   Secondly, the acceleration of

flowering which would seem possible when short days
are given subsequent to vernalisation does not take
place; furthermore there is no short day induction
in spring rye (Jewiss, 1958) which should also result
from operation of this schema. Thirdly, postulation
of a special leaf promoting reaction appears unneces-
sary since leaf production occurs at a steady rate
which is not enhanced by environmental conditions
which retard flowering. Finally, it is probably
incorrect in the light of current ideas concerning
the biochemistry of differentiation to consider each
stage in development as being controlled by a discrete
substance. To amplify this last point a brief
appraisal will now be made of what is known of dif-
ferentiation in plants with special reference to the
nucleic acids. Data concerning substances which have
been studied in relation to the vernalisation effect
will also be discussed.

Biochemical aspects of vernalisation

It is now clearly established that the bio-
chemical potentialities of all living organisms are
controlled by the nucleic acids. From the common
building blocks of living matter such as the amino
acids and sugar molecules, complex organisms are

assembled with characteristics which may only vary
within fairly narrow limits controlled by the nucleic
acids.   There is also good evidence that each cell
of a plant possesses in its DNA information concern-
ing all the processes of differentiation that occur
in every cell of that plant.   Therefore some mechanism
exists for the control of genetic activity which
results in selective repression and derepression of
gene activity thus making possible orderly develop-
ment.   Vernalisation, on account of its influence
on differentiation, is inevitably concerned with
this control.   It has been suggested that gene com-
plexes are associated with each stage in development
and that they are activated in relay-like manner
(Brown, 1963; Heslop-Harrison, 1963).   Flowering
appears to involve phasic developments of this kind
and in many plants the inception of each phase is
highly influenced by its age and by external factors
such as temperature and daylength.

Before discussing the way in which the
nucleic acids could be involved in vernalisation,
a summary will be made of existing information con-
cerning the mode of action of these substances.   Most

of what is known about the nucleic acids is derived
from studies of bacteria and to a lesser extent
mammalian tissues but there is no reason to believe
that plants differ greatly from these.

The nucleic acids control protein synthesis
and in this way they exert their influence on the
development of the organism.   In nucleic acid mole-
cules the constituent bases are in a linear sequence
and the positions of the bases in the sequence encode
the information necessary to align correctly the amino
acids during the synthesis of proteins.   The main
site of protein synthesis is the ribosomes.   These
are small ribonucleoprotein subcellular particles
which contain most of the RNA of the cell.   The
type of protein synthesized by ribosomes is determined
by the DNA.   Information necessary for the production
of specific proteins is transcribed into so called
"messenger" RNA which is formed in association with
the DNA.   This RNA is then transported to the ribo-
somes with which it forms a complex which is active
in protein synthesis.   The control of production
of specific messenger RNA molecules is possibly
exerted by DNA-associated histone protein.   When
the appropriate portion of this protein is

dissociated from the DNA, the bared DNA is then available to act as a template for RNA synthesis. The actual sequence of amino acids in the protein is apparently determined by the messenger RNA. Before incorporation into protein, an amino acid is first activated by the addition to it of AMP derived from ATP. The activated amino acid then reacts with soluble RNA of which there appears to be at least one species specific for each amino acid. The soluble RNA, after the appropriate amino acid has reacted with it, becomes associated with the ribosome-messenger RNA complex in a position determined by its characteristic base sequence. Peptide linkages form between the amino acids aligned by the various species of soluble RNA and in this way protein is formed. Although the broad outlines of nucleic acid operation appear now to be defined, many details still await elucidation.

In bacteria the cell regulatory processes have been extensively studied (see Umbarger, 1963). Two main types of regulation are recognised. In one the production of enzyme is affected and therefore the nucleic acids are directly implicated.

Production may be prevented in some instances
(repression) or stimulated in others (induction) by
certain metabolites.   In the other type of regu-
lation, activity of the enzymes themselves is al-
tered.   Control of this type is effected in bacteria
by the end products of biosyntheses which may, if
present in excess, inhibit the functioning of an en-
zyme early in its biosynthesis (end product inhi-
bition).   For instance repression of enzyme synthesis
occurs in Escherichia coli in which the production
of the first three enzymes required for pyrimidine
synthesis is stoppoed by the addition of uracil to
the growth medium.   In this way the end product of
a biosynthetic sequence may prevent synthesis of one
or more of the enzymes involved in its production.
An example of enzyme induction in E. coli is that
of β-galactosidase synthesis.   The production of this
enzyme is induced by growing these organisms in a
medium in which lactose or some similar substances
(but not necessarily a substrate of the induced en-
zyme) is the carbon source.

Genetic experiments have shown that specific
regulatory genes are involved in induction and

repression. The synthesis of the messenger RNA necessary for enzyme synthesis is controlled by a gene (operator) which is closely associated with that (or those) involved in messenger RNA production. When the operator gene is derepressed messenger RNA synthesis by the genes which it controls may proceed. The activity of the operators in initiating messenger RNA synthesis is in turn either prevented or promoted by the products of yet other genes. It is postulated that the substances regulating enzyme synthesis (such as the uracil and lactose cited above) affect these products. In enzyme repression for instance, they are thought to convert products which are not repressors into repressors of the operator genes (and vice versa in the case of enzyme induction).

In control of cell processes by end product inhibition the structure of the end product seldom has much in common with the structure of the initial substrate so that inhibition of the enzyme metabolising this substrate is most often non-competitive. In some instances the site of end product inhibition can be destroyed without substantially affecting the activity of the enzyme. Jacob and Monod (1961) suggest that when the inhibiting end product interacts

at a site on the enzyme it causes a change in the
configuration of the enzyme molecule which affects
its active site.

Unfortunately few studies of regulatory
processes have been made in higher plants but pre-
liminary experiments indicate that some of the controls
of enzyme activity in plants are similar to those in
bacteria.   In animals examples of both induction
and repression of biosynthetic enzymes have been
observed and therefore regulatory processes of this
type are likely to occur in plants as well.

A tentative scheme has been proposed by
Brown (1963) for the integrated action of the nucleic
acids in biochemical differentiation in plants.
With special reference to the growth of roots he con-
sidered the three main stages in cell development
which are the embryonic state, the stage of cell
expansion and the final state in which growth has
ceased.
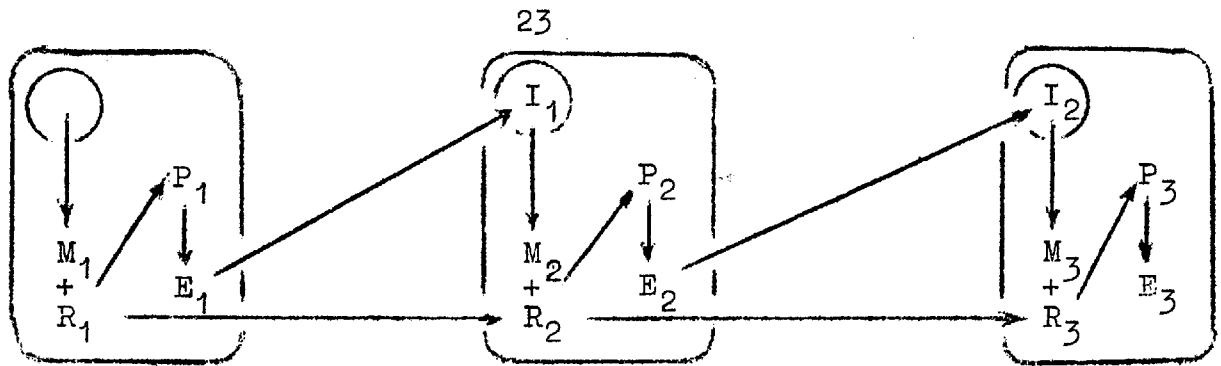
It is proposed that the changing protein complement of a cell is a consequence of a changing messenger system released from the nucleus. There is a messenger appropriate to each stage. In the initial state messenger M_1 interacts with a ribosome complex R_1. This yields a protein complex P_1 which promotes a catalytic state E_1. From this a promoter I_1 is generated in the cytoplasm and is transferred to the nucleus from which consequently another messenger M_2 is released. The second messenger reacts with a ribosome complex R_2 which has been derived directly from R_1. The product of this interaction is protein P_2 and the corresponding catalytic state E_2. In this state growth occurs and from this a promoter I_2 is generated which leads to the final state in which growth is not sustained. Such a mechanism might be visualised as occurring in any phasic plant development.

As far as experimental support for this scheme
is concerned, there is convincing evidence that the
protein complement changes as cells mature (Brown
& Robinson, 1955; Wright, 1963) and Wright also
showed that there are organ specific proteins.   The
presence of a changing messenger RNA system is less
well substantiated.   By blocking stages in flower
development using inhibitors of ribonucleic acid
synthesis Bonner and Zeevaart (1962) obtained results
strongly suggesting that specific RNA's are involved
in the various differentiation processes.   The ex-
periments of Bonner, Huang and Gilden (1963) provide
evidence that the messenger RNA's differ in different
organs of the same plant.   Concerning the promoters
of specific messenger RNA synthesis in plants, rela-
tively little is known but as in bacteria, induction
and repression of biosynthetic enzymes by metabolites
is likely to occur.

Of the compounds that profoundly affect
plant differentiation, the hormones are the most
notable.   Organised structures such as roots, leaves
and buds are initiated from unorganised pith and
callus tissue by subtle manipulations of these com-
pounds (Skoog & Miller, 1957).   Also, for instance,

auxin may markedly modify the existing differentia-
tion pattern in roots (Torrey, 1963). Recent ex-
periments with animals, insects and plants suggest
that hormones are directly concerned with the syn-
thesis of RNA. For example, the auxin mediated
expansion of cells is stopped by inhibitors of RNA
synthesis (Noodén & Thimann, 1963). Thus the role
of the hormones in differentiation could be that
of promoters (inducers of enzyme synthesis).

Cells with identical information encoded
in their DNA are affected entirely differently by
stimuli such as light, temperature and various meta-
bolites. The nature of the response depends on the
previous history of the cells. Polarity of the
fertilised egg results in dissimilarity between the
two daughter cells arising during the first division
and this difference is perpetuated during further
division. A consequence of dissimilarities of this
kind is that cells develop differing functions and
there is a division of labour among them so that they
are interdependent in maintaining normal functioning
of the whole plant. In the developing organism,
cells at one specific site influence cells (presumably

by production of chemicals) at another site in a particular relative position. Apical dominance is an example of this phenomenon.

Having now briefly surveyed what is known of biochemical aspects of differentiation in living organisms, the manner in which vernalisiation could alter such processes will be considered. One possibility is that the cold affects the nucleus causing the production of specific messenger RNA molecules which in turn promote the synthesis of enzymes of the catalytic state necessary for flower induction. Alternatively the cold could exert its influence on existing enzyme systems so that one metabolic pathway is favoured at non-vernalising temperatures and another in the cold. The resulting cytoplasmic environment may then stimulate the production of a catalytic state which would result in flowering. The various processes of biochemical differentiation include initiation of flowering but it is as yet by no means clear how many of these processes occur while vernalisation is in progress.

Floral stimulus production during vernalisation

Most schemes that have been proposed to ex-
plain vernalisation have assumed that there is pro-
duction of a floral stimulus during vernalisation
and that this stimulus is a hormone.   The involve-
ment of a growth promoting substance might be in-
timated in several ways.   For instance, by inducing
plants to flower by

> a) transmission by grafting of a flower
>   promoting substance from a plant that
>   had been induced,
>
> b) application of known hormones,

or   c) application of extracts from vernalised
>   plants.

Transmission of the stimulus

As yet grafting experiments have only been
successful with the biennial strain of henbane,
beets, cabbage scion upon annual Brassica or upon
mustard stock, carrot upon dill, and late peas upon
early peas.   In most cases, the cold effect is
strictly localized to chilled parts of the stem.
Wycherley (1952) has shown that the grass Cynosurus
cristatus when grown in long followed by short days,

produced bulbils. Detached bulbils grew vegetatively while those still attached to the vernalised mother plant produced secondary inflorescences. He concluded that the immediate vernalisation product is immobile, while the final flowering substance is transferable.

The nature of vernalisation response in rye has been studied by Purvis (1948). She removed the main shoots of vernalised rye plants and cut off the first produced tillers. The plants produced further tillers which developed from axillary buds formed after vernalisation. These new tillers behaved as if they had received a cold treatment. The cells of the auxillary buds giving rise to these tillers were, however, derived from vernalised tissue. This shows that the vernalisation response is not confined to those cells which actually underwent the cold treatment but the evidence is insufficient to decide whether a stimulus reaches the tiller cells by diffusion or whether it is self-perpetuating and is transferred to daughter cells during mitosis.

An attempt was made by Yamasaki (1947) to determine whether or not a product of wheat vernalisation

is transmissible. He grew juxtaposed halves of vernalised and unvernalised grain. Only 0.5 per cent of his sample survived and in none of these was vernalisation induced. Hurd (personal communication) attempted similar experiments with rye but had no success.

The results of the experiments cited above, although providing no direct evidence for the existence of a diffusible flowering stimulant in cereals, suggest that there is such a substance. If so the product of the processes leading to flowering in cereals could be extracted and when applied to non-induced plants should stimulate them to early flower formation. The stimulant might possibly be a known hormone since many such substances affect flowering of certain plants.

Application of known hormones

Currently the idea is favoured that flowering results from a complex of processes (Kefford, 1963) and that prevention of flowering in various plants may not always be due to the same processes. The controlling factor may for instance be a deficiency of gibberellin, auxin or kinin. Chailakjan (1959)

suggests that the flowering hormones are of two types, one type being the gibberellins which influence stem formation and growth and the other type "anthesins" which influence flower formation. Flower induction in short day species is related to the formation of deficient anthesins and in long day plants to the formation of deficient gibberellins. Although this hypothesis appears to be applicable to a large number of plants, there are long-day plants which are caulescent in short-days. It may also be noted that amongst the various species of plants examples can be found of all possible combinations of vernalisation and light requirements which lead to early flowering.

Of the plants requiring vernalisation, a few flower as a result of gibberellin treatment in the absence of chilling. This response is restricted to rosette plants and mainly those flowering on elongating terminal buds. Gibberellic acid (GA) has been shown to stimulate the progress to flowering in winter rye (Purvis, 1960; Caso, Highkin & Koller, 1960) but the stimulus was only temporary (Hurd & Purvis, 1964) even when GA was repeatedly applied.

Furthermore the GA appeared to have little effect
on the vernalisation requirement of winter rye, whether
given during the cold treatment or during the early
growth of plants before the formation of flower
initials. Early application resulted in structural
abnormalities in the ear. Although the stimulating
effect of GA on floral initiation is only temporary,
a universal feature of its action in rye plants is
the promotion of meristematic activity in the stem.
Its influence on flowering may therefore be related
to that of long day photoperiods. In the winter
rye, early application failed to promote internode
extension so it seems that the potential to extend
is restricted by some other factor which slowly loses
its influence. It may be concluded that GA is not
directly involved in vernalisation. Nevertheless
the possibility remains that one of the other gib-
berellins is concerned (Hurd & Purvis, 1964).

Photoperiodic induction is inhibited in some
short day plants by the application of IAA, NAA
2:4-D and other auxins. Conversely auxin antagonists
may cause flowering when applied under threshold
conditions (Bonner & Bandurski, 1952). Although

auxins probably play some part in flower induction, their role in vernalisation is obscure. Neither Hussey and Gregory (1954) nor Hurd and Purvis (1964) found any difference in the flowering of Petkus rye in which the auxin concentration was increased by its injection through the leaves. However, externally applied auxin increases the temperature sensitivity of various plants (Leopold & Guernsey, 1953; Lockhart, 1959) and it may therefore be involved in the perception of the cold.

The steroids have been shown to have some effect on growth and development. For instance, saponin treatment increases the growth rate of wheat embryos and promotes germination in several seeds. Of the sex hormones, several induce flowering. Bonner, Heftmann and Zeevaart (1963) demonstrated that inhibitors of steroid biosynthesis suppress flowering of Xanthium pennsylvanicum and Pharbitis nil. It is also noteworthy that steroids are found in relatively high concentrations in flowers (Heftmann, 1963). The effect of steroids on the vernalisation of cereals has not yet been studied.

Thus, despite the success that has been

achieved in affecting the flowering in some plants
by applications of hormones such methods have not
been successful in replacing the effect of vernalisa-
tion in cereals.   Of the substances studied, only
the gibberellins had any pronounced effect and this
was mainly on post vernalisation processes.

Extracts

Since there are probably numerous as yet un-
discovered hormones, a more satisfactory approach to
the problem of hormone involvement in the flowering
of winter cereals appears to be application of ex-
tracts of vernalised to unvernalised plants.   Purvis
and Gregory (1953) made water, chloroform, ethanol
and ether extracts of vernalised embryos and added
them to the substratum on which isolated unvernalised
embryos were grown.   Small accelerations of flower-
ing were obtained with the chloroform extracts, how-
ever, similar extracts made recently by Keys and
Hurd (personal. communication) did not promote flower-
ing.   Highkin (1955) reported flower promoting
activity in diffusates of peas and of spring and
winter rye.   Flowering was hastened by about 2
weeks in spring rye and the cold requirement was at

least partially replaced by the action of the
extract in winter rye. Promoting and inhibiting
fractions were separated from the diffusates and it
was suggested that guanosine was involved (see
Chouard, 1960). Recently in a similar experiment
Tomita (1962) found that a diffusate from vernalised
winter rye promoted flowering in unvernalised rye.
Subsequent attempts to isolate the active material
suggested that it had chemically much in common
with uridylic acid. Promotion of flowering was
in fact attained when uridylic acid was applied to
the leaves of unvernalised plants. The question
arises as to why, previously, aqueous extracts did
not have this effect. Hurd (personal communication)
has therefore attempted to repeat Tomita's experiments
but without success. The situation concerning effects
of the application of extracts therefore remains
confused, but recent successes in the isolation of
floral initiating substances from Xanthium (Lincoln
et al., 1964) suggest that it is worthwhile to per-
sist with this approach to the problem.

Inhibitors of flowering

Although many workers are of the opinion
that flowering follows the formation of floral in-
itiating substances, there is an increasing amount
of evidence for the existence of inhibitors of flower-
ing (Schwabe, 1956; Wellensiek, 1958; Imamura,
1959) and for the conclusion that production of the
inhibitor is reduced in the cold. It is claimed
that in short day plants an inhibitor accumulates
in the light and is lost again in darkness. For
instance Wellensiek found that when _Perilla_ plants
were kept in long days at 20°C they remained vege-
tative. If, however, the temperature was maintained
at 5°C in the light and 20°C during the 8 hours of
darkness, flower formation took place. These re-
sults can be explained by supposing that the flower
promoting process is less restricted by the cold
than the flower inhibiting process. In short day
plants also, light given to one part while the rest
is subjected to an inductive dark period partially
inhibits the flowering responses. Concerning ver-
nalisation, the inhibitory effects of heat treatments
before vernalisation and also the inhibition caused

by daily alternations of high and low temperatures
suggest that an inhibitor may be involved in this
process as well.

Before proceeding to formulate the objectives
of the work presented here, a conclusion may be
drawn from the above discussion. It is that al-
though numerous attempts have been made to under-
stand the biochemical processes which take place
during vernalisation, little evidence has been forth-
coming as to what reactions lead to the flowering of
winter cereals.

The study of nucleic acids in relation to vernalisation

At the start of this introduction several
reasons were given for believing that the nucleic
acids are involved in the processes leading to
flowering which occur during chilling. The main
purpose of the experiments described here was to
devise means of providing direct evidence of their
implication. Of the many possible approaches to
this end three were considered. They were:-

   a) to induce flowering in non-induced

      plants by the transfer of a specific

      nucleic acid from induced plants,

b) to show that there are differences in
   the nucleic acid content or in its
   composition in induced and non-induced
   plants,

and c) to prevent flowering by inhibiting the
   synthesis of RNA during the inductive
   period.

The first approach would appear to be the
only one that could provide unequivocal confirmation
of a direct effect of nucleic acids. However, tech-
nical difficulties of isolating pure, undegraded
and biologically active nucleic acid from vernalised
cereals and of administering it in a suitable way
to non-vernalised plants are very great and it was
not considered practical to do so.

The second approach is amenable to analytical
procedures and in the event most time was devoted to
investigations of techniques required for this ap-
proach. Several studies had already been made by
other workers of the nucleic acid content of vernalised
and unvernalised cereals. However, the accuracy of
the methods used is often questionable and this is
the reason that made it necessary to pay particular

attention to the selected techniques.

The third approach has been attempted
by Hurd in the same laboratory (see discussion)
and so only a single experiment is reported here
in which an inhibitor of nucleic acid synthesis was
used.

METHODS

The first objective in this investigation
was simply to compare the nucleic acid contents of
the spring and winter races of Petkus rye, before
and after vernalisation with each other and also
with that of grain germinated at higher non-verna-
lising temperatures to a stage comparable with that
of the cold-treated grain.   Later (in winter wheat)
a further study was made of changes occurring in the
nucleic acid content during a cold treatment.   The
various methods of estimating the nucleic acids are
discussed in some detail in conjunction with descrip-
tions of the methods used since considerable time was
spent in selecting suitable analytical techniques.
The nucleic acids of Petkus winter rye were fraction-
ated to see whether any change occurred in the com-
position of any one of the various types of nucleic
acid and this investigation also presented technical
problems.

By supplying radioactive nucleic acid pre-
cursors to rye embryos, it was hoped to distinguish
between the nucleic acid formed during a particular
treatment and that already present in the plants.
A chromatographic method of separating the main nucle-
otide derivatives of RNA was developed for this
project.

The methods are described in the following groupings. Growing the plant material; preparation of plant material for nucleic acid analysis; nucleic acid analysis; nucleic acid fractionation; incorporation of radioactive carbon into the nucleic acids.

Growing the plant material

Ideally the cereal seedlings should be grown aseptically at constant temperatures and moisture levels and in an atmosphere of unchanging composition. As far as was possible these conditions were realised in the first procedure devised but the method proved to be complicated and was later simplified. At first the grain was germinated in restricted moisture conditions to reduce vegetative growth and so increase the likelihood that any change in nucleic acid content or composition would be related to flower initiation, but subsequently this was also abandoned.

Method 1 About 3,000 grains each of Petkus spring and winter rye (F. von Lochow, Bergen) were selected by hand, damaged grains being discarded. Approximately 1,000 grains were weighed out into each of 6 nylon mesh bags and immersed in 50 per cent

ethanol/H_2O for 5 min.   During this time they were
transferred to a 4 x 2 x 1.75 ft. glove box, the
interior of which had been sterilised by spraying
with absolute ethanol and by the presence in it
of two Phillips 6 W. TUV u.v. germicidal tubes which
were kept on for about 2 hr. prior to placing samples
in the box.   After immersion in the 50 per cent
ethanol, the grain was washed with water and trans-
ferred to a 0.1 per cent mercuric chloride solution
for 5 min., washed again and then immersed in 0.1
per cent silver nitrate for 5 min.   The grain was
then washed with 8 changes of water.   A thousand
grains were then placed in each of 6 inverted 'Bex'
clear polystyrene refrigerator boxes, 24 x 12 x 6.7 cm.
The base of each was lined with a layer of Whatman
GF/A glass paper, and in the top and at one end,
holes were drilled into which serum caps could be
inserted.   The grain was spread out in a single
layer on the glass paper and the boxes were sealed
with a flame into polythene bags.   At the places
where there were holes in the boxes for the serum
caps, corresponding holes of slightly smaller diam-
eter were made through the bags.   The serum caps were
passed through these holes into the holes of the

boxes forming airtight seals. Each box was tested
to see if it was airtight by withdrawing air from
it through a syringe needle inserted through one
of the serum caps, any air leak being detected by a
continual flow of air from the box. After sealing
the bags the boxes were removed from the glove box and
weighed on a Mettler K7 balance (accuracy 0.03 g.).
Sufficient water was added through the serum caps
by means of a syringe to bring the moisture content
of the grain to 33.3 per cent.

Before exposing the grain to cold germin-
ation was initiated by keeping it in an oven at $15^{\circ}C$
for 24 hr. To prevent condensation of water on the
tops and sides of the boxes, they were held in posi-
tion on the underside of the oven shelves and the
sides were surrounded by a metal shield. The boxes
were then placed in the cold room (2° to $5^{\circ}C$).
Condensation of water was prevented by covering the
boxes with a layer of expanded polystyrene heat
insulating board 1 in. thick. During the cold treat-
ment which lasted 6 weeks 5 days, the boxes were
aerated at approximately 3 day intervals for the first
$3\frac{1}{2}$ weeks and then after 4 weeks 6 days and after
6 weeks. Air sterilised by bubbling through a

1 per cent mercuric chloride solution and saturated
with water vapour by passing through water, was sucked
through the boxes by means of a water pump. The
rate of air-flow was approximately 150 ml./min. for
5 min. and the air entered through syringe needles
inserted in the serum caps.

Method 2   The grain was soaked for 15 min. in a
sodium hypochlorite solution with 3 per cent avail-
able chlorine. The hypochlorite was washed away
with tap water and the grain soaked in running tap
water for 2 hr. A 10 x 14 in. piece of Whatman No. 54
chromatography paper was draped over a 10 x 10 in.
glass plate raised on four corks 0.5 in. above the
bottom of the 2.7 x 11 x 11 in. glass or polythene
dish so that both ends of the paper dipped into
water (250 ml.) in the bottom of the dish. The
imbibed grain (400 per dish) was arranged on the
paper in rows, embryos all pointing in the same
direction and facing upwards. The dishes were
covered with black polythene bags and placed in ap-
propriate constant temperature rooms to germinate.
Despite the less stringent precautions taken to
prevent infection little contamination occurred even
during lengthy cold treatments.

Method 3   Dissected embryos were grown on nutrient
agar for studies of incorporation of radioactive
carbon into the nucleic acids.   About 700 seeds of
Petkus winter rye were individually weighed and
those either much heavier or lighter than the
average were discarded.   The grain was placed in
a terylene mesh bag and immersed for 15 min. in a
3 per cent (w/w) solution of sodium hypochlorite.
During this time the grain was transferred to a glove
box which had been sterilised by u.v. light.   The
grain was then washed with about 6 changes of double
distilled water and **allowed** to imbibe for 5 hr.   The
embryos were then dissected out using a scalpel and
transferred to a nutrient agar medium (Purvis, 1944)
in 10 cm. Petri dishes, 10 per dish.   The embryos
were kept at 2° to $5^{o}C$ until they were supplied with
radioactive sucrose (see p. 93).

Preparation of material for nucleic acid analysis

In experiments in which the nucleic acid
content of cereal tissues was to be determined,
freeze-dried powders of the plant material were pre-
pared.   This facilitated collection of relatively
large samples which could be stored and analysed
when convenient.

After the grain had received the appropriate treatment it was dissected into embryo and endosperm portions and the samples immersed in liquid nitrogen in Dewar flasks. In some instances only the embryos were analysed. Freeze-dried powders of the tissue were prepared as described by Keys, Smith and Martin (1963).

Before the nucleic acids can be estimated it is necessary to remove as far as possible substances which would interfere with the reactions involved in their determination. Three different methods for the preparation of the material were used. In the first the material was transferred back and forth several times between beakers and filter funnels. In the second and third removal of interfering substances was carried out in glass columns thus obviating these transfers. The solvent systems used to extract the interfering substances were also modified several times.

Method 1 The freeze-dried material (0.3 g. embryo) contained in a beaker was extracted with boiling absolute ethanol for 2 min. and after cooling, the contents of the beaker were transferred to a Hirsch funnel in which a Celite pad had been placed. After

filtration the residue was washed with 20 ml. ethanol
and then returned to the beaker and suspended in
20 ml. 50 per cent ethanol, containing 0.01 N-acetic
acid. After standing for 1 hr. with occasional
stirring, the liquid was filtered off through the
same Celite pad and the residue washed with 20 ml.
of the ethanol/acetic acid solution and then dried
by washing with a small volume of absolute ethanol.
Finally the residue was resuspended in 20 ml.
ethanol/ether (2:1 v/v), allowed to stand for 15 min.
and filtered again on the Celite pad. The material
was then removed from the funnel and placed in the
beaker ready for nucleic acid analysis.

Method 2   The freeze-dried material (2 g. endosperm;
0.3 g. embryo) was mixed with Whatman Ashless,
Standard Grade cellulose powder, 1 g. cellulose/0.2 g.
embryo material and 1 g. cellulose/0.5 g. endosperm.
Fatty materials and some pigments were extracted
from endosperm samples with 30 ml. boiling ethanol/
chloroform (3:1 v/v). After 30 sec. the solutions
were cooled and a further 10 ml. ethanol/chloroform
added. The material and solvent were transferred to
a column of cellulose powder (9 g. packed evenly

into a glass tube 2.4 cm. diameter and 30 cm. long,
closed at the lower end by a sintered-glass plate
of porosity 1). When the liquid had drained
to the surface of the tissue powder, 60 ml. ethanol/
chloroform was added and this also drained. Sugars,
glucosides and some fatty compounds were removed by
passing 100 ml. methanol/5 per cent potassium ace-
tate (9:1 v/v pH 5.9) through the column. With
embryo material, the initial ethanol/chloroform
extraction was omitted and the material transferred
to the column with 30 ml. methanol/potassium ace-
tate and extracted on the column with a further
80 ml. of this solvent. Subsequent to the methanol/
potassium acetate extraction, the potassium acetate
was removed by passing 50 ml. ethanol through the
column. Additional extractions were necessary at
this stage with endosperm samples. Twenty-five ml.
each of a series of ethanol/water mixtures, 0.01N
with respect to acetic acid with ethanol contents of
85, 70, 55 and 40 per cent and 100 ml. of a mixture
containing 30 per cent ethanol were passed through
the column. These mixtures removed the soluble
nucleotides and a considerable amount of unidentified

material. Only the solution of 30 per cent concentration was used for embryo material. The column was dried by passing through 100 ml. ethanol/ether (1:1 v/v) and then air. The remaining plant material was removed from the top of the column and deposited in a beaker ready for nucleic acid analysis.

Method 3   The nucleic acid content in embryo material from which the soluble nucleotides had been removed for analysis was also determined (see Keys and Cornelius, 1965). A cellulose column was prepared as described in method 2 and weighed samples of freeze-dried material (approximately 0.5 g.) were mixed with 1 g. of the Whatman cellulose and packed dry onto the top of the cellulose column. The last particles of the tissue were washed onto the column by means of a 20 ml. aliquot of a mixture of methanol/1 M-ammonium acetate pH 7.5 (9:1 v/v) and consequently the elution (extraction) started of substances such as pigments, sugars, lipids and glucosides. When the solvent had drained to the surface of the tissue powder a further 60 ml. of the same solvent was added and when this had drained, 25 ml. absolute ethanol followed by a further 25 ml. were passed through the column. Nucleotides were eluted by means of 100 ml.

0.01 N-acetic acid in 30 per cent ethanol. The
material was removed from the top of the column,
placed in a sample tube and dried over calcium
chloride in a vacuum desiccator. The tissue was
extracted at room temperature (approximately $22^{o}C$)
or at $25^{o}C$ and the flow rates of the solvents were
about 1 ml. per minute.

The estimation of nucleic acids

Estimation of nucleic acid in plant tissues
presents considerable problems since large amounts
of substances are frequently present that interfere
with the determinations. As a consequence there is
at present no method which has the desirable at-
tributes of being quick, reproduceable, accurate
and simple to carry out. Chemical methods for the
estimation of nucleic acid are based on the deter-
mination of either the phosphoric acid, the purine
or pyrimidine bases, or the pentose sugars, all of
which result from DNA or RNA degradation. The
features distinguishing DNA and RNA are the pentose
constituents and the presence of thymine in the
        and
former/uracil in the latter. Unless these are
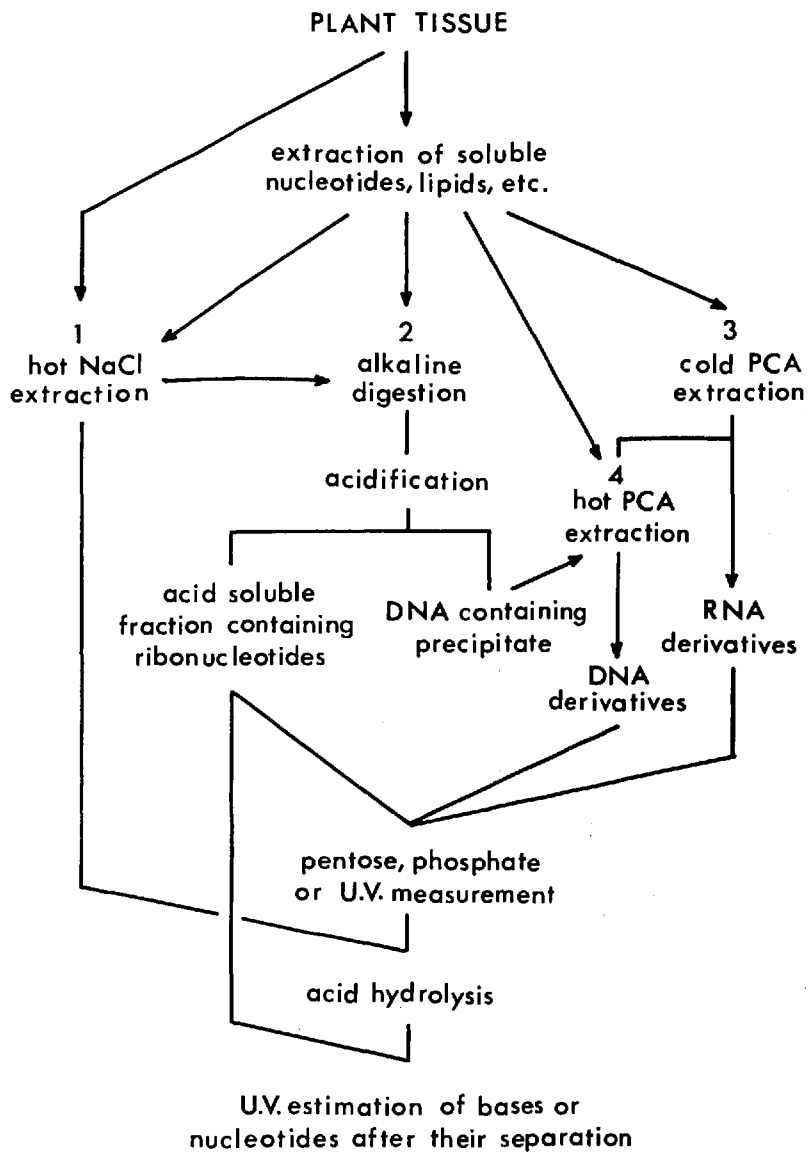specifically assayed, the DNA and RNA must be

FIGURE 2 SUMMARY OF THE METHODS THAT MAY BE USED FOR THE ESTIMATION OF PLANT NUCLEIC ACIDS

separated before determinations of phosphate content or of u.v. absorption of the bases are made. The various ways in which the nucleic acids may be estimated are summarised in Figure 2. Each of the steps numbered 1 to 4 will be discussed in turn.

1 <u>Hot NaCl extraction</u>   Nucleic acid relatively free from contamination is obtained by extracting plant tissue with hot 1.7 M-NaCl and then precipitating the nucleic acid by the addition of either cupric, lanthanum or aluminium salts, by adjusting the extract to pH 1 or by adding two volumes of ethanol. Several investigators have used this procedure for quantitative extraction of nucleic acid from plant tissues.   Martin and Morton (1956) recorded complete extraction from beet petioles with NaCl or KCl solutions (buffered to pH 4 to reduce extraction of contaminants).   Others that have used this method include Trim (1959) for the extraction of sugar beet leaves and Zscheile and Murray (1963) for the extraction of barley leaves and young wheat embryos. Kupila, Bryan and Stern (1961) compared various methods of DNA extraction from wheat embryos, tobacco leaves, wheat leaves and wheat roots.   They found

agreement between values for DNA after extraction
with either hot NaCl or alkali with those derived
from microbiological assay. Attention has been
drawn to the limitations of the method by Smillie
and Krotkov (1960). Although more than 90 per
cent of the total nucleic acid was extracted from
Euglena, NaCl failed to extract more than a trace
of DNA from Chlorella. They recommend that the
efficacy of this extraction should be tested for
each tissue to which it is applied. It does,
however, appear to be suitable for a wide range of
plant material and was therefore used.

2 Alkaline digestion   DNA and RNA differ notably
in stability in dilute alkali, RNA being degraded
to acid soluble -2'- and -3'- nucleoside phosphates
while the DNA goes into solution but is not deg-
raded. This difference in behaviour forms the basis
for the Schmidt-Thannhauser procedure (1945) for esti-
mating nucleic acids. For analytical purposes only
sodium and potassium hydroxides have been used and
there are considerable differences in the exact con-
ditions for hydrolysis described by various authors.
                                    with
Concentrations of alkali/normality between 1.5 N
and 0.05 N, temperatures between 5° and 100°C and

times between 15 min. and 48 hr. have been used.
Some controversy moreoever still exists as to whether
or not DNA is stable to alkali.   From the evidence
available Hutchinson and Munro (1961) conclude that
pure DNA is stable to N alkali at 37°C and probably
at considerably higher temperatures but that condi-
tions introduced accidentally during extraction of
lipids and acid soluble components may lead to the
formation of apurinic acid which is susceptible to
alkaline degradation.   Ingle (1963) found that 50
per cent of the DNA of three day old etiolated corn
embryos was lost as a result of alkaline digestion.

When the alkaline digestion is applied
directly to plant tissue considerable quantities of
material are removed in addition to the ribonucleotides.
Hutchinson and Munro (1961) suggest that to reduce
the amount of such "impurity" the hydrolysis time
should be reduced from the 18 hr. originally pro-
posed to 1 hr.   After this time the RNA is apparently
sufficiently degraded to be acid soluble.   Millikan
and Pickett (1963) digested plant leaf tissue with
0.33 N-KOH at 37°C.   They concluded that RNA
hydrolysis/is complete in 7 hr. but that shorter times

were insufficient. After a similar study, McLeish
(1963) decided that a 3 hr. hydrolysis period with
1 N-NaOH at 37°C was sufficient to hydrolyse the
RNA in onion cells and nuclei. The exact conditions
for hydrolysis must clearly be established for each
tissue investigated. As shown in the methods summary
chart, the precipitate obtained after hot NaCl ex-
tract of plant material may also be subjected to al-
kaline digestion.

Much of the material which interferes with
measurements of ribonucleotide concentration may be
eliminated by ion exchange resin (de Deken-Grenson
& de Deken, 1959; Smillie & Krotkov, 1960; Ingle,
1963). This procedure was therefore adopted when
whole tissue digests were being analysed. It was,
however, unnecessary after hydrolysis of RNA extrac-
ted by hot NaCl.

3 Cold perchloric acid extraction of RNA Ogur and
Rosen (1949) proposed a method for determining small
amounts of nucleic acid in plant material. The DNA
and RNA were differentially extracted, the RNA by
cold PCA and the DNA by hot PCA. There is consider-
able evidence that this procedure, although suitable

for some tissues, is not satisfactory for a large
number of others.   The main source of error appears
to be the incomplete extraction of RNA by the cold
PCA.   There seems little to recommend it and there-
fore it was not used.

4 <u>Hot acid extraction of the nucleic acids</u>   Schneider
(1945) extracted nucleic acid with 5 per cent TCA
at $90^{\circ}C$.   The DNA and RNA were thereafter measured
by reactions specific for deoxyribose and ribose.
Numerous modifications of this procedure have been
made including the use of PCA, extraction at lower
temperatures, and the use of u.v. absorption or phos-
phate measurements for estimates of total nucleic
acid content.   The Schneider procedure has proved
attractive because results are obtained relatively
rapidly.   However, it can result both in incomplete
extraction of DNA and in the destruction of deoxy-
ribose.   Furthermore other substances absorbing u.v.
light, interfering with colorimetric sugar reactions
and phosphate measurements are extracted from plant
materials (Smillie & Krotkov; Ingle).   For ins-
tance Ingle obtained RNA values 100 times too high
when the orcinol method of ribose estimation was

applied to extracts of barley embryos.   The Schneider
procedure has, however, found wide application in
combination with the Schmidt-Thannhauser procedure.
The DNA and most of the protein are precipitated
from the alkaline digest on acidification and the
DNA is extracted from this precipitate with hot PCA
and subsequently estimated.   This hot acid extrac-
tion of DNA appears to have much to recommend it
providing the DNA is subsequently estimated by a
suitable method.   Although not used here, it has
been used with success in another investigation.

Determination of RNA

Ribose   The ribose content of RNA has been determined
in a number of ways including those involving re-
action with orcinol, phloroglucinol, aniline, car-
bazole, cysteine and anthrone.   Of these only the
reaction with orcinol has been widely used.   Because
of the large amounts of polysaccharide present in
plants which also react with orcinol, no reliable
estimate can be made of the ribose in RNA (Smillie
& Krotkov;   Ingle) unless purification is effected
using ion exchange resins.   Ingle showed that even
after passage through anion exchangers, values for
RNA content based on ribose estimates were considerably

higher than those based on u.v. measurements. This
method of estimating RNA is therefore unsatisfactory
for most plant tissues and was not used.

Phosphate   Plants also contain large amounts of
substances that interfere with estimates of phosphate
content of RNA.   As with the substances affecting
ribose determinations, the interfering phosphates
also persist to some extent after ion-exchange
purification.   Values for RNA phosphate content
of plant tissues should therefore be regarded with
the same caution as those derived from ribose esti-
mation.

Ultra-violet spectrophotometry   The intense and
characteristic absorption of u.v. light provides
a method of measuring the content of undegraded RNA,
of ribonucleotides and of the purine and pyrimidine
bases.   The use of u.v. light adsorption is compli-
cated by the fact that proteins and peptides also
absorb u.v. light at similar wavelengths and u.v.
absorbing substances other than nucleic acids are
extracted by both alkali and PCA.   As yet no esti-
mate appears to have been made of materials present
in hot aqueous sodium chloride extracts which are not

nucleic acids but which contribute to u.v. light
absorption.   It has been noted that such extracts
of cereal embryos are often turbid and therefore
liable to give erroneous spectrophotometric measure-
ments.   Both Ingle and Smillie and Krotkov report
satisfactory values for RNA derived from measurements
of u.v. absorption subsequent to anion exchange puri-
fication after alkaline hydrolysis.   This method
is also recommended by Hutchinson and Munro (1961).
One difficulty lies in the fact that a suitable
standard of pure plant RNA is required.   It is per-
haps preferable to determine amounts of the individual
nucleotides or bases in the hydrolysate, and this
method was used in this investigation (see p. 66).

Determination of DNA

<u>Deoxyribose</u>   Of the numerous methods of deoxyribose
estimation, only the diphenylamine reaction appears
to have found general use (e.g. Smillie & Krotkov;
Ingle; Kupila, Bryan & Stern, 1961; Heyes, 1963;
McLeish, 1963) and is the predominant method by
which DNA is measured in plant tissues.   Although
satisfactory in most circumstances, Lee (1963) has
pointed out the need for careful checking of absorption

spectra, because carbohydrates occurring in certain plant tissues were found to lead to erroneously high results. The method, however, appears to be one of the best for the determination of DNA.

Phosphate   The estimation of DNA by its phosphate content has been little used with plant tissue.   In combination with the Schmidt-Thannhauser alkaline digest, Ingle found reasonable agreement between estimations of DNA derived from amounts of phosphate and deoxyribose with growing barley tissue but not with the endosperm.

Ultra-violet spectrophotometry   Following alkaline digestion of the RNA of barley embryos and of barley and corn endosperms, hot acid extracts of these tissues had no distinct absorption maxima at 260 mμ. Although the spectra of extracts from corn embryos and yeast extracts had slight peaks at 260mμ, estimates of DNA content based on these absorption values were up to 15 times higher than those based on the diphenylamine reaction (Ingle).   However, Nieman and Poulsen (1963) have successfully applied this method to leaves of several plants.

Estimation of individual bases

In theory, unequivocal values for the amounts of RNA and DNA present in tissue can be obtained by isolation and measurement of the bases in the constituent nucleotides. All the procedures for quantitative estimation of bases or nucleotides are more complicated than those for nucleic acid analysis and are therefore not suitable for analyses of large numbers of samples.

Both DNA and RNA may be degraded to the free bases by rigorous acid hydrolysis. RNA may also be degraded to ribonucleotides by alkaline hydrolysis and a mixture of purine bases and pyrimidine nucleotides is obtained by further hydrolysis with dilute acid. All these ways of hydrolysing the nucleic acids were used in the experiments to be described and they will therefore be discussed in some detail.

Hydrolysis to the bases A commonly used procedure for liberating the bases is that of Marshak and Vogel (1951) in which the nucleic acids are digested with 72 per cent PCA at $100^{O}C$. DNA is completely hydrolysed in 1 hr. but 2 hr. is usually required for the

hydrolysis of pyrimidine ribosides of RNA, especially
when protein is also present. Crosbie, Smellie and
Davidson (1953) hydrolysed a specimen of yeast RNA
with PCA and after paper chromatography recovered 86
per cent of the phosphorus. Most of the loss occurred
in the uracil fraction. Conditions for complete
hydrolysis of DNA by PCA have been investigated by
Emanuel and Chaikoff (1958) who established what
were the optimum conditions. Both time of heating
and volume of acid affected the amount of base re-
covered, thymine being noticeably destroyed by pro-
longed heating and both purine and pyrimidines dim-
inishing when excessive acid was present. With the
optimum conditions 98 per cent of the bases were re-
covered. Although not entirely satisfactory, this
is the method giving highest yields of the bases
of RNA.

Liberation of the bases of DNA may also be
accomplished by hydrolysis with concentrated formic
acid at 175°C. Although excellent recoveries are
obtained, practical difficulties precluded its use
here.

Mild acid hydrolysis of RNA   Hydrolysis of RNA in
1 N-HCl at 100°C for 1 hr. yields products that

include purine bases and pyrimidine nucleotides.
The pyrimidine nucleotides are degraded to some ex-
tent and deamination of the cytidylic acid at the rate
of 3 to 4 per cent, per hour has been reported by
Loring and Ploeser (1949).   The main source of
error, however, arises from dephosphorylation of the
nucleotides.   Levene and Jorpes report (1929) that
as much as 15.5 per cent of the phosphate may be
liberated from pure cytidylic acid.   The effects of
"impurities" on this breakdown does not appear to
have been studied, but since impurities have a marked
effect on the products of PCA hydrolysis of RNA, their
influence may be considerable here.   Markham and
Smith (1951) recommend the use of a correction factor
to compensate for this hydrolysis to nucleosides.
It is advantageous that HCl can be removed by eva-
poration after the hydrolysis, and the products are
readily separated by paper chromatography.   Precipitates
are moreover readily dispersed.

Alkaline hydrolysis of RNA   Although treatment with
alkali renders RNA acid soluble fairly rapidly, com-
plete resolution to mononucleotides is more difficult,
even after lengthy hydrolysis periods.   In the

hydrolysates of some organisms 3 to 5 per cent still

remain in oligonucleotides. As a result there is

no difinitive conclusion as to the hydrolysis time

and concentration of alkali necessary for total

hydrolysis to be achieved. Examples of concentra-

tions that have been used with plant tissue are

summarised below.

Concen-tration of alkali (N)	Temp. ^oC	Time hr.	Tissue	Author
0.3	37		Pea roots	Loening (1962)
1.0	25	24	Barley embryos	Ingle (1963)
0.5	30	40	Barley leaves wheat embryos	Zscheile & Murray (1963)

Action on 1 mM-monoribonucleotides of 1 N-KOH at

37°C was studied by Röttger and Fritz (1962). CMP,

UMP and AMP were all destroyed to some extent and there

was also extensive deamination of CMP to UMP.

Correction factors were devised to compensate for these

losses and it was established that hydrolysis of RNA

to mononucleotides was complete after 22 hr. The

same time was necessary for RNA from various sources

including tobacco leaves. These authors concluded

that the presence of proteins and carbohydrates does

not interfere with the hydrolysis or increase des-

truction of the nucleotides. It is difficult to

reconcile these results with those obtained by Crosbie,

Smellie and Davidson who recovered 97 per cent of

yeast RNA in the form of mononucleotides after alkaline

hydrolysis and electrophoretic separation.

The main advantage of this method of hydroly-

sis is that none of the components of the RNA are

lost, thus making possible the use of radioactive

isotopes such as P^{32} as tracers. Difficulty, how-

ever, was encountered since no convenient paper chro-

matographic method for the separation of the mono-

nucleotides was available.

In conclusion, the most satisfactory method

for estimating RNA of plants appears to be that in

which the u.v. light absorption of the individual

nucleotides or bases is measured. The plant DNA

may probably best be estimated either by the dipheny-

lamine reaction or the bases measured by u.v. spectro-

photometry after hydrolysis. Partly compensating

for the complexity of the estimation by u.v. absorption measurements of the bases is the additional information that is derived concerning the base composition.

The two procedures used are set out below. In the first only RNA content was measured and in the second both DNA and RNA.

1 <u>Estimation of RNA after alkali extraction</u>   Twenty-five ml. 0.3 N-KOH was added to the material prepared by methods 1 and 2 (pp. 46 & 47).   In early experiments the material was kept in KOH for 18 hr. at 37°C but later the time was reduced to 1 hr. at the same temperature with satisfactory results.   The beakers containing the material were cooled in an ice bath and the alkali neutralised by addition of 1 N-perchloric acid.   The supernatant was adjusted to pH 2 with HCl and when the period in KOH was 18 hr., ethanol was added to make the final concentration in the supernatant 50 per cent.   The liquid was filtered off through a Celite pad in a Buchner funnel and the residual material washed several times with small volumes of cold 0.01 N-HCl.   The combined filtrates which contained the hydrolysis products of RNA were

concentrated to about 20 ml. using a rotary vacuum

evaporator. To remove some of the impurities present,

the solution was adjusted to pH 8 to 10 with 1 N-NH$_4$OH

and passed through a Dowex-1 x 4 ion exchange resin

in the chloride form (2 g. in a column 1.2 cm. in

diameter). The nucleotides were absorbed by the

resin while a considerable amount of contaminating

material passed through the columns. The nucleo-

tides were eluted with 25, 5 and 5 ml. aliquots of

2 N-HCl. Further amounts of contaminating material

remained absorbed on the resin. The eluates were

dried using a rotary vacuum evaporator, the residue

dissolved in a little distilled water and the eva-

poration repeated. The residue was then dissolved

in either 5 or 10 ml. 1 N-HCl (depending on the

concentration of the nucleic acid derivatives present)

and the solution transferred to a Pyrex test-tube,

sealed and heated for 1 hr. at 100°C in a boiling water

bath. An aliquot of the hydrolysate was pipetted

into a small test tube and dried over calcium chloride

and sodium hydroxide pellets in a vacuum desiccator.

The hydrolysis products were then dissolved in small

volumes of 0.1 N-HCl and transferred using a capillary

melting point tube (see Keys, 1963) to form a 1 in.
streak on Whatman No. 1 chromatography paper that
had been washed overnight by elution with 1 per cent
oxalic acid followed by water. The chromatogram
was developed for about 17 hr. by isopropanol/conc.
HCl/water (170:41:39 v/v) mixture. RNA derivatives
were located on the paper under u.v. light (Markham
and Smith, 1949). Areas of paper containing the
various compounds to be estimated were cut out, cut
into strips and placed in clean, dry test tubes.
To elute the compounds, 5 ml. of hydrochloric acid
of the appropriate normality was pipetted into each
test tube, the tubes covered with 'Parafilm' (A.
Gallenkamp & Co.,Ltd.), shaken well and left to stand
overnight. Elution by this method was found some-
times to be incomplete (Keys, private communication).
Therefore in subsequent experiments the tubes were
packed into a cylindrical container and rotated over-
night at 8 revolutions per minute while inclined at
an angle of 80° from the vertical. The contents
were then mixed by inversion of the tubes to recover
water condensed at the upper ends. The absorbancies
of the solutions were determined at the appropriate

wavelengths with a spectrophotometer. For paper
blanks, paper adjacent and equivalent in area to
that containing the compounds was cut out and
similarly eluted.

The acidic extracts of chromatography
paper and the substances other than the bases and
nucleotides on the chromatograms, exhibited irregular
absorption in the u.v. For this reason it is pre-
ferable to estimate the contents of extracts by the
difference in the extinction values at the absorption
maximum and at another suitable wavelength rather
than taking the absolute extinction values at the
absorption maxima as a basis for calculations
(Vischer & Chargaff, 1948). The concentrations
of acid used for eluting the bases and nucleotides,
the wavelengths at which extinction values were de-
termined and the difference between these values for
standard solutions containing 1μmole/ml. are shown
overleaf.

Substance	Conc. HCl (N)	Absorp. Max. mμ	Ref. wave-length mμ	Diff. in 1 μmole/ml. E_1 cm. at the two wavelengths
Guanine[1]	1.60	249	290	8.15
Adenine[1]	0.1	262.5	290	12.64
Cytosine[1]	0.1	275	290	6.07
5-Methylcy-tosine[2]	0.1	283	310	7.09
Uracil[1]	0.1	259	280	6.83
Thymine[2]	0.1	265	290	6.93
Cytidylic acid[1]	0.01	278	300	10.7
Uridylic acid[3]	0.1	260.5	280	6.94

References: 1. Crosbie, Smellie & Davidson (1953)

2. Bendich (1957)

3. Wyatt (1955)

Each determination was carried out several times and any result greatly different from the rest of the replicates was rejected. A typical set of results is shown below. The content of the predominant bases in the RNA of rye embryos is expressed in μmoles per g. dry weight.

G	A	U	C	$\dfrac{G + U}{A + C}$
11.6	11.4	10.1	12.6	1.02
13.1	10.7	10.8	12.2	1.04
16.4	12.1	9.9	12.7	1.06
14.8	11.1	9.8	12.2	1.05

The variation in estimates of cytidylic acid is the least and in estimates of guanine the most. It has been shown for RNA of a large number of tissues including plants that the ratio of 6-keto (guanine and uracil) to 6-amino (adenine and cytosine) bases is unity and therefore any estimates which diverge greatly from this value must be viewed with suspicion. The ratios calculated from the above results approximate closely to unity, but at the same time the proportions of the bases in each pair may vary considerably, less of one compensating for more of another. It seems advisable therefore to take into account both the precision of replication and the base ratio when evaluating results derived by a method such as this.

While the quoted results are sufficiently

accurate to show any major change in either the base
composition or the amount of RNA, they would not
reveal the type of subtle change that might be ex-
pected to be involved in vernalisation.   Several
attempts were therefore made to develop a more
satisfactory method.   A major source of variability
was the u.v. absorbing material eluted with the
nucleotides.   Despite the use of short hydrolysis
times and ion-exchange purifications, contamination
by such substances was still marked.   Undoubtedly
extraction of the nucleic acids with hot NaCl greatly
reduced this contamination and was therefore chosen
as being the most likely means by which the results
could be improved.

2 Estimation of DNA and RNA after hot sodium chloride
   extraction   Whatman cellulose powder (1 g.) was
packed evenly into a glass tube, 30 cm. long and
2.4 cm. diam., closed at the lower end by a sintered
glass plate of porosity 1.   The capillary outlet at
the lower end of the tube was bent into a U-shape
so that the outlet was about 20 cm. above the lowest
part of the tube.   The tube was placed in a water-
bath about 15 cm. deep with a length of polythene
tubing leading from the outlet to a receptacle

outside the water-bath. The tissue as prepared by
method 3 (p. 49) was packed onto the top of the
cellulose column and the last particles of tissue
were washed onto the column with about 20 ml. ab-
solute ethanol. (In some cases the preparation of
the material using the solvent system described in
method 3 was carried out on this column thus obviat-
ing the transfer from one column to another). The
ethanol was expelled by increasing the air pressure
on the top of the column by means of a Dymax Mk 1
pump (Charles Austen Pumps Ltd.). An ethanol/ether
mixture (50 ml.) was added and allowed to permeate
the column for 5 min. before being expelled, then
50 ml. ether were added and expelled after the same
time. Air was blown through the column for about
30 min. during which time the temperature of the water
bath was raised to 100°C. Extraction of the nucleic
acids was accomplished by adding 20 ml. 1.7 M-NaCl
to the material and mixing it with this and the under-
lying cellulose powder using a glass rod with an ex-
panded end of diameter slightly less than that of the
tube. After 30 min. at 100°C. the liquid was expelled
from the tube and collected in a 250 ml. centrifuge

bottle.   The material on the column was extracted
a further 4 times using 12 ml. NaCl solution for 30 min.
on each occasion.   The combined eluates were mixed
with an equal volume of absolute ethanol and stored
overnight at 3° to 5°C.   The precipitated nucleic
acid was collected by centrifugation (MSE 'Magnum'
Centrifuge 1610 x g. for 15 min.) and dried by wash-
ing with acetone and placing in a vacuum desiccator.

The nucleic acids were hydrolysed with
0.2 ml. 72 per cent perchloric acid by heating for
2 hr. at 100°C in the centrifuge bottle which was
stoppered with a cork after the perchloric acid had
been added.   To avoid cracking the bottle it was
placed in cold water which was then brought to 100°C.
When the hydrolysis was complete the bottle was
cooled, 5 ml. water added and the acid neutralised
with 50 per cent KOH.   The liquid containing the
bases was then separated from the charred residue by
filtering through a sintered glass funnel of porosity
4.   The residue was washed 4 times with 3 ml. aliquots
of 1 N-HCl and the combined filtrates dried using
a rotary vacuum evaporator.   The relative amounts of
RNA and DNA were determined by measuring the quantities

of uracil and thymine produced.   The amount of cyto-
sine was also determined as a measure of total nuc-
leic acid content.

The bases were separated by paper chroma-
tography (Whatman 3MM) in experiment 2 (p. 111) and
by column chromatography in experiment 3 (p. 111) using
the system described by Cohn (1949) and Dowex-1 x8
ion exchange resin in the chloride form (200-400
mesh in a column 8.5 x 0.74 cm^2).   The bases were
transferred to the column with 1 x 4 ml. and 2 x 2 ml.
aliquots of the eluting buffer (0.2 M-NH$_4$OH + 0.025 M-NH$_4$Cl,
pH 10.6) and elution carried out at room temperature
(about 22°C) with buffer flow rate of 0.25 ml./min.
The fractions containing each base were collected
in 150 ml. beakers and dried at 96°C in an oven in
which the air was circulated by means of a fan (A.
Gallenkamp & Co., Ltd.).   The cytosine and uracil
were each taken up in 5 ml. 0.1 N-HCl and appropriate
aliquots diluted with acid of the same normality so
as to give O.D. readings of between 0.5 and 1 at the
absorbancy maxima.   Thymine was dissolved in 5 ml.
0.1 N-HCl and estimated without further dilution.
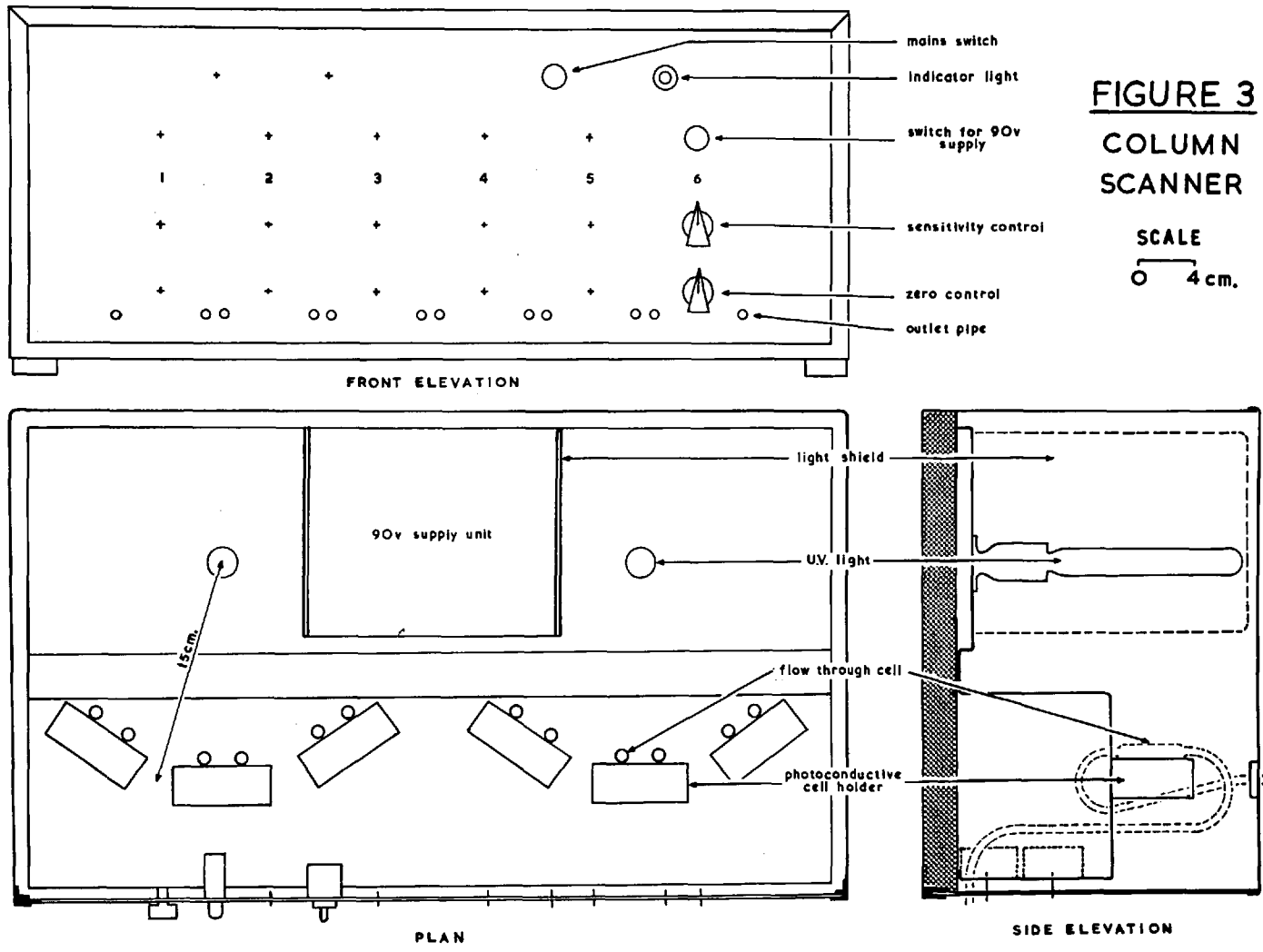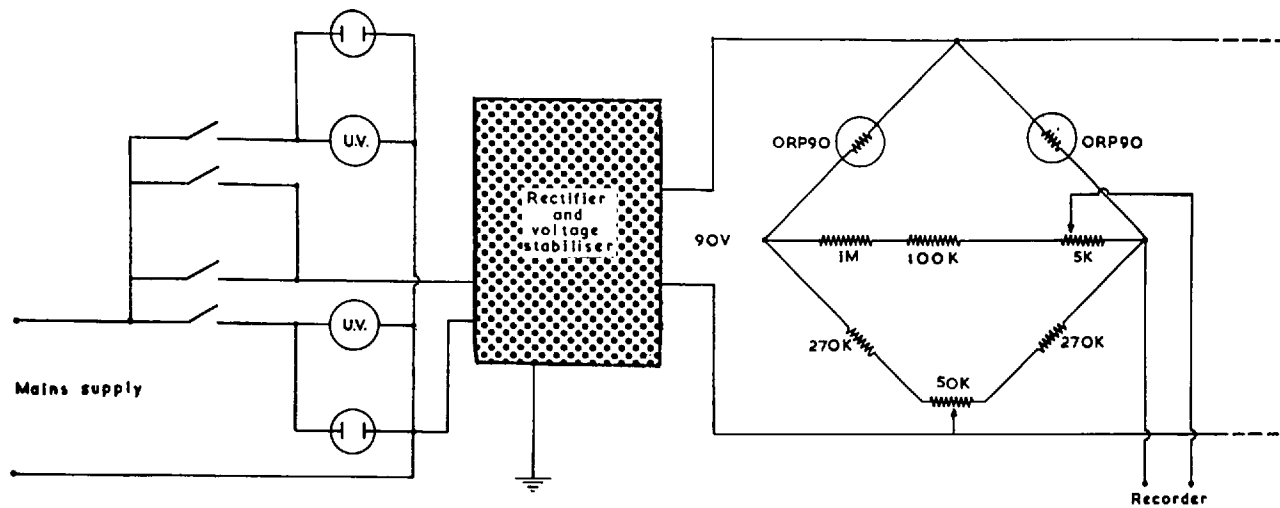The wavelengths at which measurements were made are

FIGURE 3

COLUMN
SCANNER

SCALE

○———— 4 cm.

FRONT ELEVATION

- mains switch
- indicator light
- switch for 90v supply
- sensitivity control
- zero control
- outlet pipe

PLAN

- light shield
- 90v supply unit
- U.V. light
- 15cm.
- flow through cell
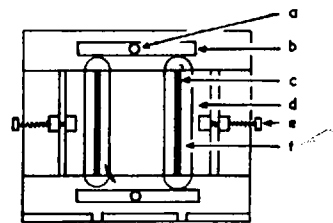- photoconductive cell holder

SIDE ELEVATION

given on page 70.

Detection of the nucleic acids and their
derivatives as they are eluted from a column presents
some difficulties. Fractions of the effluent may
be collected and then their absorbancies determined
using a spectrophotometer. A more suitable method
is to record at frequent intervals absorbancy of
the effluents and apparatus for this purpose was
therefore constructed. The main elements of the
apparatus were a 6-channel pen recorder and a column
(effluent) scanner by which the absorbancy of the
effluents of up to six columns could be determined
simultaneously. The design of the scanner was based
on the description of Begg (1961). For each channel
(Figure 3) a u.v. light source (Phillips TUV
6 W. low pressure mercury lamp) was sited 15 cm. from
two photoconductive cells (Mullard ORP 90) included
in a Wheatstone bridge circuit. Flow-through cells
of quartz tube (Heralux 5 mm. I/D, 7 mm. O/D,
Engelhard Hanovia Lamps, Bath Road, Slough, Bucks.)
were situated in the light path between the u.v.
lamp and photoconductor cells. The u.v. light was not
absorbed by solutions in the flow-through cells

CIRCUIT DIAGRAM

Mains supply

ORP90   ORP90
1M   100K   5K
90V
270K   270K
50K
Recorder

Rectifier and voltage stabiliser

U.V.

a screw
b batten to hold the flow cells in place
c slit
d sliding plate by which slit width is adjusted
e screw to adjust slit width
f sloping edge
g compartment to house photoconductive cells

SCALE
0   2cm.

FRONT ELEVATION
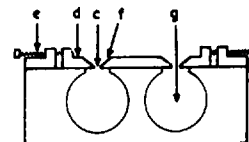
CROSS SECTION

DETAIL OF PHOTOCONDUCTIVE
CELL HOLDER

FIGURE 4

COLUMN

SCANNER

was converted to wavelengths at which the photo-
conductive cells responded (maximum response is at
670 μm). This was accomplished by the red-
fluorescence, in u.v. light, of activated cadmium
borate (AB Lumalampen, Stockholm 20). Filters
of red cellophane and films of polyethylene impreg-
nated with cadmium borate were attached by their
edges to the glass envelopes of the photoconductive
cells by means of transparent adhesive tape. The
cells and attached filters and polyethylene films
were housed in compartments in aluminium blocks
(Figure 4) with slits 3 cm. long and of variable width
in the sides facing the u.v. light. The slits were
set so that with water in the cells, the resistances
of the photoconductive cells were about 100 K Ω
when exposed to light. The polyethylene film imp-
regnated with activated cadmium borate was prepared
as follows. A 0.25 per cent solution of polyethy-
lene in boiling heptane was sprayed onto a micro-
scope slide and the slide then heated in an oven at
110°C for 5 min. After cooling, a suspension of
cadmium borate in the polyethylene solution was sprayed
on top of the polyethylene layer and dried as before.

Application of the suspension was repeated until an
even deposit was obtained, the slide being dried
between each application. A further coat of the
polyethylene alone was applied, dried and the film
then peeled gently off the slide.

The detailed layout of the apparatus is
shown in Figure 3. The switches and neon indicator
lamps were on the front panel and all the other
components were accommodated on an aluminium chassis
housed in a box with a wooden base, 2.4 cm. thick,
aluminium sides and a removable lid. The front
panel was made of 3 mm. thick Trafolite and slid
into position in channelling on the sides and base.
It was attached to the chassis so that when the lid
was removed and incoming leads disconnected, both
could be removed as a unit. Electrical components
including the smoothed, voltage-stabilised power
pack giving 90 V D.C. at 10 mA from 230 V mains,
were supplied by Radio Servicing Co., London, W.5.
Signals from the scanner were transmitted to the
recorder by twin core shielded cable. The Kent
Mk3 mV recorder with type 2M amplifier (George Kent
Ltd., Luton, Bedfordshire) had a range of 0 to 5 mV

and each of the 6 channels of the scanner was moni-
tored in succession at 5 sec. intervals and the re-
lative strengths of impulses recorded on the chart
by dots with a characteristic colour for each channel.

The column eluates flowed in polyethylene
tubing through holes in the lid of the scanner to
the lower ends of the cells. The outlet tubes
from the upper ends of the cells passed out of the
scanner through the front panel to collecting re-
ceptacles. As elution commenced, absorption due to
the eluates was recorded and the total amount of each
substance could therefore be collected in a single
fraction.

A constant flow of solvent was maintained
by a peristaltic pump (Sigmamotor pump Model T8
with Model E Zero-Max variable speed gearbox and
Hoover Mark IV A 1/6 h.p. motor, supplied as a unit
by V. A. Howes & Co. Ltd., London, W.11). The pump
was modified so that liquid could be pumped through
7 Tygon tubes (U.S. Stoneware Co., Akron, Ohio) of
I/D 1/16 in. and O/D 3/16 in. This was achieved
by replacing the springs with ones of greater strength
and fitting plates at either end to hold the tubing
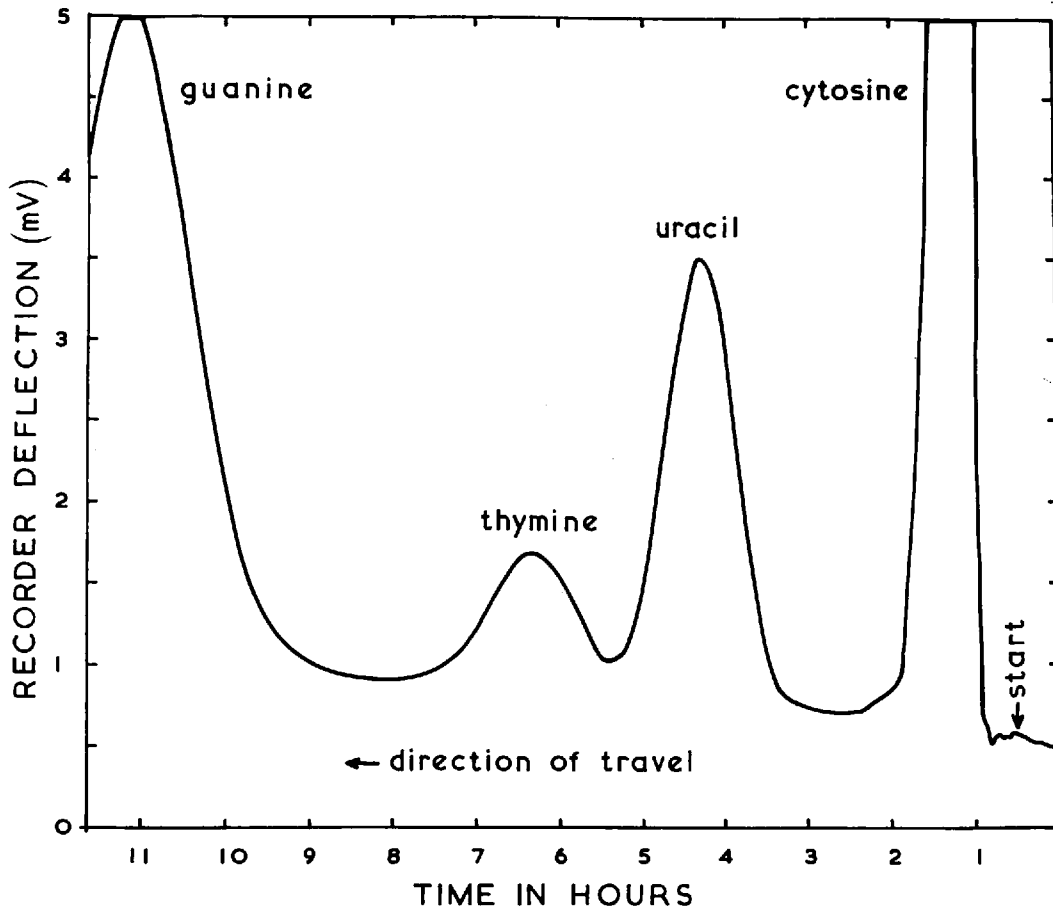in position.

FIGURE 5 PEN-RECORDER TRACE SHOWING THE
SEPARATION BY COLUMN CHROMATOGRAPHY
OF BASES DERIVED FROM D N A AND R N A

It was envisaged that after the hot NaCl extraction DNA and RNA could be separated by alkaline hydrolysis of the RNA and the base compositions of both determined. Unfortunately a certain amount of material (possibly mostly polysaccharide) sediments with the nucleic acid when ethanol is added to the initial extract. This material gelatinises on addition of alkali making the dispersion of the precipitate very difficult and as a consequence results were highly variable. The difficulty was later overcome by first dissolving the precipitate in water and then adding alkali until the appropriate concentration was attained.

In the PCA hydrolysates of nucleic acids there were relatively large amounts of guanine, adenine and cytosine, less uracil and only small amounts of thymine. Separation by column chromatography was attempted first since it seemed better suited to unequal quantities than is paper chromatography as column size may easily be adjusted until satisfactory resolution is obtained. Although many satisfactory separations were achieved (Figure 5), estimates of thymine were erratic and therefore

← STARTING LINE

← GUANINE

← ADENINE

← CYTOSINE

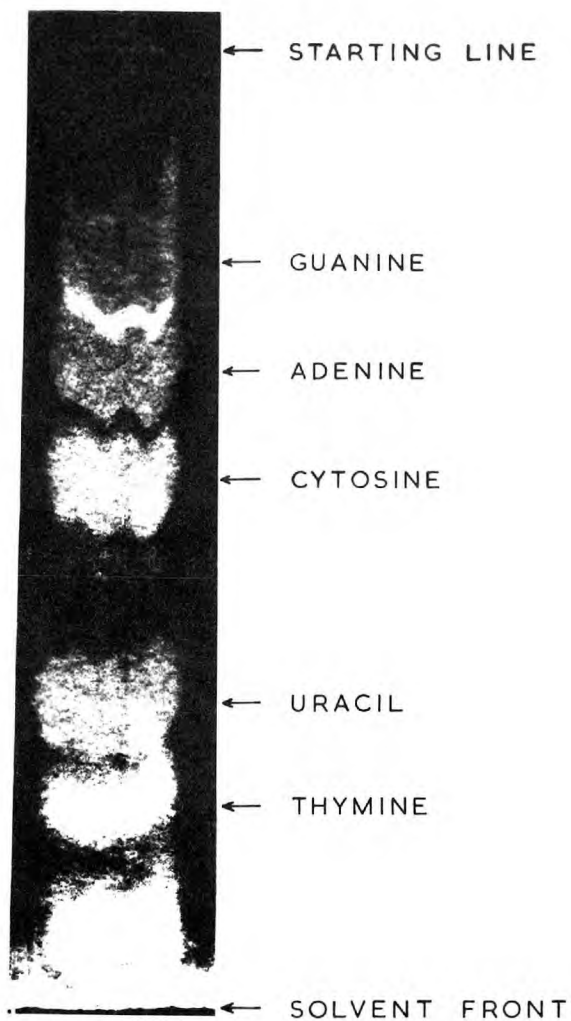← URACIL

← THYMINE

← SOLVENT FRONT

FIGURE 6   THE U.V. PRINT OF A CHROMATOGRAM ON WHICH THE BASES FROM DNA AND RNA WERE SEPARATED

paper chromatography on Whatman 3MM paper was used subsequently. A u.v. print of a paper chromatogram is shown (Figure 6) and it is evident that good separation of the cytosine, uracil and thymine was achieved. Some overloading was necessary in order to have sufficient thymine for satisfactory estimation and as a consequence the guanine and adenine were not completely separated. This chromatogram also shows that there was little other u.v. absorbing material in the region of the nucleotides. The hot NaCl extraction is therefore suitable for this type of work.

To ensure that extraction as described was complete, some of the residues were further treated as described in method 1 for RNA determination (p. 66). Only trace amounts of RNA were detected. To determine the number of extractions necessary to remove the nucleic acids, ethanol was added to the solutions resulting from successive extractions. The precipitates were then hydrolysed with 1 N-HCl at $100^{\circ}C$ for 1 hr., the resulting derivatives separated by paper chromatography (see p. 67) and the adenine estimated. Less than 2 per cent of the total nucleic acid was removed after the fourth
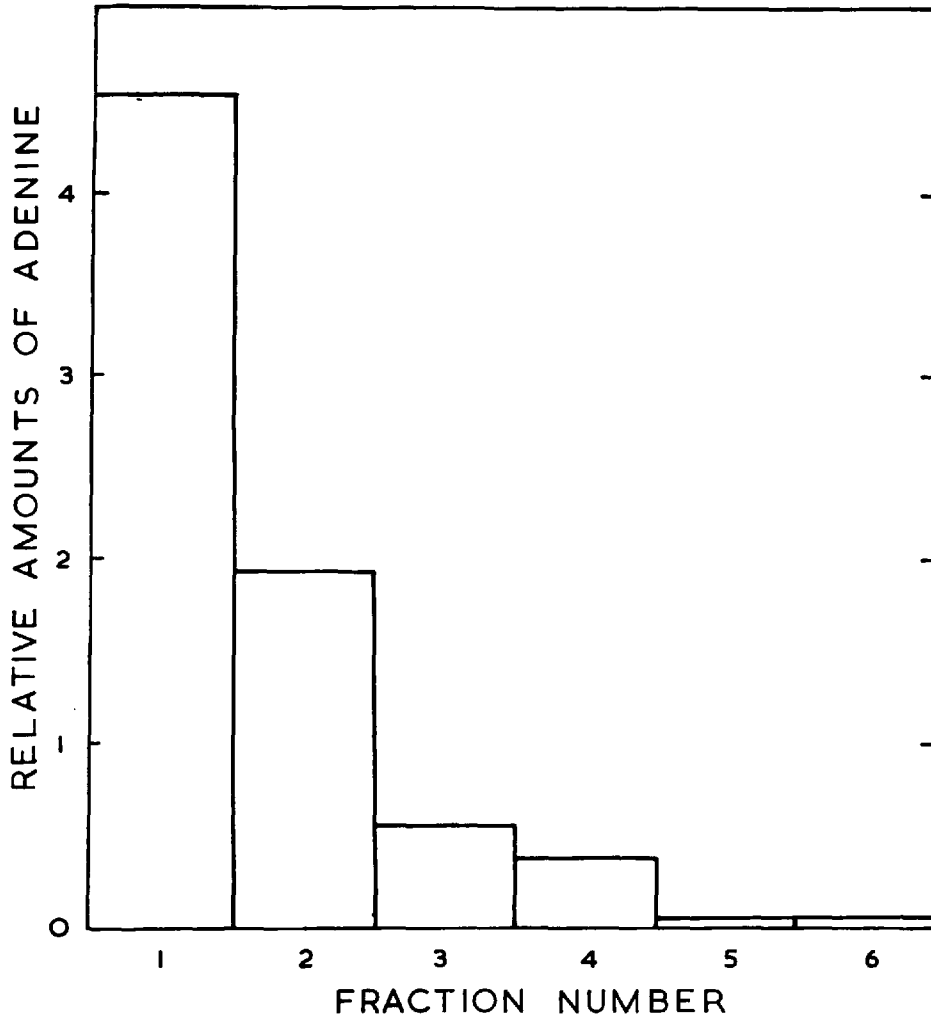
FIGURE 7   THE AMOUNTS OF ADENINE
DERIVED FROM THE NUCLEIC ACIDS IN
EACH OF SIX SUCCESSIVE HOT SODIUM
CHLORIDE EXTRACTS OF WHEAT EMBRYO
TISSUE

extraction (Figure 7). To ensure that the ethanol precipitation was complete, sodium was removed from the combined supernatants of four precipitations by passing them through a cation exchange resin column in the hydrogen form and evaporating the effluent to dryness. The residue was hydrolysed for 1 hr. at 100°C with 1 N-HCl/then removed by evaporation and the hydrolysate chromatographed using an iso-propanol: HCl: water solvent (see p. 68). Only a faint u.v. absorbing spot was observed in the region in which adenine is usually found.

In conclusion, it must be said that this method as used here still leaves something to be desired but with perseverance it could certainly be further improved.

Fractionation of the nucleic acids

A study was made of the base compositions of RNA in the nucleus, in the microsomes and in the non-particulate fraction of the cells of embryos of Petkus winter rye. The fractionation procedures were based on those described by Bonner et al. (for chromatin; 1961) Loening (microsomes; 1962),

Harris et al. (soluble RNA; 1963) and Tissières
et al. (messenger RNA; 1961).

Fifteen ml. buffer solution (0.5 M-sucrose,
5 mM-MgCl$_2$ and 0.05 M-Tris buffer, pH 7.4) was added
to 10 g. wet weight of embryos. The material was
macerated for 2 min. at maximum revolutions in an
Omnimixer (Ivan Servall Inc., Norwalk, Conn. U.S.A.)
with 50 ml. capacity maceration chamber cooled in an
ice bath. The homogenate was then transferred for
about 2 min. to a Potter-Elvehjem type glass homo-
geniser (BS. 745 B, Townson & Mercer Ltd., Croydon)
and the material finely ground. Cell wall material
was filtered off through two layers of fine nylon
mesh. The larger subcellular components such as
mitochondria, nuclei and starch granules were pre-
cipitated from the filtrate by centrifugation at
30,000 x g. for 4 min. (MSE 'Superspeed 40' & 25 ml.
angle rotor). The gelatinous layer covering the
starch granules was removed with a spatula and the
chromatin which it contained purified by resuspend-
ing it in the buffer and centrifuging for 30 min.
at 5,000 x g. (MSE 'High-speed 13', angle rotor).
This process was repeated twice each time centrifuging at

10,000 x g. for 10 min.   Nucleic acid was extracted

from the chromatin with 4 ml. 1.7 N-NaCl at 100°C

for 4 min. and precipitated in the cold by the ad-

dition of 1 vol. glycine buffer (glycine-NaCl-HCl;

all 0.1 M) and 1 vol. of 0.1 M-$Al_2(SO_4)_3$.   To

hydrolyse the RNA, 0.5 ml. of 0.3 N-KOH was added

to the precipitate and left overnight at 37°C.

The liquid was then cooled, adjusted to pH2 with

PCA and the DNA and perchlorate precipitated by cen-

trifugation.   The precipitate was washed twice with

2 ml. cold 0.01 N-HCl.   The supernatant and washings

were dried and hydrolysed with HCl and the base com-

position determined as described previously (p. 67).

The DNA containing precipitate was dried and then

hydrolysed with 0.1 ml. 72 per cent PCA at 100°C

in a sealed Pyrex tube.   After cooling, 0.5 ml.

water was added to the hydrolysate and the super-

natant containing the nucleic acid derivatives ad-

justed to pH 2 with KOH.   About half of the super-

natant was decanted into a 5 ml. test tube and dried

in a vacuum desiccator.   The bases were separated

chromatographically and estimated as described pre-

viously (p. 67).

The supernatant fraction remaining after
the chromatin had been precipitated was centrifuged
at 140,000 x g. for 90 min. (MSE 'Superspeed 40',
8 x 25 ml. angle rotor) to precipitate the microsomes.
To liberate the 'messenger' RNA, the surface of the
microsomal pellet was carefully rinsed with a buffer
of low magnesium ion concentration (0.1 mM-MgCl$_2$,
0.05 M-Tris buffer, pH 7.4), the rinses discarded
and the microsomes suspended in 10 ml. of the same
buffer. The microsomes were reprecipitated by cen-
trifuging for 5 hr. at 100,000 x g. (MSE 'Superspeed
40', 8 x 25 ml. angle rotor) and the 'messenger'
RNA in the supernatant precipitated, like chromatin
nucleic acid, by the addition of aluminium ions.
The precipitate was collected by centrifugation,
dried in a vacuum desiccator and the RNA hydrolysed
and its base composition determined as before (p. 67).
Soluble RNA which remained in solution after the
microsomal fraction had been precipitated was puri-
fied as follows. Five per cent dodecyl sulphate
solution was added to the solution to make a final
concentration of 0.5 per cent. Water-saturated
phenol (1 vol.) was added and the mixture stirred for

15 min.   After centrifugation the RNA in the aqueous

phase was precipitated/ the addition of 2 vol. eth-
            by
anol.   The precipitate was redissolved in tris

buffer (10 mM-Tris, pH 7.4; 0.1 mM-$MgCl_2$) and cen-

trifuged at 140,000 x g. (MSE 'Superspeed 40',

8 x 25 ml. angle rotor) for 10 min. to precipitate

polysaccharide material.   Finally the RNA was pre-

cipitated with 2 vol. ethanol and hydrolysed and its

base composition determined as before.   All opera-

tions preceeding hydrolysis were carried out bet-

ween 3° and 5^oC.

The main difficulty in this fractionation

was to prepare nuclear and soluble RNA in a suffi-

ciently pure state to obtain accurate values of the

base composition.   The hydrolysis products of nuc-

lear RNA streaked severely on the chromatograms.

This streaking was later ascribed to the use of alu-

minium for precipitation of the nucleic acids after

extraction from the chromatin.   Unfortunately, by

the time that this was discovered it was not possible

to repeat the experiment and so get a reliable value

for the base composition of this fraction.

8a

8b

SL · · · · · · · · · · · · · · · · G · · A · · · · · · · · · · · CA · · · · · · · · UA · · · · · · · · · SF

8c

FIGURE 8   THE U.V. PRINTS OF CHROMATOGRAMS
OF MILD ACID HYDROLYSATES OF sRNA
PREPARED BY THREE DIFFERENT METHODS
SL = STARTING LINE   G = GUANINE
A = ADENINE   C = CYTIDYLIC ACID
U = URIDYLIC ACID   SF = SOLVENT FRONT

Initially the soluble RNA was also precipitated with aluminium ions and here large quantities of u.v. absorbing material were precipitated as well. This made it difficult to distinguish the RNA derivatives in u.v. light (Figure 8a). Phenol extraction of the RNA solutions removed only part of this interfering material (Figure 8b). Phenol extraction, ethanol precipitation then re-extraction with hot NaCl and reprecipitation with aluminium ions further reduced the contaminating u.v. absorbing material. The precipitation by aluminium again resulted in streaky chromatograms but not to such a severe degree as with the nuclear RNA. Substitution of this step by an ethanol precipitation resulted in completely satisfactory chromatograms (Figure 8c).

Values for ribosomal RNA and the DNA varied little but the very small amount of RNA in the messenger fraction made determination of base composition of this fraction difficult.

Incorporation of radioactive carbon into nucleic acids during cold treatments

Two experiments were carried out in which the incorporation of C^{14} into the nucleic acids was

studied. In the first the radioactive carbon was supplied in sucrose and in the second in uridylic acid.

Petkus rye embryos, grown as described in Method 3 (p. 45) for a suitable length of time, were removed from the nutrient agar and the radioactive substances supplied to them at 2° to 5°C and at 15°C, through the scutella. Twelve or thirteen drops (volume 2.5 μl.) of the aqueous radioactive solutions were deposited on glass microscope slides (2.5 x 7.5 cm.) using an Agla syringe. As was appropriate, each embryo was placed on a drop of solution containing either 1.46 μg. sucrose (4.04 x 10^{-1} μc.) or uridylic acid (7.15 x 10^{-2}μc., sp. act. approx. 4mc./mM., from Schwarz BioResearch, Inc., Orangeburg, New York). The slides were placed on moist filter paper in Petri dishes.

Samples of embryos (25 at both 2° to 5°C and at 15°C) were removed from the radioactive solutions after 9 hr. and replaced on the nutrient agar for a further $6\frac{1}{2}$ days in the case of embryos in the cold and 33 hr. for embryos at 15°C. All operations except the initial excision of the embryos were

carried out in green light and the embryos were grown
in darkness to minimise the chances of responses to
light masking those brought about by the cold treat-
ment.

In the experiment in which sucrose was
supplied samples of 25 embryos were dropped into
about 5 ml. boiling ethanol in a 20 ml. Potter-
Elvehjem type glass homogeniser. After cooling
the embryos were homogenised and the resulting tissue
suspension precipitated by centrifugation. The
supernatants (ethanol fraction) were transferred
to sample tubes and the precipitates extracted twice
(2 x 5 ml.) with 50 per cent ethanol and the washings
retained (50 per cent ethanol fraction). The eth-
anol and 50 per cent ethanol fractions were dried
in a vacuum desiccator. The tissues remaining
were dried by washing first with ethanol and then with
ether, the last traces of the solvents being removed
under reduced pressure. The nucleic acids were ex-
tracted at $100^{\circ}C$ with 2 ml. 1.7 M-NaCl and the volume
of the solution determined by weighing. The nucleic
acids were precipitated by the addition of 1 vol. of
glycine buffer, pH 3.0 (glycine-NaCl-HCl all 0.1 M)
and 1 vol. of 0.1 M-$Al_2(SO_4)_3$. The mixture was

kept overnight at 0°C, the nucleic acid precipitate removed by centrifugation and the supernatant discarded. The precipitate was washed with 50 per cent ethanol, then suspended in concentrated NH_4OH in which it was transferred to duralumin planchets for the determination of radioactivity. Before applying the samples, 0.2 ml. of a 1 per cent solution of Teepol in 50 per cent ethanol was spread over the planchets (0.75 in. diam.) and dried to facilitate even spreading.

Radioactivity estimates were made by means of an end-window Geiger-Muller counter (Ekco Automatic Scaler Type N530F with E.R.D. lead castle and Mullard M.X. 123 G.M. tube). Corrections were made for background radioactivity and lost counts. Since the weight of material placed on the discs was greater than 1 mg./cm.^{2}, corrections were applied for self absorption and results expressed as c.p.m. at infinite thinness. Counting was carried out on the second shelf of the lead castle, a distance of about 2.5 cm. from the end window of the G.M. tube. Of the total number of disintegrations taking place, 1.62 per cent was counted.

When uridylic acid was supplied to embryos
the sample of 25 embryos was dropped into about 5 ml.
boiling ethanol in a 20 ml. Potter-Elvehjem type
glass homogeniser.   After cooling, the embryos
were stored at -20°C.   The embryos were then
homogenised and the tissue suspension precipitated
by centrifugation and the supernatant discarded.
The precipitate was extracted three times (3 x 10 ml.)
with 50 per cent ethanol and was resuspended each
time using a Vortex Jr. Mixer (Scientific Industries
Inc., New York).   The supernatant was dried using
a rotary vacuum evaporator.   In order to recover
the soluble nucleotides relatively free of other
substances, the residue was taken up in 20 ml.
0.01 N-HCl and 50 mg. charcoal added to absorb the
nucleotides (about 75 mg. charcoal per μmole nuc-
leotide).   The charcoal was collected by filtration
on a Hirsh funnel and washed with a small amount of
water.   The nucleotides were eluted by suspending
the charcoal in 5 ml. ethanol/ammonia sp. gr. 0.880/
water (25:0.5:74.5).   The charcoal was removed by
filtration and washed with 1 ml. of the eluting
solvent.   The filtrates were collected in sample
tubes and dried in a vacuum desiccator.   The

nucleotides were transferred to form 1 in. streaks

on Whatman No. 1 chromatography paper and the

chromatogram developed with ethanol/M-ammonium

acetate solvent (7.5:3.0 pH 3.8).   The substances

into which radioactive carbon from the uridylic acid

was incorporated, were detected by autoradiography.

Kodirex X-ray film (25.4 x 30.5 cm.) was placed in

contact with the chromatogram in an X-ray exposure

holder and left for 10 days.   The film was then

developed in Kodak D-19b developer for 4 min. and

fixed with Kodak acid fixing salt for 30 min. and

washed in running water for 30 min.

The residue remaining after the 50 per cent

ethanol extraction was dried by suspending it in ace-

tone and then precipitated by centrifugation.

The tube containing the material was placed in a boil-

ing water bath and the nucleic acid extracted with

3 x 4 ml. 1.7 M-NaCl.   The extracts were placed in

50 ml. centrifuge tubes and the nucleic acid pre-

cipitated by leaving overnight in the cold after the

addition of 1 vol. ethanol and a small crystal of

$MgCl_2$.   The precipitate was washed twice with cold

0.01 N-HCl and the supernatant containing the ribo-

nucleotides was dried in a vacuum desiccator.   The

material was loaded onto a 1 in. streak on Whatman

No. 1 chromatography paper and the chromatogram

developed using the iso-propanol/HCl solvent

(p. 67). A sample of the uridylic acid was also

chromatographed in this way and radioactive com-

ponents located by autoradiography. Areas of the

chromatography paper containing the radioactive

components were cut out and the radioactivity de-

termined with an IDL scaler, type 1700c (Isotope

Developments Ltd., Bath Road, Beenham, Reading)

with type 2014A automatic sample changer and type

2007A programme and read out unit. The bits of

paper from the chromatograms were placed on the

2 in. diameter planchet holders of the automatic

sample changer and the number of disintegrations

determined twice for each side of the paper for

400 sec. on each occasion. Initial identification

of the radioactive substances was by their Rf's

in the propanol - HCl solvent. This was con-

firmed by eluting them from the paper with water and

transferring them to a sheet of Whatman No. 1 chro-

matography paper and developing the chromatogram with

n-propanol/ammonia sp. gr. 0.880/water (60:30:10)

solvent and again determining their Rf's. As a

further check the substances were eluted once more
and their u.v. spectra determined using an Optica
CF4R recording spectrophotometer.

In the experiment in which sucrose was
used, it did not prove possible to remove the nuc-
leic acids from the planchets, so that a project
for locating the C^{14} in the nucleotides following
hydrolysis was abandoned.   Separation of the
nucleotides by thin-layer chromatography had been
envisaged by the following technique (Dyer, 1963).

The thin layers were prepared by sus-
pending 0.5 g. of a DEAE-cellulose powder suitable
for thin-layer chromatography (Serva-Entwicklungslabor,
Heidelberg, Germany) in 4 ml. of water.   The slurry
was poured onto an 18 x 6 cm. glass plate and
spread in a uniform layer along the plate using a
glass rod.   The rod was kept at a fixed distance
above the plate by wrapping around it two pieces of
wire (310 μ diam.) 5.5 cm. apart, the diameter
of the wire determining the thickness of the layer
of   cellulose.   The layer was dried at room
temperature and solutions of nucleotides applied
about 2 cm. from one end.   Two developments of

the chromatograms were necessary, the first with
n-propanol/ ammonia (sp. gr. 0.880-ANALAR)/water
(60:30:10) at 40°C allowing the front to reach
at least 7 cm. beyond the point of application of
the nucleotides (45 min.). After drying, the
plate was developed again in the same direction
with 0.24 M-acetic acid at 20° to 25°C until the
solvent front just reached the same position as
that reached by the first solvent (15 min.). The
nucleotides were located by examination of the
chromatogram in u.v. light.

The distances travelled by the individual
nucleotides from the point of application relative
to the position reached by the solvent fronts are:
guanylic acid 0.21; uridylic acid 0.34: cytidylic
acid 0.82; (the -2'- and -3'- phosphates of each
of these are not resolved) and adenylic acid 0.53,
0.62 (the -2'- and -3'- phosphates separate). The
use of a single development with dilute weak acid
(0.16 or 0.24 M-acetic acid) did not separate
guanylic from uridylic acid but did separate the
two adenylic acid isomers and the cytidylic acid from
one another and from the mixture of guanylic and
uridylic acids. In the double development the

first solvent separates guanylic acid (nearest the origin) from the other nucleotides and thus makes possible the separation of the four nucleotides on subsequent development with acid.   Satisfactory separations were not achieved if the first solvent was freshly prepared using an ordinary reagent grade of 0.880 ammonia.   After standing overnight satisfactory results were obtained.   Using ANALAR ammonia (British Drug Houses Ltd.), a freshly prepared solvent gave a good separation.

Ribonucleotides from both yeast ribonucleic acid (L. Light & Co.) and from samples of nucleic acid extracted from rye seedlings by the hot NaCl method (p. 72) have been separated.   The RNA samples were hydrolysed in 0.3 N-KOH at 37° for 18 hr.   Potassium was removed either by precipitation as the perchlorate or by passing the hydrolysate through a small column of cation exchange resin in the hydrogen form.   The separated components of the hydrolysates separated by thin-layer chromatography were identified by their u.v. absorption spectra and by the fact that the position of each one corresponded on the chromatograms to that of the appropriate pure nucleotide (British Drug Houses Ltd.).

EXPERIMENTS AND RESULTS

When this investigation was started, little
was known of the changes in nucleic acid occurring
during either cereal grain germination or vernalisa-
tion. The first experiment therefore attempted a
straightforward comparison of the total RNA content
and base composition before and after a cold treat-
ment with that of plants grown at 25°C to a similar
stage in development as those in the cold. Both the
spring and winter races of Petkus rye were studied in
this way. The RNA of the two races changed in a
similar fashion during the various treatments but
there was indication of a change in base composition
during germination. The second and third experiments
were designed to confirm this latter point and to ob-
tain a more detailed account of both DNA and RNA
changes during cold treatments. It was decided to
use only winter grain grown at relatively high and
low temperatures. As already described (p. 83)
difficulty was encountered in the determination of
base composition and therefore the results of ex-
periments 2 and 3 record only changes in content of
DNA and RNA. Although these results provided a
clear account of how the nucleic acids changed, there

was no difference between the cold and warm grown
samples that could be attributed to the processes
leading to flower initiation that take place in
the cold.

From these first experiments it was evi-
dent that it was not sufficient to determine only
total nucleic acids content particularly in view of the
fact that if the ribosomal and soluble RNA, which con-
stitute the bulk of the total RNA, remained unchanged
any changes occurring in a minor fraction of the
RNA would tend to be masked. An attempt was there-
fore made to determine the base composition of nuc-
lear, microsomal, messenger and soluble RNA's and of
DNA of rye embryos both before and after germination
either in the cold or warm.

Preliminary experiments (5 and 6) were
carried out in which the incorporation into the
nucleic acids of C^{14} from sucrose and uridylic acid
was studied. The fate of uridylic acid, not in-
corporated into the nucleic acids, was also of
interest since Tomita (1962) reported that uridylic
acid promoted flowering when applied to leaves of un-
vernalised rye plants. The distribution of

radioactivity in the soluble nucleotides was there-
fore examined as well as that present in the nucleic
acids.

An unexpected feature of the processes
occurring in cereals in the cold and which lead to
flowering is that they do not appear to be hindered
by inhibitors of nucleic acid synthesis.   This is
in striking contrast to the behaviour of a number of
other plants in which flowering may be prevented by
the application of such inhibitors.   When such
antimetabolites were applied to the winter rye
plants during cold treatments they did not prevent
flowering and so it appeared possible that critical
changes in nucleic acid synthesis might take place
not during chilling but in the period immediately
following it, the cold somehow preparing the plants
for this synthesis.   In experiment 7 therefore
thiouracil was supplied to wheat embryos either on
the day preceeding removal from the cold or during one
of the following three days when at higher tempera-
tures.

Experiment 1

The nucleic acid content of germinating rye grain

About 3000 grains each of spring and winter rye were germinated (Method 1, p. 41) in the cold in restricted moisture conditions. After 6 week 5 days at 3 to 5°C, the grain was removed from the germination boxes and the moisture content of a sample determined. A few grains of both spring and winter rye were planted in pots in a greenhouse (temperature 20° to 25°C) and kept under long day lighting conditions. Grain was also grown by the second method described (p. 44) at 25°C for 48 hr. by which time it had reached approximately the same stage in morphological development as that grown in the cold.

Freeze-dried powders (p. 45) of the dissected embryo and endosperm portions of the grain were prepared for nucleic acid analysis (Method 1, p. 46 or 2, p. 47) and the RNA content determined by a modification of the Schmidt and Thannhauser procedure (Method 1, p. 66).

Table 1 Base composition of RNA in the embryos of Petkus spring and winter rye grain

	Treatment	Race	G	A	C	U	6-keto/6-amino	pur./pyrim.
μmoles per g. dry weight	5 hr. soaking	spring	10.8	9.0	8.4	5.5	0.94	1.42
		winter	11.9	9.4	8.6	6.3	1.01	1.43
	7 weeks at 2-5°C	spring	15.2	11.2	12.6	9.3	1.03	1.21
		winter	14.0	11.3	12.4	10.2	1.02	1.12
	48 hr. at 25°C	spring	8.0	6.5	6.9	5.8	1.03	1.14
		winter	7.3	6.0	6.6	5.4	1.02	1.11
moles per cent	5 hr. soaking	spring	32.0	26.6	25.0	16.4		
		winter	32.8	26.0	25.7	17.5		
	7 weeks at 2-5°C	spring	31.5	23.1	25.4	19.3		
		winter	29.2	23.6	26.1	21.3		
	48 hr. at 25°C	spring	29.1	23.9	25.1	21.3		
		winter	29.0	23.6	26.0	21.5		
μmoles per 100 embryos	5 hr. soaking	spring	1.73	1.44	1.35	0.88		
		winter	1.69	1.34	1.22	0.90		
	48 hr. at 25°C	spring	3.51	2.87	3.02	2.56		
		winter	3.88	3.16	3.50	2.87		

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Estimates of the base compositions of RNA
of embryos of Petkus spring and winter rye are given
in Table 1. The amount of each base present per
unit dry weight of material was very similar in the
two varieties when subjected to the same temperature
and moisture conditions. A change in moles per cent
of each base during germination is indicated by com-
parison of values for grain soaked for 5 hr. with those
of grain germinated at $25^{\circ}C$ and in the cold. However,
this result is not substantiated by the experiment in
which the nucleic acids were fractionated (4). In
common with the RNA from several other sources the
amount of nucleotide carrying an amino group in the
6 position (in adenine and cytosine) is approximately
equal to that having a 6-keto group (guanine and ura-
cil). Comparison of RNA values per 100 embryos for
the $25^{\circ}C$ grown and 5 hr. soaked embryos show that
there was an increase in the total amount during germ-
ination. Since the results of the cold treated sample
could only be expressed on a dry weight basis it is
not possible to conclude with certainty how much change
there was in the total amount of RNA in the cold.
Nevertheless, in the embryos kept in the cold the
marked increase in concentration of RNA suggests

Table 2 Base composition of RNA in the endosperm of Petkus spring and winter rye grain

Treatment	Race	μ moles per g. dry weight				$\dfrac{G + U}{A + C}$	$\dfrac{Purines}{Pyrimidines}$
		G	A	C	U		
7 weeks at 2-5°C	spring	.42	.32	.34	.26	1.03	1.23
	winter	.38	.34	.33	.27	0.97	1.20
48hr. at 25°C	spring	.34	.24	.24	.19	1.09	1.35
	winter	.30	.21	.20	.16	0.88	1.42

considerable synthesis since the embryos increase
in weight during germination.

In the endosperms (Table 2) there is a
much lower concentration of RNA than in the embryos,
but the levels are again similar in the spring and
winter varieties in both grain grown at 25°C and
in the cold.

Growth in the cold was small and by the
end of 6 weeks in the cold the roots and shoots had
only just emerged. Growth of the winter grain was
slightly greater than that of the spring. Of the
grain that was planted out in a greenhouse, the
spring variety formed ears by the 100th day from
the beginning of the experiment whereas the winter
variety showed no signs of flowering.

Experiments 2 and 3

The nucleic acid content of germinating wheat embryos

Material for both these experiments was pre-
pared by Dr. A. J. Keys and M. J. Cornelius and was
used for both nucleotide (Keys & Cornelius, 1965)
and nucleic acid analysis. Wheat, variety Cappelle
was germinated (Method 2, p. 44) at 25°C for 48 hr.

Table 3 Data relating to the wheat embryos used to prepare freeze-dried powders analysed in experiments 2 and 3

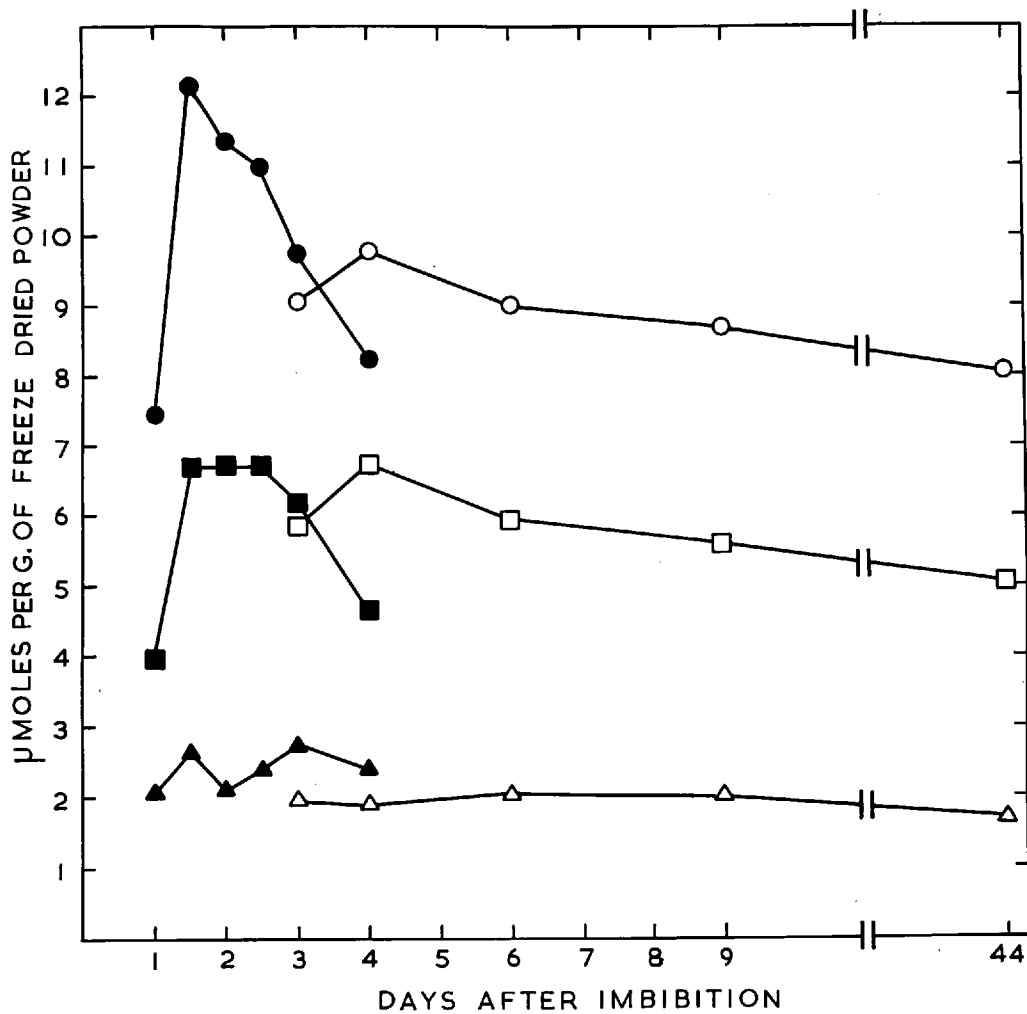| Experiment 2		
Growth at 25°	Subsequent growth at $2^{\circ} - 5^{\circ}C$	Dry wt. $(105^{\circ}C, 24hr.)$ of 100 embryos
Hr.	Hr.	g.
24	-	0.249
36	-	0.316
48	-	0.453
60	-	0.569
72	-	0.779
96	-	1.220
48	24	0.560
48	48	0.546
48	96	0.590
48	168	0.748
48	1008	2.138
Experiment 3		
Growth at 25°	Subsequent growth at $2^{\circ} - 5^{\circ}C$	Dry weight (Vacuum over $CaCl_2$) of freeze dried powder from 100 embryos
Hr.	Hr.	g.
4	-	0.278
24	-	0.303
48	-	0.513
72	-	0.932
96	-	1.300
120	-	1.718
168	-	2.00
48	-	0.517
48	24	0.619
48	48	0.648
48	96	0.747

FIG.9 RESULTS OF ANALYSES MADE IN EXPERIMENT 2. AMOUNTS OF BASES DERIVED FROM THE NUCLEIC ACIDS. ● CYTOSINE, ■ URACIL, ▲ THYMINE. SHADED SYMBOLS REPRESENT VALUES OBTAINED AFTER GROWTH AT 25°C AND OPEN SYMBOLS ARE THOSE OBTAINED AFTER PERIODS AT VERNALISING TEMPERATURES FOLLOWING AN INITIAL PERIOD AT 25°C FOR TWO DAYS

after which some of the grain was transferred to the
cold (3° to 5°C). Samples for analysis were taken
at appropriate time intervals and freeze-dried pow-
ders of the embryos prepared. The dry weights per
100 embryos at each stage examined were determined
either by drying two samples of 50 embryos for 24 hr.
in an oven at 105°C or by drying to a constant weight
over $CaCl_2$ freeze-dried powder from at least 350 embryos.
After the embryo material had been prepared (Method 3,
p. 49) the DNA and RNA were estimated respectively
by their thymine and uracil contents after extraction
from the tissue with hot sodium chloride solutions
(Method 2, p. 72).

In contrast to experiment 1 with Petkus rye,
the wheat was grown in unrestricted moisture conditions.
In Table 3 are given the details relating to the embryos
used for the preparation of freeze dried powders. The
estimates of cytosine, uracil and thymine derived by
PCA hydrolysis of the nucleic acids are plotted in
Figure 9 (experiment 2) and Figure 10 (experiment 3)
as μmoles of base per gram of freeze-dried powder.
Cytosine values indicate the changes that occurred
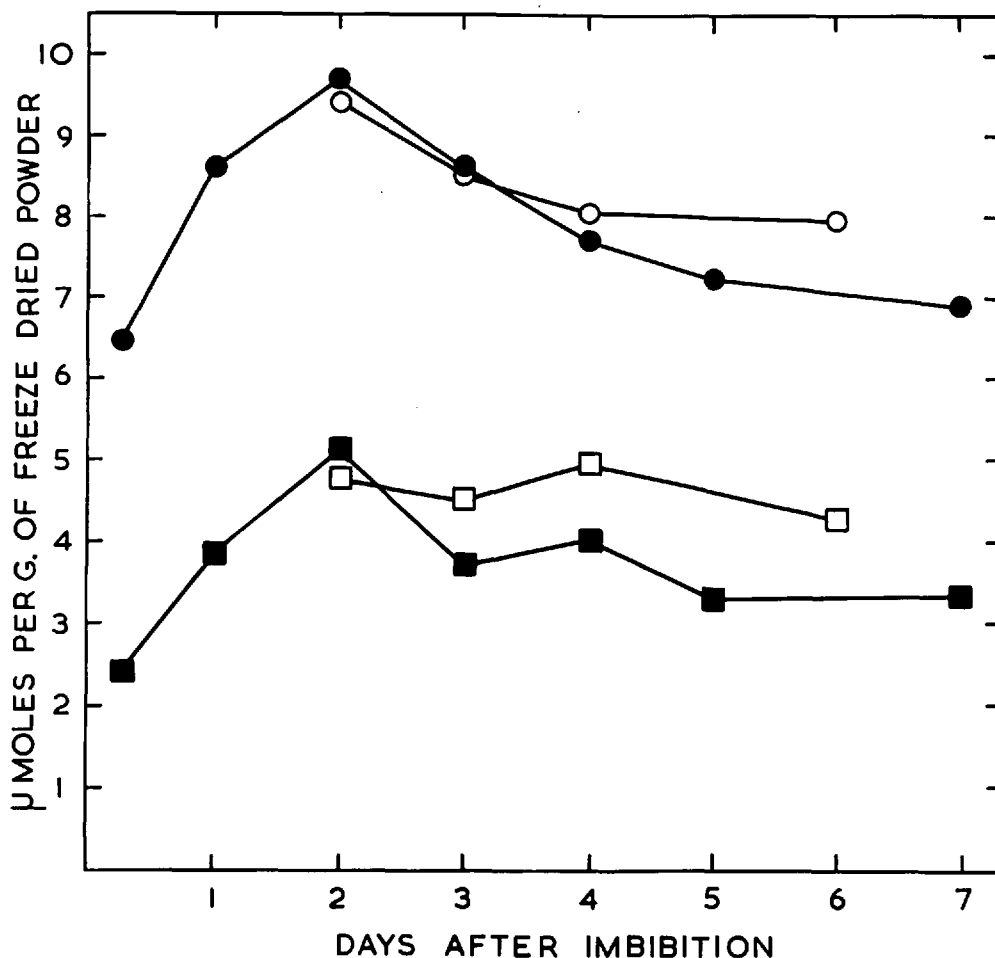in the combined nucleic acids, uracil the changes

FIG.10 RESULTS OF ANALYSES MADE IN EXPERIMENT 3.
AMOUNTS OF BASES DERIVED FROM THE NUCLEIC
ACIDS. ●CYTOSINE,■URACIL. SHADED SYMBOLS
REPRESENT VALUES OBTAINED AFTER GROWTH AT
25°C AND OPEN SYMBOLS ARE THOSE OBTAINED
AFTER PERIODS AT VERNALISING TEMPERATURES
FOLLOWING AN INITIAL PERIOD AT 25°C FOR TWO DAYS

in RNA and thymine the changes in DNA.   The amount
of these bases per unit weight of dry tissue rapidly
increased initially and reached a maximum about 36 to
48 hr. after imbibition.   The amounts of cytosine and
uracil thereafter declined both in plants grown at
$25^{\circ}C$ and at 3° to $5^{\circ}C$.   The thymine concentration
(Figure 9) increased in embryos for 36 hr. after
imbibition the^{h} declined by 48 hr. and increased
again up to 72 hr. and then decreased again.   The
concentration in embryos remained fairly constant
for the first 6 days following the transfer of grain
from $25^{\circ}C$ to 3° to $5^{\circ}C$ and subsequently decreased.
Unfortunately no satisfactory values for thymine
were obtained in experiment 3 in which ion-exchange
resin columns were used to separate the bases.

Since the cold treatment modifies mor-
phological development of the winter cereals (Purvis,
1961), there are no stages of growth of chilled emb-
ryos that may be compared directly with those of
embryos grown at higher non-vernalising temperatures.
A compromise is therefore necessary in choosing a
suitable basis for comparison.   Following the pro-
cedure employed by Keys and Cornelius (1965) for com-
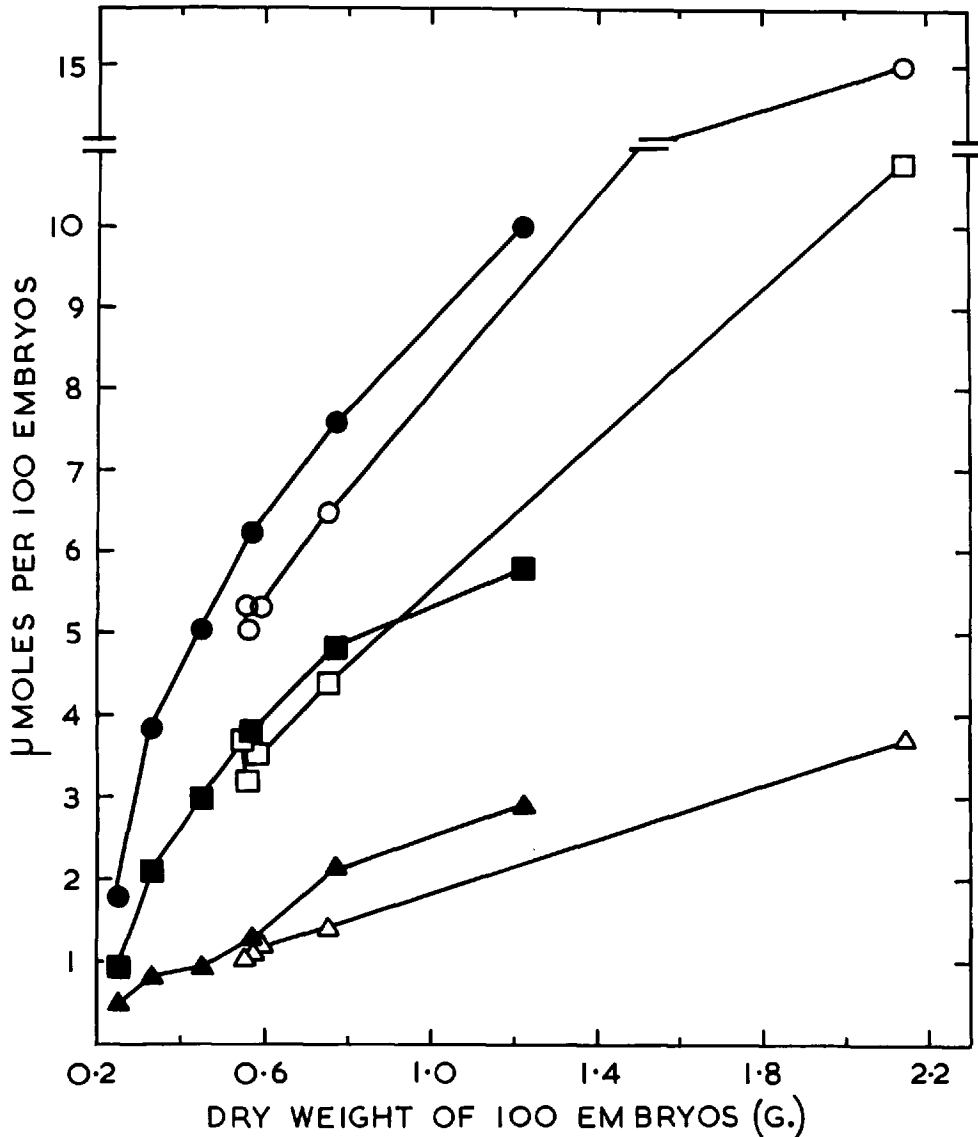paring soluble nucleotide levels, a plot was made of

FIG. 11 CHANGES IN THE AMOUNTS OF CYTOSINE, URACIL
AND THYMINE DERIVED FROM THE NUCLEIC ACIDS
IN ONE HUNDRED EMBRYOS. VALUES ARE CALCULATED
FROM THE RESULTS OF EXPERIMENT 2. ●CYTOSINE,
■URACIL, ▲THYMINE. SHADED SYMBOLS REPRESENT
VALUES OBTAINED AFTER GROWTH AT 25°C AND OPEN
SYMBOLS ARE THOSE OBTAINED AFTER PERIODS AT
VERNALISING TEMPERATURES FOLLOWING AN INITIAL
PERIOD AT 25°C FOR TWO DAYS

the results obtained in experiment 2 in which the
horizontal axis represents embryo dry weight, and the
vertical axis μmoles per 100 embryos to give values
for the total amount of each base (Figure 11).   There
was a steady increase in the total amount of each base
during germination in both cold and warm grown embryos
but   the amount in cold treated embryos was
slightly less than that in the embryos grown at 25°C.

It is necessary to know the base composit-
ions of DNA and RNA in order to make estimates of
amount of these from the values for thymine and ura-
cil.   Ergle and Katterman (1961) report a base com-
position for wheat DNA in moles per cent of each
base of A 27.2, T 27.4, 5-MeC 6.2, C 16.6 and G 22.6.
Lane and Allen (1961) give values of A 24.3, U 18.9,
C 25.5 and G 31.3 for wheat RNA insoluble in 3 M-NaCl
which is probably mostly of ribosomal origin and
close in composition to that of the total RNA.   The
total base content and hence RNA to DNA ratio was
determined using these values (Table 4).

Table 4   Total base content of the nucleic acids of
germinating wheat embryos

| Treatment | μmoles per g. | | RNA/DNA |
	RNA	DNA	
24 hr. at 25°C	20.5	7.6	2.7
36 "	35.5	9.7	3.6
48 "	35.5	7.7	4.5
60 "	35.7	8.8	4.0
72 "	32.9	10.2	3.5
96 "	24.6	8.8	2.8
48 " + 24hr. cold	31.6	7.5	4.1
" 48	36.7	7.3	5.0
" 96	32.3	7.9	4.1
" 168	30.4	7.6	4.0
" 1008	27.4	6.6	4.1

There is an increase followed by a decrease in the
RNA to DNA ratio in the embryos of grain grown at
25°C whereas this ratio remains fairly constant in
that grain grown in the cold.   Maximum nucleic acid
content in embryos at 25°C is about 35 μmoles RNA

Table 5 Results of analyses of the base compositions of nucleic acids in different cell fractions of Petkus winter rye embryos

	Fraction	Moles % of each base				6-keto / 6-amino	pur. / pyrim.
		G	A	C MeC	T (U)		
60hr. at 25°C	DNA	23.8	26.2	17.5 6.5	25.9	.99	1.00
	microsomal RNA	31.7	24.6	25.5	18.2	1.00	1.29
	messenger RNA	32.3	24.1	25.9	17.7	1.00	1.30
	soluble RNA	33.4	23.5	26.4	16.5	1.00	1.32
48hr. at 25°C + 6 days at 30°C	DNA	24.1	26.0	17.1 7.2	25.6	.99	1.00
	microsomal RNA	31.2	24.3	25.9	18.6	1.02	1.20
	messenger RNA	34.0	23.6	25.0	17.2	1.06	1.36
	soluble RNA	33.8	23.6	25.7	17.0	1.03	1.34
5hr. soaked 25°C	DNA	24.0	26.0	16.5 7.3	26.1	1.01	1.00
	microsomal RNA	30.8	24.9	25.9	18.4	0.97	1.31
	messenger RNA	33.6	21.5	28.1	16.8	1.07	1.24
	soluble RNA	32.8	20.7	26.3	20.2	1.13	1.15

TREATMENTS

nucleotide and about 10 μmole DNA nucleotide per g.
of the material and 32 μmole RNA nucleotide and 7.6
μmole DNA nucleotide in the cold.

Experiment 4

Fractionation of the nucleic acids

The Petkus winter rye grain for this experi-
ment was grown by the second method described (p. 44)
and the techniques used in the fractionation of the
nucleic acids given on p. 87.

The results given in Table 5 confirm those
of experiment 1 (p.108) which demonstrated that in
rye RNA the bases with an amino group in the 6 posi-
tion are equal in number to those having a 6-keto
group. These are equal in the DNA as well and also
the number of purine bases is equal to the number of
pyrimidine bases in this nucleic acid. Values for
the base composition of ribosomal RNA are very similar
to those for the wheat RNA which is insoluble in
3 M-NaCl (G 31.3, A 24.3, C 25.5, U 18.9; Lane and
Allen, 1961), and the wheat RNA soluble in 3 M-NaCl
is similar in composition to the soluble RNA
(G 31.9, A 23.4, C 26.5, U 18.1). The DNA values

differ slightly from those recorded by Ergle & Katterman
(1961) for wheat DNA (G 22.6, A 27.2, C 16.6,
5-MeC 6.2 and T 27.4).

There is a clear distinction between the
base compositions of DNA and of cytoplasmic RNA.
While in the RNA, the proportions of guanine and
cytosine are greater than those of adenine and ura-
cil, the reverse is true for the DNA (substituting
thymine for uracil).

Investigations of other workers using
material such as bacteria and pea root tips, have
shown that a small fraction of RNA associated with
the ribosomes and that may be dissociated from them
by lowering the magnesium ion concentration, has a
base composition the same as DNA (substituting thymine
by uracil). Also associated with the ribosomes is
soluble RNA, at least part of which is also removed
by lowering the magnesium ion concentration. Together
these two RNA's make up the messenger RNA fraction.
The messenger RNA's isolated in these preparations
were very similar in composition to the other cyto-
plasmic fractions (Table 5). This implies either
that there is no RNA with DNA-like composition in

this fraction or that it is present only in extremely small amounts.

The molar proportions of each base are similar among nucleic acids of cytoplasmic origin and similar to those recorded for the total RNA of Petkus rye (experiment 1). There were no pronounced differences in base composition of the RNA of the embryos of grain grown under different conditions.

Very approximately the percentages of the total nucleic acid of each fraction are ribosomal RNA 76, DNA 12, soluble RNA 8, nuclear RNA 3 and messenger RNA 1 per cent. Values for the base composition of the nuclear RNA were unfortunately erratic and are therefore not recorded here.

Experiments 5 and 6

Incorporation of radioactive carbon into the nucleic acids

Petkus rye embryos used in these experiments were grown as described in method 3 (p. 45) and the techniques used are given in the methods section on p. 93. The sucrose was supplied to the embryos at the beginning of the 1st, 3rd, 5th and 7th weeks of
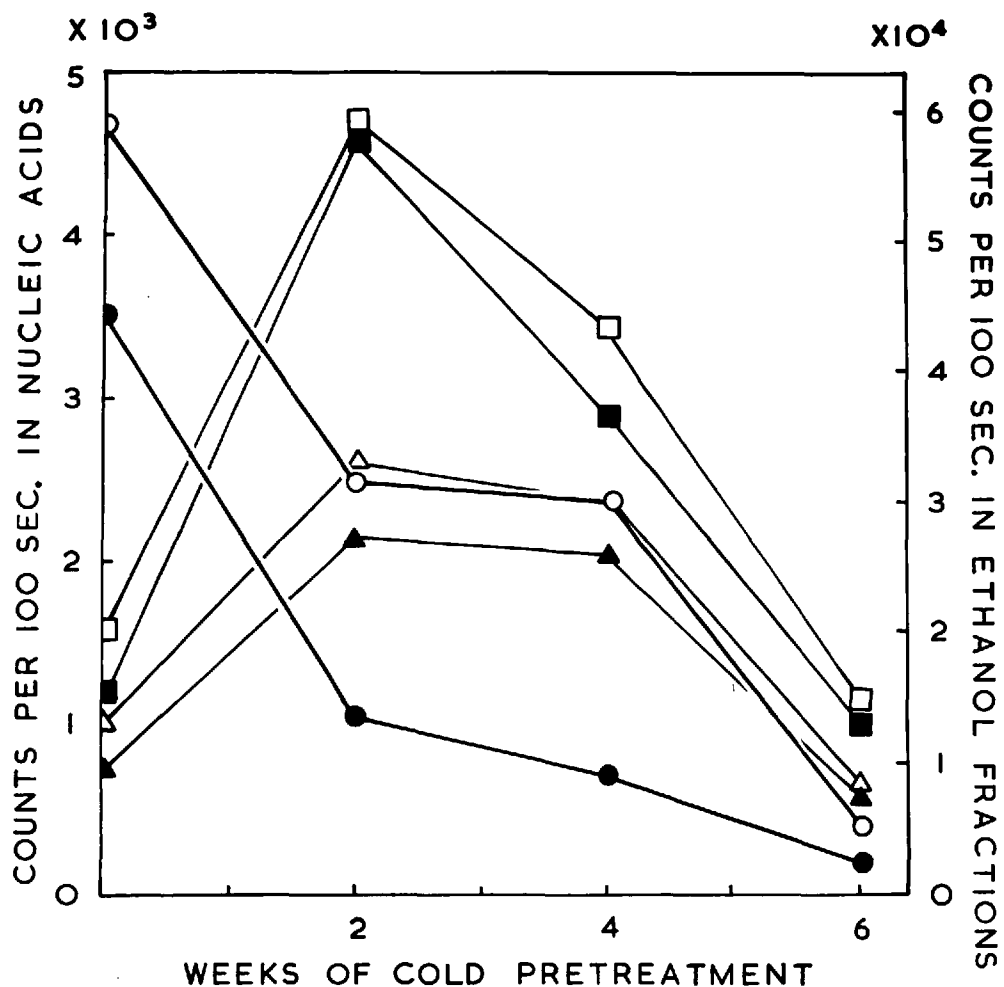
FIGURE 12   INCORPORATION OF RADIOACTIVE
CARBON FROM SUCROSE INTO THE NUCLEIC
ACIDS AND ETHANOL SOLUBLE MATERIAL IN
PETKUS RYE EMBRYOS. ●NUCLEIC ACIDS
▲ABSOLUTE ETHANOL ■ 5O PER CENT
ETHANOL. THE SHADED SYMBOLS REPRESENT
VALUES OBTAINED FOR EMBRYOS IN THE
COLD AND OPEN SYMBOLS FOR THOSE AT 15°C

the cold treatment and the uridylic acid after the
2nd and 5th weeks.

The results (Figure 12) show that carbon
from radioactive sucrose was incorporated into the
'nucleic acid' fraction of the embryos and that the
amount incorporated decreased with increase in the
time in the cold before supplying sucrose. Although
less in the cold than at $15^{\circ}C$, the labelling of the
nucleic acid at the two temperatures was similar in
pattern except after four weeks when the trend of
decreasing incorporation halted at $15^{\circ}C$. Radioactivity
in the ethanol fractions (containing amongst other
things sugars, amino acids and soluble nucleotides)
was maximal after two weeks and decreased thereafter.

When uridylic acid was supplied to the
embryos most of the radioactivity in the soluble nuc-
leotides was in UDPG. The other radioactive subs-
tances present only in extremely small amounts were
not identified.

The components of the alkaline hydrolysate
of the RNA gave 6 u.v. absorbing bands on the chroma-
togram when developed with the isopropanol/HCl/water
solvent. Adenine and guanine were present as well as

Table 6 Radioactivity in the nucleic acids of Petkus rye embryos supplied with radioactive uridylic acid

Conditions under which uridylic acid was supplied.	Weeks of cold pre-treatment	counts per 100 sec.			C/U
		C	U	G	
In cold	2	1817	1629	858	1.11
	5	2098	2012	1485	1.04
At 25°C	2	768	758	651	1.02
	5	2054	2038	1312	1.01

the corresponding nucleotides indicating some hydro-
lysis of the latter during chromatography.   There
was radioactivity in uridylic, cytidylic and guanylic
acids and guanine but not in the adenine derivatives.
The amount of radioactivity was approximately the same
in the cytidylic and uridylic acids but only about
half the quantity in the guanylic acid (Table 6).
There was a small amount of radioactive guanylic acid
present as an impurity in the uridylic acid supplied
to the embryos but the amount was probably insufficient
to account for that found in the nucleic acid.   There
was no radioactivity present that did not correspond
to a u.v. absorbing substance.

Some of the RNA of the first of the warm
grown samples was lost which accounts for the low
value for radioactivity of this sample.   However,
in contrast to the sucrose experiment there was greater
incorporation of C^{14} into the older embryos of cold
grown samples and in comparable warm and cold grown
embryos there was little difference in the amounts
of radioactivity present.

Experiment 7

Application of 2-thiouracil to germinating winter wheat

Five samples of 8 grains of Cappelle winter

wheat were removed from the polythene dish in which
they had been germinated in the cold (3° to 5°C)
for 7 weeks and 3 days in unrestricted moisture con-
ditions (Method 2, p. 44).   They were transferred
to moist Whatman No. 54 filter paper in inverted
plastic refrigerator boxes one or two samples per
box.

Drops (0.01 ml.) of a saturated solution
(600 μg/ml.) or 2-thiouracil (L. Light & Co. Ltd.,
Colnbrook, Bucks.) containing 0.1 per cent Tween 20
were placed in contact with the coleoptiles of the
first sample and all samples retained in the cold for
a further day.   They were then removed from the cold
and placed in an oven at 13° to 15°C for 3 days.
A further sample was treated with thiouracil on each
of these 3 days, leaving one sample untreated.   On
the day that the samples were first placed in the
oven 8 grains were soaked for 5 hr. in water then
transferred into a plastic box and also placed in
the oven.   After 3 days each sample was planted
out (on May 2nd, 1964) into a 10 in. clay pot.   The
wheat was grown to maturity in a greenhouse in
natural long days and a note made of the days to
anthesis and the number of leaves produced by each

plant.

The results in Table 7 show that the 2-thiouracil applied to Cappelle winter wheat directly after a cold treatment did not prevent its flowering or its vegetative growth.

Table 7   Number of leaves produced and days to anthesis of Cappelle winter wheat treated with 2-thiouracil. The experiment was concluded after 140 days

Temp. after thiouracil application				Un-treated	Un-vern-alised
Days at 3°C 1	-	-	-	Un-treated	Un-vern-alised
Days at 15°C 3	3	2	1		
Leaf number 10	9	10	9	10	Still vegeta-tive
Days to anthesis 124	128	124	132	132	

DISCUSSION

The basic processes underlying cell differentiation, as tentatively defined in the introduction, may be the selective activation of latent gene DNA followed by transcription of the nucleotide sequence of DNA to that of RNA and ultimately to the amino acid pattern of protein. The direct participation of the nucleic acids in the processes of flowering is indicated by the inhibition of this process by base analogues which disrupt normal functioning of the nucleic acids into which they are incorporated and by inhibition of nucleic acid synthesis. Salisbury and Bonner (1960) and Bonner and Zeevaart (1962) showed that in Xanthium buds application of 5-fluorouracil during the first part of an inductive dark period prevented flower induction by inhibiting RNA synthesis. However, in Pharbitis nil 5-fluorouracil inhibited flowering by suppressing DNA synthesis in the shoot apex (Zeevaart, 1962). There was also inhibition of flowering by 2-thiouracil in Cannabis sativa (Heslop-Harrison, 1960). Xanthium, Pharbitis and Cannabis are all short day plants. In Streptocarpus wendlandii in which low temperature (10°C) during short days are required for flower induction, flowering is prevented and vegetative growth

slightly stimulated by 2-thiouracil (0.1 to 0.4 mg.

per week) applied to the leaf during the 8 weeks of an

inductive cold period.   Larger doses of the

thiouracil disrupted the vegetative growth as well

(Hess, 1959).

In rye plants germinated in short day con-

ditions flower initiation was earlier than in plants

grown in long days and in this respect the photoperiod

simulates the effect of vernalisation.   If changes

occurring during light treatments at non-vernalising

temperatures are comparable with those in the cold

it might be expected that high temperatures during the

light treatment would delay flowering.   This aspect

has not been investigated in rye but Purvis (unpublished

results) has shown that high night temperatures delay

flowering.   In Hordeum bulbosum (a perennial grass

with responses to heat and light similar to those

of winter rye) high temperatures (26°C) given with

long day treatments completely suppress flowering and

greatly diminish the effect of short day inductive

periods (Koller & Highkin, 1960).   Since therefore

a vernalisation treatment is similar to an inductive

photoperiod, it seemed possible that base analogues

might also prevent progress to flowering in the cold.

Application of 2-thiouracil or azaguanine to winter
rye plants, however, had no effect on the response to
cold (Hurd, personal communication).   Also
Ketallapper (communication from Zeevaart) found
that of the many base analogues applied none affected
this process.   Application during this investigation
of 2-thiouracil to winter wheat plants on each of
three days subsequent to an 8 week cold treatment
(experiment 7, p. 127) also did not prevent flowering
and if anything slightly hastened this process.
Since heavy doses of 2-thiouracil (embryos were grown
on solution of 60 µg./ml. for 1 week) severely in-
hibited the growth of winter wheat it is unlikely
that its entry into cereal plants is prevented.
From this it appears that the nucleic acid concerned
in floral initiation is neither synthesised during
vernalisation nor during the period shortly there-
after.

Involvement of the nucleic acids in vernalisa-
tion would alternatively be indicated if there were a
difference in either their content or composition
in vernalised and unvernalised plants.   Several
studies have been made in order to detect differences

of this kind but unfortunately the methods used are
suspect in some cases.   Finch and Carr (1956) found
no change in the RNA phosphorus per unit dry weight
in Petkus rye embryos of grain grown under restricted
moisture conditions during vernalisation but there
was an increase in RNA phosphorus per embryo with inc-
reasing embryos size.   Similar results were obtained
for the DNA phosphorus fraction.   The validity is in
doubt of both the version of the Ogur and Rosen method
which was used to extract the nucleic acids and of the
phosphate estimation by which the nucleic acids were
measured (see discussion on nucleic acid determination,
p. 50).   Sechet (1962) also using the Ogur and Rosen
method of extraction, found in winter wheat that
after an initial decrease in both DNA and RNA (per
unit dry weight) the content remained fairly con-
stant.   When, however, amounts of nucleic acid per
embryo were expressed in terms of coleoptile length,
increase in nucleic acid content was apparent as growth
took place.   In a comparable study made during this
investigation (experiment 1, p. 108) the RNA concen-
tration per unit dry weight of spring and winter
rye embryos was found to increase to approximately

the same level in both races during germination in
the cold in restricted moisture conditions.   RNA
level was lower in embryos grown to approximately
the same stage in development in unrestricted mois-
ture conditions at $25^{\circ}C$ and again it was comparable
in the spring and winter races.   The lower level of
RNA in the embryos at $25^{\circ}C$ would be explained if res-
tricted moisture conditions suppressed cell wall syn-
thesis more than RNA synthesis.   Such a situation
exists in corn seedlings germinated under water
stress in which West (1962) reports enhanced RNA
synthesis and diminished increase in dry weight pro-
tein and nucleotides.   Also an increase in RNA
level has been recorded during the dehydration of
olive and ligustrum leaves (Kessler & Frank-Tishel,
1962).

    The nucleic acids per unit dry weight of
embryos of Cappelle winter wheat increased in concen-
tration for about 48 hr. after imbibition of water
(experiments 2 & 3, p. 11) and subsequently decreased.
This trend follows a similar course to that found
by Barnell (1937) for the respiration rate per gram
of barley embryo material.   The trend is also very

similar to that found by Keys and Cornelius (1965)
for soluble nucleotides in the same wheat material used
in experiments 2 and 3.   The latter authors suggest
that the decrease in the concentration of soluble
nucleotides after the maximum level had been reached
was the result of an increasing contribution of cell
wall material to the dry matter.   A similar explana-
tion of the nucleic acid results seems likely since
it has been shown that during cell expansion in the
pea root, RNA content per cell increases fourfold and
the DNA content twofold (Heyes, 1960) while the
increase in dry weight per cell is greater than this
(Brown & Broadbent, 1950).   Wright (1961) has shown
that cell division in the coleoptile ceases between 48
and 60 hr. and that after this time growth is entirely
due to cell expansion.   The coleoptile comprises as
much as one third of the dry matter of wheat embryos
after 48 hr. germination at $25^{\circ}C$ in the dark.   Similar
cell expansion possibly takes place in the scutellum
and expanded, differentiated cells comprise an increas-
ing proportion of the roots.   In wheat embryos of the
same dry weight, grown either in the cold or warm,
the amount of nucleic acid present is similar al-
though slightly less in those embryos grown at the

lower temperature (Figure 11, p.117). The smaller
amount in cold grown embryos may be due to the cold
reducing the rate of cell division more effectively
than it does the rate of cell expansion and differen-
tiation. Haber and Luipold (1960) showed that in
lettuce embryos, the inception of mitosis is delayed
more by low temperature than is cell expansion. The
main effect of the cold, however, is to reduce the rate
of growth. For example, wheat embryos grown in the
cold after having been at $25^{\circ}C$ for 48 hr. took approx-
imately 16 days to increase in weight to an equivalent
extent to those grown at $25^{\circ}C$ for a further 2 days.
The changes occurring in the nucleic acid content,
although similar to those occurring at $25^{\circ}C$ take
place at a much slower rate in the cold. It would be
of interest to see whether the metabolism of the nuc-
leic acids is totally suppressed at the sub-zero
temperatures which stop growth processes (Grif, 1958)
but at which there is still progress to flowering.

Evidence is at yet inconclusive as to whether
or not the base composition of the nucleic acids of
flowering tissues differs from that of vegetative
tissues. Ross (1962), in a study of the vegetative

and flowering buds of <u>Xanthium pennsylvanicum</u>, found
no difference in the base compositions of the RNA
but Hess (1961) reported a small difference of this
kind in the total nucleic acid composition of vege-
tative and flowering plants of <u>Streptocarpus wendlandii</u>.
No difference was found in this investigation (ex-
periment 1, p. 108) in the base composition of the RNA
of 7 weeks cold treated rye embryos and of those
grown at 25°C for 48 hr. Thus it seems unlikely that
the induction of flowering in rye by a cold treatment
causes a change in a major fraction of RNA. However,
the possibility cannot be excluded that changes in base
composition of a minor fraction might occur. This is
suggested by the experiments of Oota (1964) who found
that in the long day duckweed <u>Lemna gibba</u> G 3 inductive
photoperiods modify solely a minor RNA species.
Also, in <u>Streptocarpus wendlandii</u> as mentioned pre-
viously, relatively low concentrations of thiouracil
inhibited flowering and much larger doses were neces-
sary before vegetative growth was impaired (Hess, 1959).
The important RNA fraction involved in flower initia-
tion is probably messenger RNA and since it constitutes
only a very small proportion of the total RNA, changes

in its composition are difficult to detect.   In the
cell fractionation studies described in experiment 4,
(p. 12) no difference was found between the 'messenger'
RNA fractions of cold and warm grown rye embryos.
Unfortunately the identity of a messenger RNA with
the expected DNA-like base composition was not est-
ablished in these fractions and therefore modified
experimental techniques are probably necessary in order
to investigate this particular problem further.

A change in the composition of the RNA
of winter wheat embryos during vernalisation was found
by Teraoka et al. (quoted by Oota, 1964) using the
technique described by Heyes (1960) in which two
fractions of RNA are obtained, the first (RNA_1) by
extracting the tissue with cold PCA and the second
(RNA_2) by hydrolysing the residual RNA with alkali.
They found a drop in the ratio of RNA_1 to RNA_2 during
vernalisation.   Heyes showed RNA_1 to be the most ab-
undant form in young meristematic cells and suggests
that RNA_2 is formed during the course of normal meta-
bolism from RNA_1 as a result of the action of ribo-
nuclease and is therefore more prevalent in older
tissues.   Oota (1964) therefore suggested that

vernalisation may be 'a cold-promoted ageing in embry-
onic tissues'.   There is a marked departure from
unity in the ratio of 6-amino to 6-keto bases in RNA_2
and therefore a shift from unity in the total base
composition might be expected during vernalisation as
the RNA_2 accumulated.   However no such change was
found in experiment 1 (p. 108).

In a study of nucleic acid metabolism during
vernalisation, it is an advantage to be able to dis-
tinguish those nucleic acids formed during a cold
treatment from those present in tissue before the
treatment commenced.   For this reason radioactive
precursors of the nucleic acids were supplied in ex-
periments 5 and 6 to winter rye embryos.   These
experiments were of a preliminary nature to determine
the feasability of this approach to the problem.   The
incorporation of C^{14} from both sucrose and uridylic
acid into the nucleic acids confirms that there was
nucleic acid synthesis at low temperatures.   Also the
patterns of incorporation of both precursors were
similar in the cold and at higher non-vernalising
temperatures indicating further that any change oc-
curring in the cold as a result of flower initiation

must be slight.

The pattern of incorporation of the C^{14} from the uridylic acid into the nucleic acids, although not directly of interest as far as vernalisation is concerned has unusual features, namely the labelling of guanylic acid and the absence of labelling in the adenylic acid. Buchowicz et al. (1963) in a study of the incorporation of radioactive uracil into the nucleic acid of wheat leaves found no radioactivity in the purine derivatives of the nucleic acid. Only 4 to 7 per cent of the radioactivity of the entire RNA hydrolysate was found in the purines by Sebesta et al. (1964) in cucumber seedlings infiltrated with radioactive uracil whereas in experiment 6 (p.126) 20 to 30 per cent of the activity was in the guanine fraction.

The main conclusion that may be drawn from this investigation is that there is no large overall change in cereal nucleic metabolism that can be attributed to the initiation of flowering processes in the cold. This was shown in experiments in which concentration, base composition and the incorporation

of radioactive precursors of the nucleic acids was
compared in cold and warm grown cereal embryos. The
methods used in these experiments were critically
tested and where necessary appropriate precedures
evolved, much more time being spent on technique than
was first envisaged. It seems unlikely that any
additional straight analytical study will result in
further information about vernalisation. Possibly
the development of this investigation from which most
might be learned would be the fractionation of the
nucleic acids following incorporation into them of a
radioactive precursor supplied exogenously to the
cereal seedlings.

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