12-1970

The Application and Refinement of Thin Layer Chromatographic Procedures in Analyzing the Ribonucleic Acids Present During the Morphological Development of Two Varieties of Avena Sativa L.

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THE APPLICATION AND REFINEMENT OF THIN LAYER CHROMATOGRAPHIC PROCEDURES IN ANALYZING THE RIBONUCLEIC ACIDS PRESENT DURING THE MORPHOLOGICAL DEVELOPMENT OF TWO VARIETIES OF AVENA SATIVA L.

by

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A Thesis
Submitted to the
Faculty of the Graduate College
in partial fulfillment
of the
Degree of Master of Arts

Western Michigan University
Kalamazoo, Michigan
December 1970

ACKNOWLEDGEMENTS

The writer would like to express her gratitude to the members of her Graduate Committee consisting of Dr. Imy V. Holt, Dr. Leo C. Vanderbeek, and Dr. William C. Van Deventer for their time and effort in regard to this study.

Because of the writer's association as a graduate student under the guidance of Dr. Holt she would like to extend a particular note of appreciation for his patience and encouragement during the failures as well as the successes of this investigation.

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INTRODUCTION

The nucleic acids and the proteins are two major divisions of molecules that form the underlying basis upon which the functions of living systems are dependent for a continuing existence. DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) occupy a central position in molecular biology as the directive agents for control of genetic processes and protein synthesis.

Gene expression resulting in protein syntheses involves numerous chemical steps, many of which are now known (Epstein and Beckwith, 1968). Transcription of the genetic code into a complimentary single-stranded mRNA molecule is the initial step (Hayashi, et. al., 1963; Marmus and Greenspan, 1963; and Bautz, 1963). The ribosomes and tRNA's, with the cooperation of a variety of enzymes, coordinate the translation of the genetic message carried by the short lived mRNA into a sequence of amino acids to form a protein (Berger and Yanofsky, 1967).

The ontogeny of an organism can be viewed as the result of a sequential and systematic change in the amount, type and function of protein present, and, therefore, the morphological spatial alterations within any organism's development should be foreshadowed by a chemical alteration within the mRNA which is transcribing the genetic code inherent within the DNA molecule into cytoplasmic protein.

This study was designed as one of a series of steps within an investigation attempting to demonstrate a correlation between the morphogenesis of the oat plant (Avena sativa L.) with a chemical

variation involving the mechanisms of protein systhesis.

An investigation has been completed in which a quantitative and qualitative analysis of the amino acid content during developmental stages was undertaken (Halgren, 1969). This present investigation was designed to determine if a pattern of chemical alteration could be demonstrated in the mRNA of the same species under the same conditions of growth and development, and the refinement of techniques necessary to achieve this.

The shoot apex was the only tissue involved in these studies because of many experimental investigations which have led to the concept that this embryonic meristematic region is of primary importance for development (Sinnott, 1960).

LITERATURE REVIEW

The principles of adsorption chromatography were first discovered by Michael Tswett, a Russian botanist, in 1903 (Sakodynsky, 1970; Akhrem and Kuznetsova, 1964). The technique, however, was not fully utilized for almost three decades when a substantial development of real methods was introduced to the scientific field in 1931 by Kuhn, Winterstein, and Lederer. Since that time column adsorption chromatography has rapidly developed, generally for the separation of lipophilic substances, followed by the invention of partition chromatography with methods suitable for the separation of hydrophilic molecules (Martin and Synge, 1941). Paper chromatography was introduced as a system of chemical analysis in 1944 and has been used extensively for amino acid and protein differentiation (Consden, et. al., 1944).

Thin layer chromatography (TLC) has been extensively utilized since Stahl (1958) standardized the basic procedures and demonstrated the wide applicability of separations based on the micro adsorption principles which were first described by two Russian authors, Ismailov and Shraiber (1965).

TLC methodology was introduced commercially in the United States in 1960 and has become one of the most widely accepted analytical procedures in research and control laboratories (Stahl, 1969). The original applications are being constantly expanded and refined, the most significant advantages at this point and time consisting of the following favorable features.

TLC incorporates speed with efficiency since the development time

is faster than either column or paper chromatography. The average separating time with standard solvents ranges from 20 to 40 minutes. Pure inorganic adsorption layers are used which permit the application of corrosive reagents for visualization and the separations resulting are usually sharper than those obtained with either paper or conventional column procedure with sample volumn varying from as little as 0.5 micrograms to as much as 55 micrograms. TLC results may be correlated with column separations since the same materials are generally used and it is a practical complement to preparative and ultra micro methods.

Chromatography encompasses a procedure in which the material or mixture to be resolved is repeatedly distributed between two phases. The technique of TLC involves the spreading of a thin layer of sorbent on one side of a glass plate, applying a sample at a starting point, and dipping the edge of the plate into a solvent system. The liquid is drawn upwards by capillary forces with the separation of the sample attained by adsorption, partition, and/or ion exchange based on the nature of the reaction between the dissolved substances and the solid with which they come in contact.

Polyethylenimine-(PEI)-cellulose coated plates are anion-exchangers and the results are based on the formation of ionic bonds between the material to be resolved and the electrically charged groups of the sorbent. Nucleotides exhibit pH-dependent charge differences with similar solubilities and are therefore ideal for ion-exchange chromatographic analysis.

The application of TLC procedures to analytical investigations

involving the nucleic acids of both DNA and RNA has progressed rapidly in the last few years with one of the major contributors being Dr. Kurt Randerath (1963) of the John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University. Detailed information and laboratory guides for the technical procedures and specific applicabilities involved in this form of analytical investigation are available (Stahl, 1964; Bobbitt, 1963).

The grasses (Gramineae), which includes the oat cereal plant Avena sativa L., exhibit one of the more complex embryonic development found in the monocots. Growth is accomplished by apical meristems which, although small and inconspicuous initially, are the organizing centers for all axial development and the laying down of future secondary growth areas (Wardlaw, 1955). The shoot apex has been considered a region of continuing embryonic tissue which gives rise to all ensueing cells, leaves and stems which form the above ground shoot system of the plant (Torrey, 1967).

The shoot apex has been studied intensively as the organizing center of morphological development and the focal point of self-determination and autonomous perpetuation in both an anatomical and physiological approach (Reeder, 1953). Anatomical considerations of the grasses commenced in the seventeenth century with the investigations formulated by Malpighi, the emphasis of research shifting from structual and mathematical growth curve analysis only to an understanding of internal control mechanisms in the last four decades. Much of the recent work along these lines has been aimed at hormonal controls in terms of internal metabolic influences and their interdependent

relationships. It has only been recently, due to the impetus provided by advanced technological procedures and more workable models of nucleic acid involvement, that the area of genetic control through the chemical process of protein synthesis has been under intensive investigation (Neuhard, 1965).

One of the most extensive investigations of the nucleotide composition of plant ribonucleic acids was that of Vanyushin and Belozersky (1959) who analysed the RNA of seed, pollen and thallus tissue from a wide range of plants. Trim, Baker and Leah (1964) concentrated their efforts toward a quantitative analysis of RNA content found in plant leaves. The ribonucleotide composition of preparations of sugar beet material was determined in 1959 (Trim, 1959) and analyses of RNA preparations of another member of the Gramineae family, wheat, was finished in 1963 (Trim, et al., 1964).

A very high proportion of the whole RNA of the above investigations has been shown to be composed of the four major nucleotides. An accumulation of RNA was shown to be greater in the transition from a vegetative to flowering state which correlated with anatomical and cytological changes within the apical meristem by Shvedskaya and Kruzhilin in an investigation concerned with changes in the nucleic acid content in biennial plants during differentiation of the buds (Shvedskaya and Kruzhilin, 1968). An effect of kinetin concentration on RNA metabolism in detached leaves of Italian ryegrass has also been demonstrated (Oritani, et al., 1969), as well as a rise in RNA content before flowering of various taxonomic plants by the use of autoradiographic methods (Knox and Evans, 1968). A microphoresis technique

has been used to determine an alteration of nucleic acid content in the initiation of bud primordia in other plant species, the total amount of RNA increasing fifteen fold in kinetin induced cells to that of protonematal cells (Shneider, et al., 1969).

The above investigations which combined the techniques of electrophoresis and chromatography of plant hydrolysates, followed by the measurement of spectral characteristics, showed the presence in quantity of only the four major nucleotides, AMP, GMP, CMP and UMP with little or no evidence that there was a presence of other nucleotides in more than trace quantities.

METHODS AND MATERIALS

The laboratory plant

Rodney and Clintwood, two varieties of commercial oats (Avena sativa L.), were used in this study. Seeds selected for firmness and large size were planted from each variety in 12¼" x 17¼" plastic trays containing prewashed vermiculite for the removal of mineral and organic impurities. The plants were then placed in the greenhouse to germinate. The following three-day-sequence for nutritive watering was continued throughout the study: distilled water the first day; 1/10,000 per cent iron tartrate solution the second day; and Hoagland's solution (See Table I.) the third day. Minor elements were omitted as nutritional factors, the endosperm retaining an adequate amount for healthy growth of the embryo and developing seedling for the 28 day span of this study.

TABLE I. Molar Formula for Hoagland's Solution (One Liter)*

Constituents		ml per liter
KH_2O_4	Potassium phosphate	1
KNO_3	Potassium nitrate	5
$Ca(NO_3)_2$	Calcium nitrate	5
$MgSO_4$	Magnesium nitrate	2

* Hoagland and Arnon, 1950.

Twenty-five plants were collected of each variety at six different stages of morphological development (See Table II.) based on a histological investigation of Avena organogeny (Holt, 1955). The shoot apices were carefully dissected, placed in 95 per cent ethanol and refrigerated until used.

TABLE II. Schedule of Sampling and Comparison of Plant Ontogeny

Days after planting	Vegetative organogeny	Plant appearance
6	Third Foliage Leaf primordium	Emergence from soil
10	Fifth Foliage Leaf primordium	Coleoptile and one foliage leaf
14	Sixth Foliage Leaf primordium	Three leaves
18	Seventh Foliage Leaf primordium	Four leaves
23	First Order Branches of the panicle	Four leaves
28	Spikelet Initiation	Five leaves

The shoot apices were prepared for chromatographic procedure in the following manner. Twenty-five apices for each sample were crushed with a sterilized glass homogenizer in 3 ml of ethanol. Ribonuclease was added and the solution allowed to incubate at room temperature for approximately 20 minutes.

Thin-layer chromatography materials and procedure

Commercially coated glass plates of 20 cm x 20 cm were obtained from the Brinkman Instruments Incorporation. The sorbent used was a suspension of cellulose in a poly(ethylenimine) hydrochloride solution (PEI-Cellulose). The plates were stored at -20° C. until used to prevent degradation. All plates used were from the same lot to insure uniformity throughout the experiment. Each plate was given a preliminary development with distilled water to remove any impurities and dried before the application of any experimental or control solutions.

First dimension: After applying five Lambda of the hydrolysate solution of each sample at a starting point 2 cm from both edges of the plate the chromatograms were developed in a closed circular tank containing a 60 per cent saturated $(NH_4)_2SO_4$, neutral pH, solution until the solvent front had risen to 14 cm. This development took approximately 50 minutes.

Second dimension: Before carrying out the second dimension the plates were first dried in a stream of air and placed for arproximately 15 minutes in a flat dish containing 800 ml of methanol to remove any salts which might interfere with development in the second direction. The dissolution of any salts present was accelerated by occasional agitation. The plates were then dried before being placed in a solution of 2 per cent Boric Acid: 1.0 M LiCl (pH 6.8). The solvent front was again allowed to reach a height of 14 cm. The plates were again dried in a stream of air before being viewed and photographed under ultraviolet light. This phase of development required approximately 50 minutes.

Standards

In order to identify the four major nucleotides constituting mRNA, an equimolar solution was prepared using commercial salts obtained from the Nutritional Biochemicals Corporation of the four major bases. A .002 M solution of adenine, cytosine, uracil, and guanine was prepared with distilled water and five Lambda of the solution was applied to a PEI-cellulose plate as a control for each chromatographic run.

Photography

Spots were detected under ultra-violet light (260mu) and photographed with a Leica MD-1 camera attached to a Leitz-Reprovit 2-A instrument. The film was tri-X ASA200 and the exposure time was 1/2 second with a shutter opening of F11. The camera was approximately 20 cm from the plates.

RESULTS

Photographs of the chromatographic plates resulting from this investigation are shown in Figures 1-7. It is evident upon examination of these that the double aims of this investigation have been satisfactorily completed. A technique for the separation of the RNA contained within the shoot apex of Avena sativa L. has been developed, including a workable solvent system for two dimensional chromatography, that will demonstrate a pattern alteration of ribonucleic acids during the ontogenical development of this species.

Three factors that must be taken into account in the selection of proper procedures for preparing cells were considered in this study. The first of these, the hardness of tissue to be used, was not a determining factor as meristematic cells are soft. They contain no secondary wall growth or any appreciable amount of storage material and can therefore be crushed easily. The second factor concerns itself with those cells which are highly vacuolated, the fluid of which is often acidic and causes a rapid denaturation of macromolecules upon cell destruction. Ethanol, the solvent used for homogenizing the cells from meristematic tissue, is less acidic than water and would tend to neutralize any chemical reaction along these lines even though this type of plant tissue is not regarded as highly vacuolated. The last major factor to be considered is that of nucleic acid content as this varies from tissue to tissue. A rough guide of nucleic acid content has been given in the literature (Stern, 1968) and for growing tissues it has been determined that 0.2 per cent of the dry

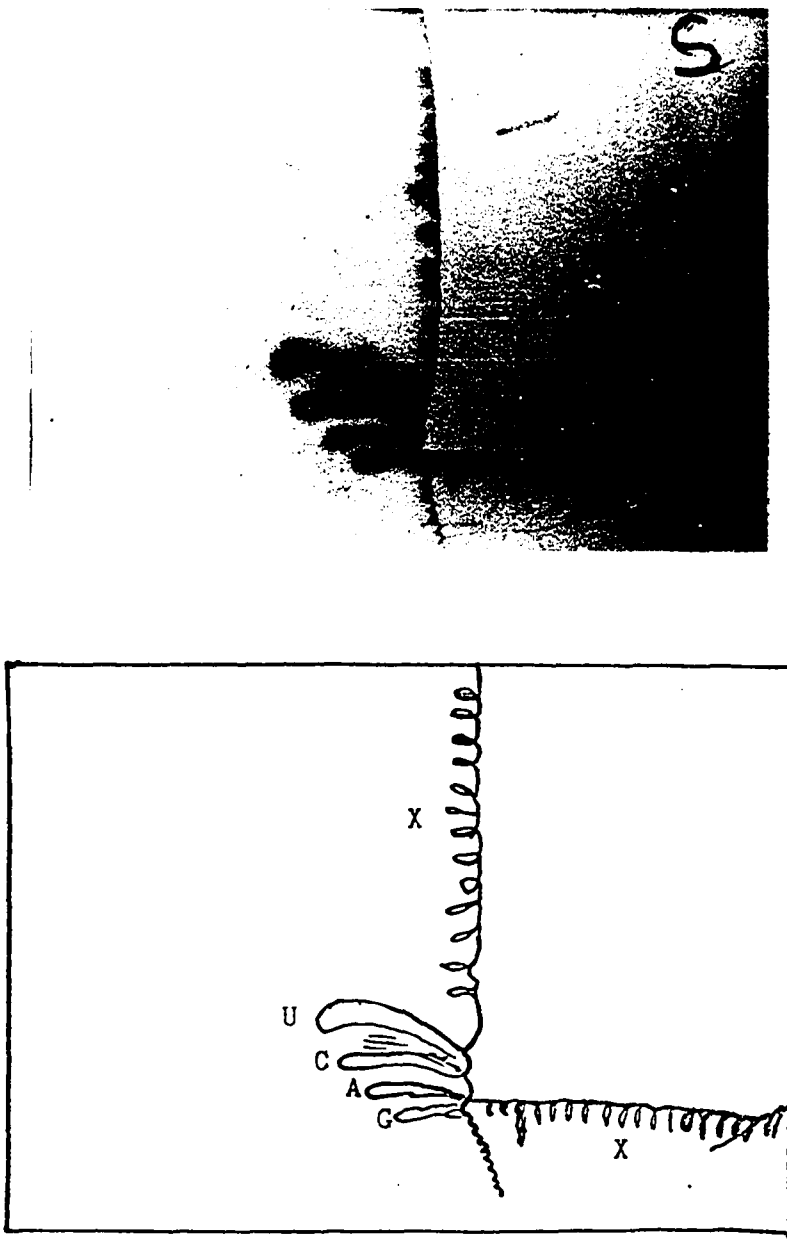

Figure 1. Photograph with schematic interpretation of nucleic acid salts.

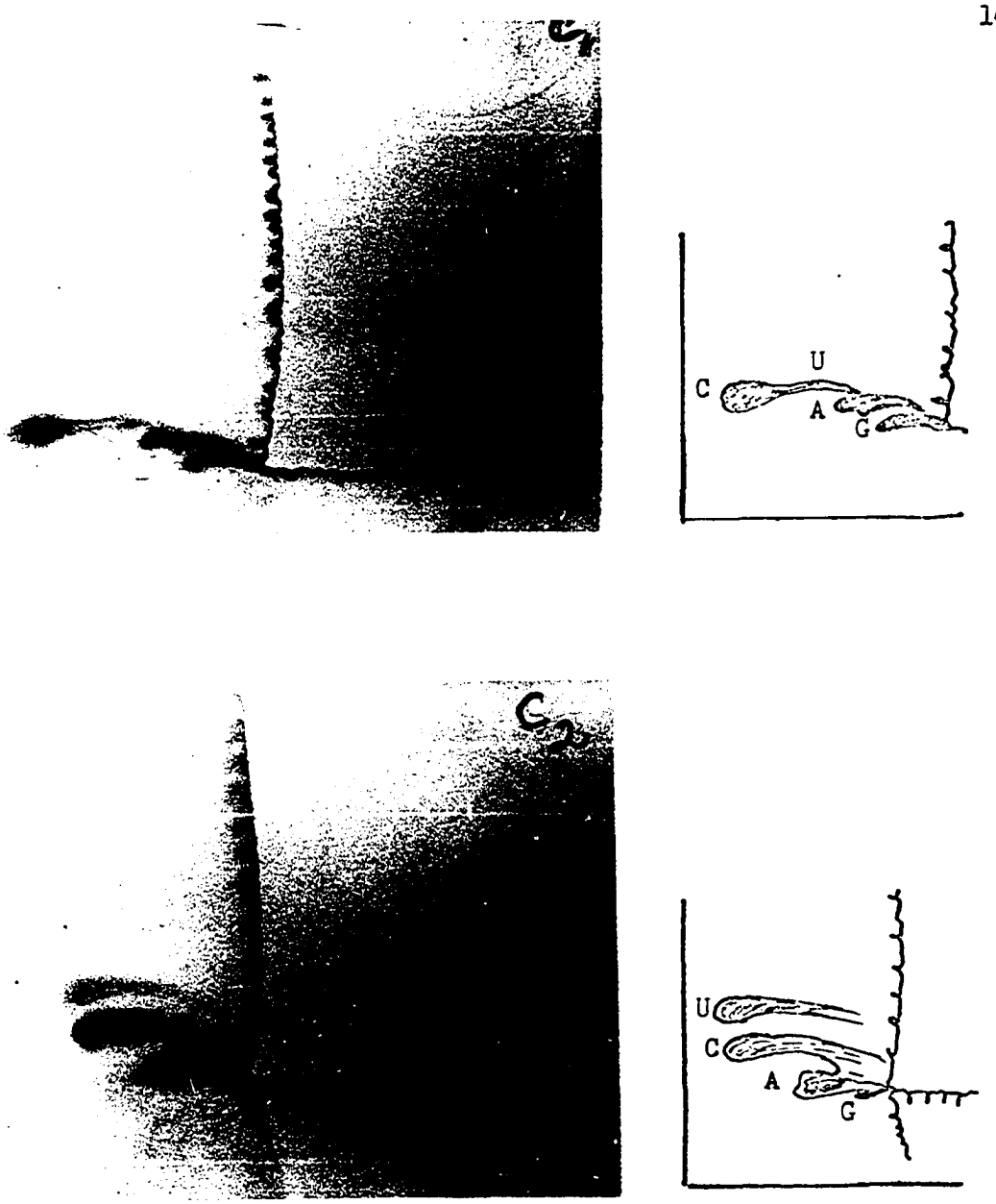

Figure 2. A - Photograph with schematic interpretation of Clintwood: 6th day.

B - Photograph with schematic interpretation of Clintwood: 10th day.

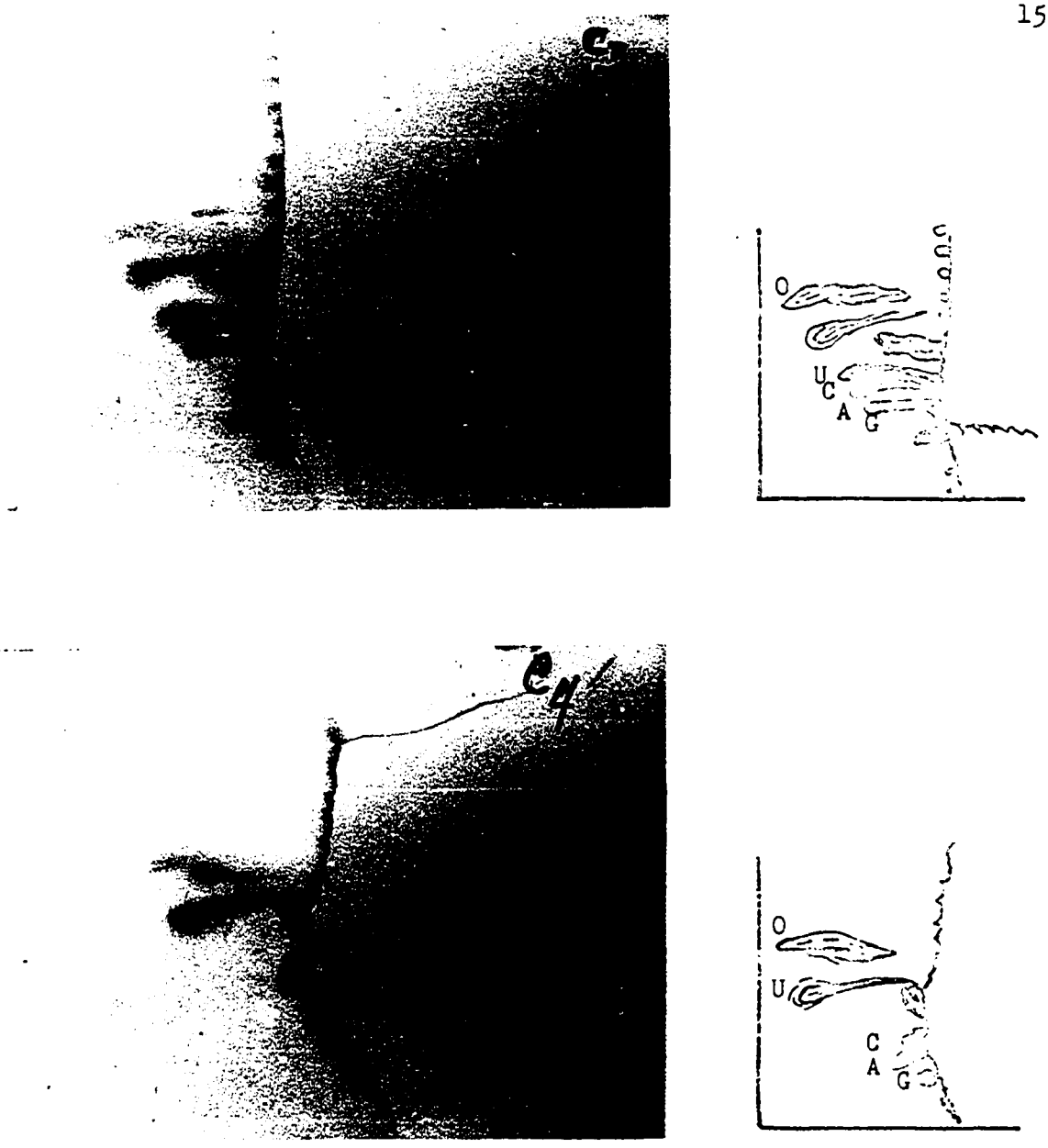

Figure 3. A - Photograph with schematic interpretation of Clintwood: 14th day.

B - Photograph with schematic interpretation of Clintwood: 18th day.

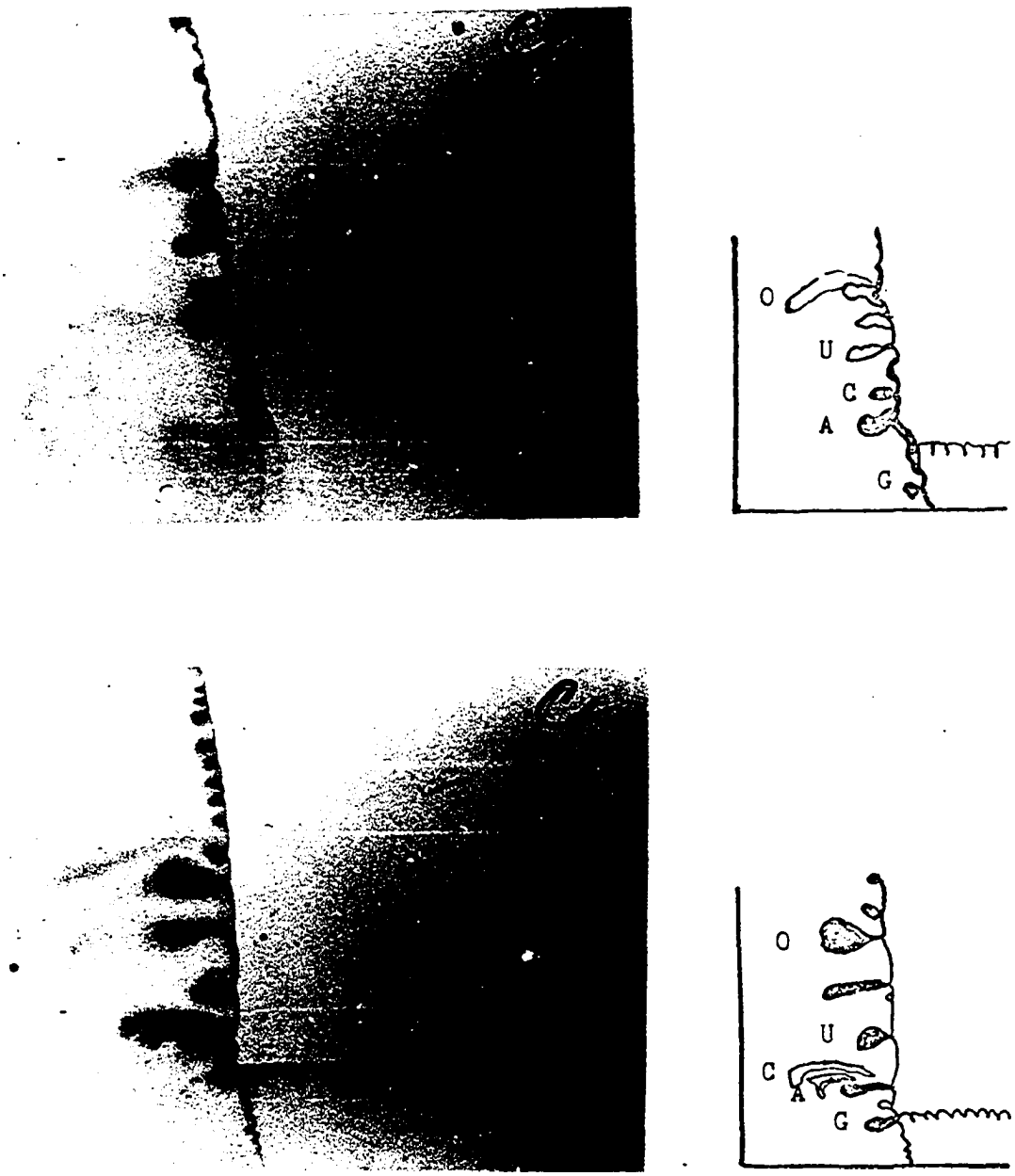

Figure 4. A - Photograph with schematic interpretation of Clintwood: 23rd day.

B - Photograph with schematic interpretation of Clintwood: 28th day.

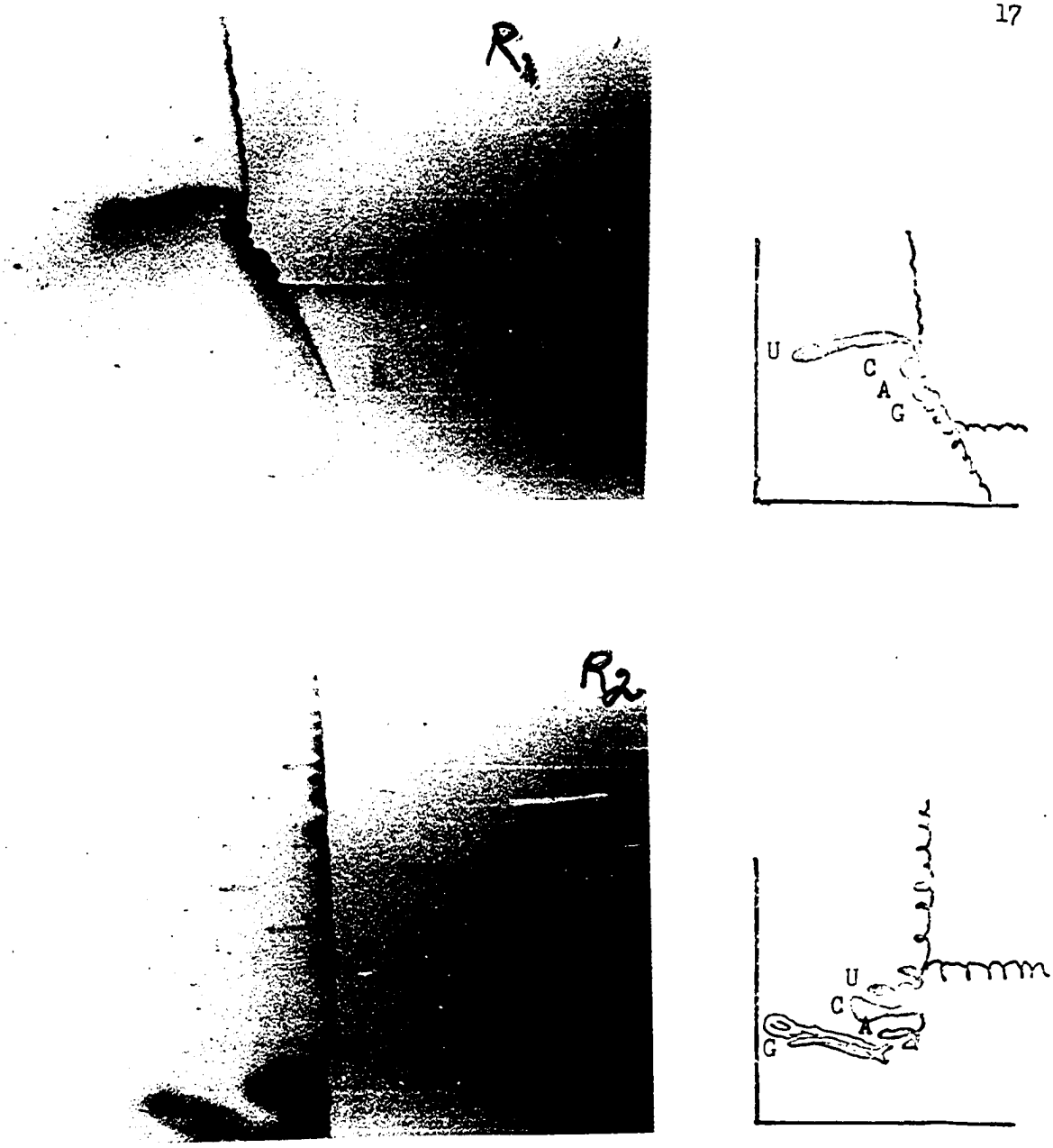

Figure 5. A - Photograph with schematic interpretation of Rodney: 6th day.

B - Photograph with schematic interpretation of Rodney: 10th day.

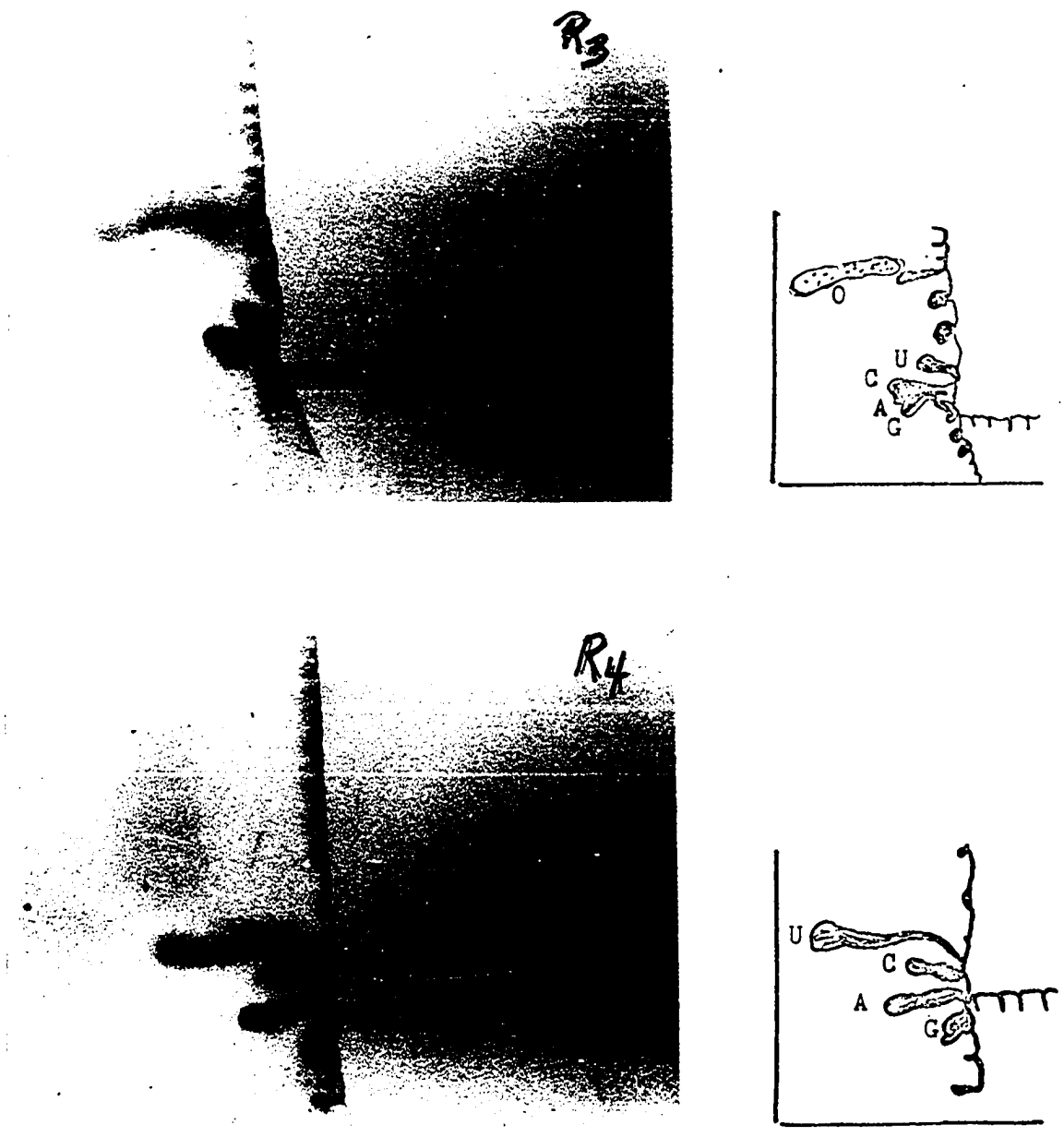

Figure 6. A - Photograph with schematic interpretation of Rodney: 14th day.

B - Photograph with schematic interpretation of Rodney: 18th day.

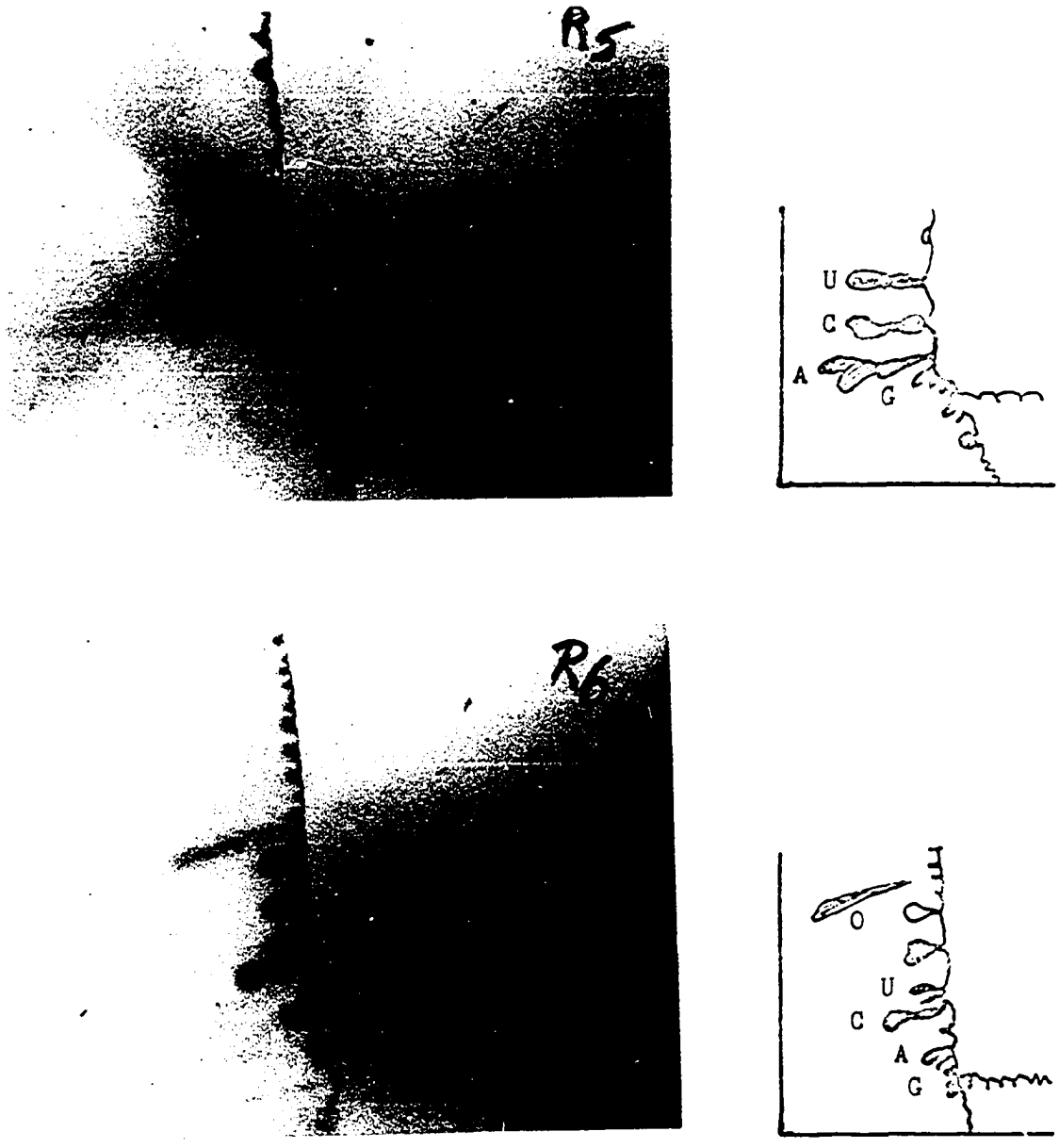

Figure 7. A - Photograph with schematic interpretation of Rodney: 23rd day.

B - Photograph with schematic interpretation of Rodney: 28th day.

weight is DNA with the RNA four to eight times that.

Discrimination between the three types of RNA was not felt to be vital in this preliminary investigation of pattern analysis. Transfer RNA is present in small amounts, approximately 5 per cent of the total, and remains basically stable throughout the life of the organism. Ribosomal RNA, even though it makes up almost 85 per cent of the total RNA present in the cytoplasmic fluid, is also relatively stable and any alteration of content would be in the form of quantity and not quality in terms of nucleotides present. The greatest alteration, therefore, in the chemical pattern of ribonucleotides would consist mainly of changes within the short-lived messenger RNA which varies according to the dictates of genetic information contained within the DNA which controls the morphological alterations through the transcription of structural genes.

The choice of solvent used for the first dimension of chromatography was selected primarily due to its ability to separate nucleotides on the basis of their containing a particular purine or pyrimidine base (Randerath, 1964). At a neutral pH the rate of migration has been shown to decrease in the order of uracil, cytosine, adenine and guanine derivatives (Randerath and Randerath, 1964). This order of elution is predicted by net charge considerations as well as size, physical properties and arrangement of nonionic substituents which influence the bonds established between the compound and plate adsorbent.

The solvent selected for the second dimension was chosen because of the pronounced effects that incorporating borate ions into a LiCl

solution displays on the anion-exchange behavior of cis-glycols in general (Khym, et al., 1957), and ribonucleotides in particular (Cohn and Bollum, 1961). Borate complexes add a net negative charge to these compounds and nucleotides differing only with regard to their sugar moieties may be separated (Randerath, 1963; Randerath and Randerath, 1965). The use of this system eliminates any identical migration of deoxyribo and ribonucleotides of the same type, thus insuring that the spots resulting would not be contaminated with DNA constituents that might possibly be present due to the mechanical crushing of the cells and subsequent hydrolysis.

The fact that the spots resulting were diffuse, and hence poorer resolutions were obtained, could be accounted for by several factors. Chromatography on commercial cellulose ion-exchangers gives more diffuse spots than on laboratory made plates (Randerath and Randerath, 1964). The elongation of the ribonucleotide spots may also be due to partial dissociation of the borate complexes (Randertah, 1963), which was included in the second dimensional solvent. The fact that the ribonucleotides did not result in as sharp a resolution pattern desired does not detract from the fact that a pattern was displayed.

DISCUSSION

The importance of gene action resulting in development and differentiation is now accepted and basic understanding of morphogenesis must include an analysis of biochemical controls found in the genetic material, DNA, which acts upon biosynthesis and cellular metabolism in terms of protein produced. This investigation has been concerned with refining a technique to demonstrate one step of many which leads from a genotype to expressed phenotypic macromolecules resulting from the mechanisms of differentiation and cellular determination.

Messenger RNA, coded for the creation of specific proteins, plays a vital role in the translation of the inherent chemical code for cellular specialization. An alteration in the chemical composition of this class of molecules will dictate an alteration in the sequence of amino acids which are bound together to construct the various proteins, both structural and enzymatic in nature, that enter into the cells morphological change and metabolic potential. The fact that a chemical variation has thus been demonstrated correlates this facet of an overall study pertaining to these particular species of grasses to that of the previous investigation already completed which demonstrated a chemical basis of change concerning the amino acid subunits of protein present.

This investigation originally concerned itself with an attempt to produce a workable system by employing the techniques of paper

chromatography. These attempts failed to produce any concrete results, even though several solvents were tried for two dimensional runs. Paper chromatography was not sensitive enough for the separation of nucleic acids from such minimal amounts of tissue that were utilized in this study. Thin layer chromatographic procedures were then investigated for the reasons of increased sensitivity and efficiency that this research tool afforded.

Several plates were developed with sample solutions of varying amounts of tissue hydrolysates to determine a concentration level that would produce the clearest and most reproducible separations. Whereas 40 shoot apices had been used for the paper chromatographic system without producing any spots, this concentration was found to result in large overlapping areas on this layer plates. The number of apices was reduced to 25, a quantity which minimized the amount of overlapping while maintaining an effective separation that could still demonstrate a shift in nucleic acid composition.

The techniques of thin layer chromatography are basically similar to those employed in paper development. However, as the system is so highly sensitive to minute quantities of substances and the plates so delicately coated with adsorptive materials, certain precautions were discovered during the refinement of this laboratory procedure that should be heeded. The plates must be kept cold, (-4°C) to prevent degradation when not in use. They should be handled as little as possible and then only by the edges when necessary. Contaminates can adhere to the adsorption layer and the presence of any purine or pyrimidine based substances should

be accomplished in such a manner that the hyrolysate is applied on the same point. The nucleic acids will remain at the center of the spot due to charge attractions but the solvent will spread rapidly, a factor controlled by regulating the flow of application. Caution must be observed when setting the plates for drying. A clean square of glass can be used to set the plates upon as metal stands allow residues to adhere and move upward on the coating, thus contaminating any plates which come in contact with them. The flow of air should pass near the plates but never directly hit them as absorbent flaking and forced solvent flow can result. Care must also be taken when agitating the plates during the methanol wash, a gentle action needed to eliminate any possibility of loosening the adsorbtive layer which is easily disturbed when wet.

The variation of spots resulting from this two dimensional thin layer system demonstrated a pattern of nucleotide change in the direction of complexity for both the varieties of Rodney and Clintwood under investigation. The control run of the nucleic acid salts (See Figure 1.) demonstrated the pattern of the four major bases of the ribonucleic acids. The schematic diagram included in Figure 1 identifies these as uracil (U), cytosine (C), adenine (A), and guanine (G). The areas marked X are the results of components contained within the solvent system which absorb ultraviolet light and are therefore considered irrelevant in the context of this discussion.

Figures 2, 3 and 4 visually display the results obtained from the oat variety Clintwood. Figure 2a clearly shows three of the

four basic nucleotides, uridylic acid displaying only a faint haze which indicates that it is not present in any comparable concentration at this point of morphological development. Uridylic acid does increase, however, as the plant develops from six to ten days after planting with a decrease in guanylic acid which tends at this point to overlap with adenylic acid (See Figure 2b.).

Fourteen days after planting the nucleic acid pattern for Clintwood shows a trend toward complexity (See Figure 3a.). The areas marked O are additional nucleic acid compounds other than nucleotides of U, C, A, and G. These large diffuse spots could be the result of mRNA bound to rRNA, a faction which would increase as cellular differentiation became more complex due to an increase in the number and types of protein required for increased specialization. Ribosomal bound mRNA is difficult to hydrolyse with an enzymatic ribonuclease and could therefore lead tc the production of oligonucleotides which would migrate in a slightly different manner due to a change in intrinsic charges, molecular size and configuration. Figure 3b, 18 days after planting, definitely shows a predominance of uridylic acid, a decrease in cytidylic and adenylic acid, with guanylic acid remaining in small concentration.

Figure 4 portrays the 23rd and 28th days after planting. Figure 4a displays an increase in the amount of oligonucleotides which have migrated together, creating darker spots under ultraviolet light. Cytidylic acid predominates at the morphological stage of spikelet initiation (See Figure 4b.).

The second variety of grasses under investigation, Rodney, also

displayed a pattern of simplicity with a predominance of uridylic acid on the sixth day after planting (See Figure 5a.). The diffuse areas of oligonucleotides (O) begin to show up on the 14th day, just as they appeared with Clintwood (See Figure 6a.) indicating an overall similarity between the two varieties in their mechanisms of differentiation. Uridylic acid predominates on the 18th day (See Figure 6b.) and cytidylic acid again increases at spikelet initiation (See Figure 7b.).

The following similarities between the two varieties can therefore be pointed out. Uridylic acid predominates in both varieties at the morphological stage of the seventh foliage leaf primordium, 18 days after planting. Guanine never predominates as a mucleotide and tends to remain stable throughout the study in both varieties. Spikelet initiation resulted in an increase in cytidylic acid, indicating that a definite shift has occurred in the genetic message as the plants matured toward flowering.

An overall similarity does exist between the two varieties of Rodney and Clintwood, results which were not unexpected. The fact that the patterns of nucleotide alterations are not identical enforces the concept of genetic differences between varieties of the same species creating morphological variations within a general framework of cellular differentiation.

Further work needs to be completed in connection with these investigations and the next step toward a more complete understanding of morphological change would be the elucidation and quantitative analysis of the ribonucleotides present during the same periods of

maturation. This study has laid the groundwork for such an undertaking.

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